Universidade de Aveiro Departamento de Química
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Carlos Alberto Cruz Pinto

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biotecnologia, ramo Alimentar, realizada sob a orientação científica do Doutor Jorge Manuel Alexandre Saraiva, Investigador Auxiliar do Departamento de Química da Universidade de Aveiro e da Professora Doutora Ivonne Delgadillo, Professora Associada com Agregação do Departamento de Química da Universidade de Aveiro

Dedico este trabalho aos meus queridos pais, irmã, avó e tia

o júri

presidente

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"Sê todo em cada coisa. Põe quanto és/ no mínimo que fazes"
Ricardo Reis – heterónimo de Fernando Pessoa

palavras-chave

Armazenamento hiperbárico, refrigeração, endósporos, *Bacillus subtilis*, *Alicyclobacillus acidoterrestris*.

resumo

Pretendeu-se, com este trabalho, avaliar o efeito de uma nova metodologia de conservação alimentar, designada armazenamento hiperbárico (AH), à temperatura ambiente variável (≈ 18-23 °C, AH/TA) como possível alternativa aos convencionais processos de refrigeração (RF), em estruturas de resistência bacteriana, nomeadamente endósporos de *Bacillus subtilis* e *Alicyclobacillus acidoterrestris*.

Os endósporos de *B. subtilis* foram inoculados em sumo de cenoura (alimento perecível) enquanto os endósporos de *A. acidoterrestris* foram inoculados em sumo de maçã comercial. As amostras foram armazenadas à pressão atmosférica (PA) e TA variável (≈ 18-23 °C, PA/TA), RF (≈ 4 °C, PA/RF e sob AH/TA (25, 50 e 100 MPa), até 60 dias. Adicionalmente, inocularam-se esporos de *B. subtilis* em duas matrizes nutricionalmente distintas (tampão McIlvaine e meio de cultura líquido, *BHI-broth*, a pH 6.00) a fim de averiguar se a composição da matriz de inoculação poderia influenciar o comportamento dos esporos em condições de AH/TA (50 e 100 MPa).

A composição da matriz de inoculação provou influenciar o comportamento dos endósporos de *B. subtilis* em condições de AH/TA. Em tampão McIlvaine, os endósporos não germinaram nem se desenvolveram em condições de PA/TA e PA/RF devido à carência de nutrientes, verificando-se o mesmo a AH/TA a 50 MPa. No entanto, uma pressão de armazenamento de 100 MPa resultou na redução da carga microbiana total e de esporos.

O sumo de cenoura armazenado a 50 e 100 MPa, sofreu uma redução da carga microbiana total (vegetativos e esporos) de ≈ 5.4 unidades logarítmicas e abaixo do limite de quantificação (2.00 log CFU/mL), respetivamente, ao sexagésimo dia de AH/TA, permitindo aumentar o prazo de validade quando comparado com o armazenamento à AP/TA e AP/RF, em que os esporos germinaram e se desenvolveram ao final de 9 e 60 dias, respectivamente, enquanto o AH/TA a 25 MPa despoletou a germinação e desenvolvimento dos esporos logo no primeiro dia de armazenamento, resultando na deterioração do sumo.

Quando inoculados em *BHI-broth*, os esporos germinaram e desenvolveram-se à PA/RT e PA/RF ao final de 1 e 9 dias, respetivamente, enquanto sob pressão (50 e 100 MPa) se verificou uma redução da carga microbiana total de ≈ 5.1 unidades logarítmicas e abaixo do limite de quantificação, respetivamente.

Os esporos de *A. acidoterrestris* em sumo de maçã foram inativados em todas as condições de AH/TA, especialmente a 50 e a 100 MPa (em que e atingiu o limite de deteção, 1.00 log CFU/mL, ao fim de 2 e 30 dias de armazenamento, respetivamente). A 25 MPa, a carga microbiana total também diminuiu, contrariamente ao observado no sumo de cenoura, possivelmente devido à acidez da matriz. Estes resultados indicam que o AH/TA é uma alternativa potencial aos processos convencionais de PA/RF relativamente ao controlo da presença de esporos nos produtos alimentares.

keywords

Hyperbaric storage, refrigeration, endospores, *Bacillus subtilis*, *Alicyclobacillus acidoterrestris*.

abstract

This work aimed to evaluate the effect of a new preservation methodology, known as hyperbaric storage (HS) at variable/uncontrolled room temperature (≈ 18-23 ºC, HS/RT) as a possible alternative to the conventional refrigeration (RF), in bacterial resistance structures, namely endospores, of *Bacillus subtilis* and *Alicyclobacillus acidoterrestris.*

The *B. subtilis* endospores were inoculated in carrot juice (highly perishable food product), while *A. acidoterrestris* endospores were inoculated in commercial apple juice. Then, samples were stored at atmospheric pressure (AP) at variable RT (≈ 18-23 ºC, AP/RT), RF (≈ 4 ºC, AP/RF) and under HS/RT (25, 50 and 100 MPa) up to 60 days. Moreover, *B. subtilis* endospores were also inoculated in two nutritionally distinct matrices (McIlvaine buffer and brain-heart infusion broth, BHI-broth, at pH 6.00) to ascertain if the composition of the inoculation matrix could influence the endospore behaviour at HS/RT conditions (50 and 100 MPa).

The matrix composition has proved to influence the *B. subtilis* endospores behaviour under HS/RT conditions. In McIlvaine buffer, the endospores did not undergo germination and outgrowth at both AP/RT and AP/RF storage conditions, due to the absence of nutrients, similarly to HS/RT at 50 MPa. A storage pressure of 100 MPa yielded total microbial and endospore loads reductions.

The carrot juice kept at 50 and 100 MPa underwent total microbial load (vegetatives and endospores) reductions along storage, being reduced ≈ 5.4 log units and to the quantification limit (2.00 log CFU/mL), respectively, at the 60^{th} day of HS/RT, allowing a shelf-life extension when compared with AP/RT and AP/RF storage, wherein endospores germinated and outgrowth after 9 and 60 days of storage, respectively, while HS/RT at 25 MPa quickly triggered endospore germination and outgrowth right on the first day of storage, thus yielding juice spoilage.

While inoculated in BHI-broth, the endospores germinated and outgrew at AP/RT and AP/RF conditions right after 1 and 9 days, respectively, while HS/RT at 50 and 100 MPa resulted in total microbial loads reductions of ≈ 5.1 log units and to the quantification limit, respectively.

The *A. acidoterrestris* endospores in commercial apple juice faced a sharp total microbial load reduction at 100 MPa (below the detection limit, 1.00 log CFU/mL) after 2 days of storage, while at 50 MPa the detection limit was reached by the thirtieth day of HS/RT. A storage pressure of 25 MPa was also feasible to preserve apple juice, contrarily to the carrot juice, probably due to the acidity hurdle of the matrix.

These results hint HS/RT as a possible preservation procedure, and a potential replacement of the conventional AP/RF processes, regarding the presence of endospores on food products.

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List of abbreviations

Abbreviation	Designation
ANOVA	Analysis of variance
AP	Atmospheric pressure
AP/RF	Atmospheric pressure and refrigeration
AP/RT	Atmospheric pressure and room temperature
ATP	Adenosine triphosphate
a_W	Water activity
BAT-agar	*Bacillus acidophilus* agar
BHI-agar	Brain heart infusion agar
BHI-broth	Brain-heart infusion broth
Ca-DPA	Calcium dipicolinate
CFC's	Chlorofluorocarbons
CFU	Colony forming unit
CLE's	Cortex lytic enzymes
CO_2	Carbon dioxide
DPA	Dipicolinic acid
FDA	Food and drug administration
GAM	Gifu anaerobic broth
Gpr	Germination protease
GR's	Germination receptors
h	Hours
HPLC	High performance liquid chromatography
HPP	High pressure processing
HS	Hyperbaric storage
HS/RT	Hyperbaric storage at room temperature
HSD	Honest significand differences
MPa	Mega Pascal
min	Minutes

Abbreviation	Designation
PCA	Plate count agar
PDA	Potato dextrose agar
PDB	Potato dextrose broth
PHS	Post-hyperbaric storage
PME	Pectin-methylesterase
RF	Refrigeration
RT	Room temperature
SASPs	Small acid soluble proteins
spp.	Species
TEL	Total endospore load
TML	Total microbial load
UHT	Ultra-high temperature

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Contextualization and thesis structure

This thesis is composed of six chapters, wherein the first one comprises a literature review regarding the subject of food preservation techniques, with special focus on storage under pressure (hyperbaric storage, HS), as well some features regarding endospores and their response against hydrostatic pressure, followed by the work objectives and the schedule adopted. Then, on the second chapter, a detailed description of the materials and methods used in the aim of this thesis is provided. The third chapter presents and discusses the results obtained for *B. subtilis* endospores inoculated in three different matrices. On the fourth chapter, the results obtained for *A. acidoterrestris* endospores in commercial apple juice are presented and discussed, followed by the main conclusions (chapter fifth), proposed future work (chapter six) and the list of the consulted literature in the aim of the present work. Finally, an appendix section is provided, concerning data that, due to its extension, could not be presented on the corresponding chapters.

CHAPTER I – LITERATURE REVIEW

THIS SECTION COMPRISES AN EXTENSIVE, BRIEFLY COMPILED LITERATURE REVIEW REGARDING THE SUBJECTS OF FOOD PRESERVATION TECHNIQUES AND ENDOSPORES

I. Introduction

The survival and evolution of mankind was possible due to several factors, being one of them the ability of feeding themselves, as well as the development of techniques to preserve food products for later consumption. The food preservation techniques used back then (and still being used) relied, mainly, on water content depletion, food matrix acidification, temperature control and, more recently, pressure control. This literature review will briefly mention the basis concept inherent to some of the conventional food preservation techniques, with special focus to pressure control (hyperbaric storage, HS), then, a second topic regarding the role of endospores on food industry and its behaviour under different pressure conditions will be discussed.

The salting process consists on keep food products involved on edible salt in order to dry perishable food products. This process relies on the reduction of the water activity (a_W) of a food product in order to inhibit the development of both deteriorative and pathogenic microorganisms, as well preventing degradative biochemical reactions (Sperber, 1983). It is thought to be the oldest preservation technique (Li et al., 2016), which was used by the Egyptians to preserve fish and meat products in clay plots full of salt (Ordóñez et al., 1998).

Dehydrating food products using the fire heat or the sun light was also used as a food preservation technique on dried fruits, since the water of the foodstuff is evaporated, which causes the reduction of the a_W to levels that do not allow the development of deteriorative and pathogenic bacteria and moulds (Sperber, 1983).

The exposure of food products to wood smoke is quite popular among food preservation procedures, allowing to obtain products with exceptional organoleptic characteristics, such as chorizos, along with other smoked meat and fishery products. The shelf-life improvement of smoked food products is due to a combined effect of the bacteriostatic effect of phenols on the endogenous microflora (Løvdal, 2015), as well by the reduction of the a_W of such products.

Acidification is also a very popular food preservation technique, that relies on the depletion of the food product endogenous pH to a lower value (usually below 4.5), by direct addition of an acidic matrix, such as vinegar (pickling) or by consortia of microorganisms, in a process known as fermentation, to avoid the proliferation of pathogenic microorganisms, such as *Bacillus cereus* or *Clostridium botulinum*.

Also known as an ancient preservation technique for edible products, pickling consists on the direct addition of an acid solution, such as vinegar, or a salt solution as brine, to lower its endogenous pH or to change the osmolarity, respectively, to inhibit the development of both deteriorative and pathogenic microorganisms. The pickling process is commonly used to preserve vegetables, meat and fishery products. Despite its feasibility for food preservation, it is responsible for severe changes on food texture, flavours, aromas, etc., and loss of nutritional content (Montaño et al., 2016). Fermentation was also popular between the firstly employed food preservation strategies, mainly on milk, fruit juices, meat products and cereals. It is thought to have been discovered by accident, probably by storing sour milk for a period of time long enough to obtain a fermented product (Ordóñez et al., 1998), being this process based, as aforementioned, on the depletion of the original pH of the food product to a lower value, carried out by a consortia of microorganisms such as bacteria (lactic acid, propionic, acetic bacteria) and fungi (*Saccharomyces* spp., *Aspergillus* spp., etc.), which inhibits the development of pathogenic bacteria and moulds (Russell and Diez-Gonzalez, 1997).

The aforementioned food preservation techniques, in spite of being generally safe and cheap to use, are also responsible for changes on texture, flavour, aromas and nutritional content of foodstuff, especially on meat and fishery products. These were the factors, along with many others, that led to the development of alternative and with minor impact on food products, relying on temperature control.

Food storage below 0 ºC yields longer shelf-lives when compared to other food preservation techniques, since the available water undergoes its solid state, allowing to inhibit microbial proliferation, the majority of biochemical reactions, as well the death of worms and other organisms that can contaminate food products. Although, this process is responsible for structural changes on animal and vegetal cells (lysis) caused by the ice crystals, resulting in loss of water and solutes after thawing, a phenomenon known as drip loss (Hundy et al., 2016).

Food preservation at low temperatures is thought to have been firstly performed using ice from the mountains by the Persians, later by the Egyptians and then by the Romans. Only in 1800 the first refrigeration (RF) units were developed (Briley, 2004) and revolutionized the cold-chain preservation industry, that was still dependent on the ice harvest from the mountains and glaciers. The RF feasibility as a preservation strategy relies

on the effect of low temperatures (1–7 ºC) on the endogenous microflora of food products, retarding its development, as well the majority of deteriorative biochemical reactions (both enzymatic and non-enzymatic reaction) (Ashie et al., 1996).

Quickly, RF began to replace the conventional preservation techniques, since it allowed to preserve foodstuff with minimal impact on flavour, texture, nutritional value and aromas, when compared to salting, smoking and fermentation, and became the most used preservation technology (along with freezing) for both industrial and domestic use, with a current (2016) market value of 167.24 billion dollars, being thought that it will have an economic impact of 234.49 billion dollars by 2020 (Marketandmarkets, 2017).

Nonetheless, with the increase of Earth population, which is predicted to reach 9 billion people by the end of 2045 (Van Bavel, 2013), the environmental concerns regarding the use of fossil fuels to generate energy to sustain the food cold-chain industry are being considered, since 35–50% of the total energy consumed in super and hypermarkets is attributed to both RF and freezing equipment, contributing for approximately 1% of the carbon dioxide (CO_2) emissions worldwide (James and James, 2010), being the third major source of CO_2 of all food industry (with 490 megatons of CO_2 released to the atmosphere in 2008) (Gilbert, 2012). For so, it is convenient to invest on strategies that can decrease energy consumption in order to reduce the carbon footprint associated with food preservation, without compromising food safety, quality, and sustainability, being the storage pressure control a possible alternative, as will be furtherly discussed.

I.1. Food preservation by pressure control

Recently, a new preservation methodology was proposed, relying on an emergent food processing technology. Under the name of HS, it states that instead of controlling the temperature of the container where the food product is stored, it is advantageous to control the pressure level that the food product is subjected (Fernandes et al., 2014). But first, a contextualization regarding the involved technology (high pressure processing, HPP) will be given and discussed.

I.2. High pressure processing

HPP is a non-thermal food processing technology that makes use of elevated hydrostatic pressure (up to 600 MPa) for inactivation of vegetative microorganisms on

food products. This technology relies on two essential principles. The first one is the *Le Chatelier's* principle, which states that any change made in an equilibrium system (chemical reaction, phase transition or modifications of molecular configurations) accompanied by a volume decrease is compensated by a pressure increase, and vice-versa. The second one is the isostatic principle, which claims that pressure is uniformly distributed by the entire sample whether in direct contact or in a flexible container, regardless of its shape or size (Smelt, 1998).

HPP is currently being employed as a cold pasteurization procedure of acidic fruit juices, ready-to-eat meals, fishery, and meat products worldwide (Bermúdez-Aguirre and Barbosa-Cánovas, 2011). The next topics will focus a few historic facts concerning HPP, as well its evolution throughout time and its application as an assistant of sterilization procedures to destroy endospores (that will be detailed on the endospore features section).The first scientific report concerning the effect of high hydrostatic pressure on microorganisms belongs to Certes (1884), who found viable bacteria on deep sea sediments collected at a depth of 5,000 m (≈ 50 MPa). Bert Hite was the first author reporting the use of high pressure for food processing. In fact, Hite (1899) proved that it was possible to extend raw milk shelf-life for 4 days after a pressure treatment of 1 hour at 600 MPa at room temperature (RT), inasmuch the milk suffered a microbial reduction between 5 to 6 logarithmic cycles. It was also observed that the souring process was retarded for about 24 h after a pressure treatment of 200 MPa.

Later, in 1914, Hite, Giddings, and Weakley (1914) verified that several HPP pre-treated fruits (400–820 MPa) remained commercially stable at least for 5 years, not being found the same effect in vegetables due to spore-forming bacteria that were able to survive over the pressure treatment and grew in low acidic vegetables.

Many other studies were performed concerning the use of HPP to inactivate both deteriorative and pathogenic microorganisms, although, it took almost a century between Bert Hite's studies and the commercialization of HPP food products (Patterson, 2005) due to the lack of adequate of commercial equipment (Torres and Velazquez, 2005). In fact, the first food product processed by HPP was commercialized in 1990 by the Japanese retail company *Meidi-ya*, which introduced a line of acidic jams that, according with the Japanese patent no. 1991-219844 (Hori et al., 1991), were processed at a pressure level between 392–588 MPa during 10 to 30 minutes (min) (Meidi-ya, 1991).

From that moment, many other companies worldwide rushed to introduce new food products processed by this innovative technology, being even initiated research programs in Europe and in the United States of America between industrial consortia and research institutes (Williams, 1994). Since the first HPP industrial application, the number of equipment and products processed by this technology have been increasing, mainly due to consumer requirements for fresher, tastier and minimally processed foods (Huang et al., 2013). Moreover, the HPP effects are instantaneous and independent of the shape and size of the food or the package, resulting in a more smooth transition from laboratorial studies to industrial applications (Thakur and Nelson, 1998).

Regarding HPP feasibility against vegetative cells, it relies on the interruption of cellular functions that are essential for reproduction and survival of microorganisms. In fact, HPP is responsible for changes on the microorganism's membranes, resulting in leakage of the inner cell content and interference on nutrient uptake mechanisms (Mújica-Paz et al., 2011), as well solute lost during the pressurization steps, protein denaturation and enzymatic inactivation (Shimada et al., 1993). The inactivation of gram-positive bacteria normally requires more intense pressure treatments than gram-negative bacteria due to the rigidity of teichoic acids present on the peptidoglycan layers of gram-positive bacteria (Heinz & Buckow, 2010).

Nowadays, HPP is a well-established technology that has been widely used as a non-thermal food pasteurization procedure, with particular emphasis on fruit juices and beverages, meat and fishery products, vegetables and ready-to-eat meals, as aforesaid (Bermúdez-Aguirre and Barbosa-Cánovas, 2011), since it allows the maintenance of foods attributes (vitamins, proteins, flavour and taste) inasmuch heat is not applied.

At an industrial level, among other novel processing technologies, HPP seems to be one of the most promising due to the high number of equipment operating worldwide (Mújica-Paz et al., 2011), as seen in **Figure 1**. Furthermore, Visiongain (2016) predicted that, by the end of 2016, the HPP food marked worth more than 11 billion dollars and the equipment market of about 0.47 billion dollars.

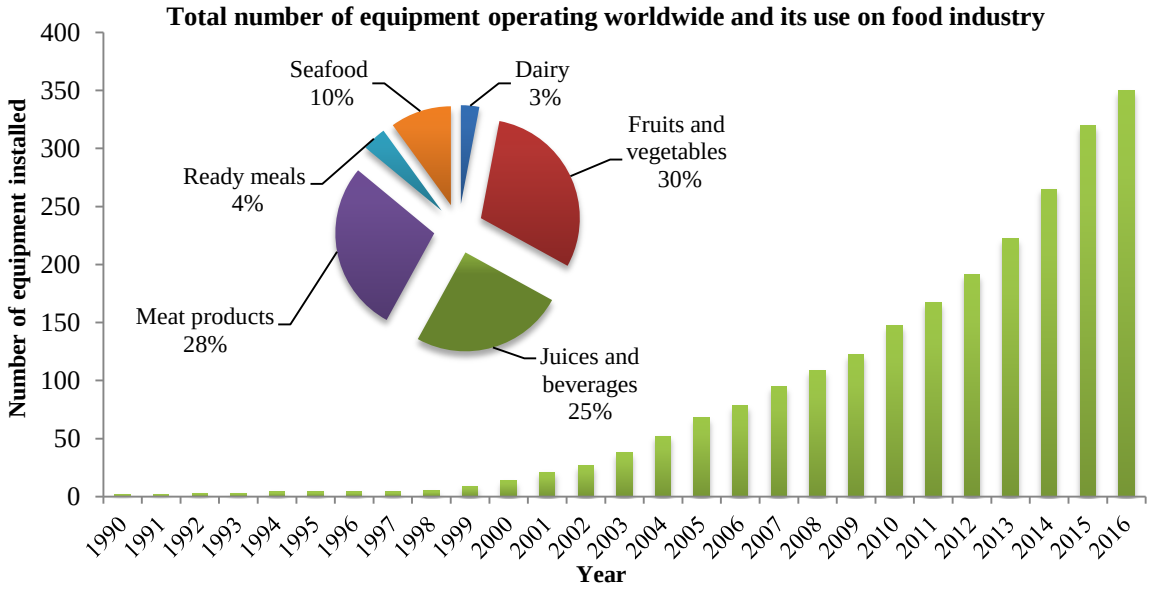


Figure 1: Total number of high pressure processing equipment currently operating on food industry and its use according with product typology. Courtesy of Hiperbaric.

The major HPP disadvantage still relies on the equipment costs, although this can be surpassed as new manufacturers starts to operate on the market, as well new and cheaper materials are developed (Gupta and Balasubramaniam, 2012).

HPP at cold or RT, similarly to thermal pasteurization, only inactivates vegetative microorganisms, which means that both fungal and bacterial spores are able to survive, germinate and outgrowth once the conditions are more propitious (Kort et al., 2005), even after intense processing at very high pressures (up to 1,200 MPa) (Reineke et al., 2013a). Thus, food products processed by HPP are, generally, to be kept at RF conditions (1–7 ºC) to inhibit the development of spores on high and low-acidic food products (Mújica-Paz et al., 2011). Moreover, acidic food products, such as apple or orange juices, have the acidity hurdle that intrinsically allows a longer shelf-life under RF and require less intense HPP treatments (Heinz and Buckow, 2010).

As food industries are interested on the development and commercialization of shelf-stable high perishable food products with superior quality, while ensuring microbial safety (Barbosa-Cánovas et al., 2014), research on food sterilization using different technologies (sequentially or simultaneously) have been intensified to test its feasibility (Park et al., 2014). The possibility of combine HPP along with other thermal and/or non-thermal technologies will be a reality on the food industry, with potential economic and financial gains, since new food products, with greater quality, will be available on the markets.

I.3. Hyperbaric storage

I.3.1. Definition and background

HS is a new preservation methodology that consists on food storage under a pressure range up to 220 MPa for a certain period of time, from several hours (h) to days or even months, being pressure the hurdle to the microbial development, as well it enhances the preservation of several biochemical characteristics and other attributes (Fernandes et al., 2014).

In October of 1968, the research submarine Alvin sank due to a failure on its support cables while being launched. Almost one year later (September of 1969), Alvin was recovered and, surprisingly, after being on deep seawater at 1540 meters (≈ 15 MPa and 3-4 ºC), some foodstuff from the crew (namely two thermos bottles containing bouillon and a plastic box with apples and sandwiches) seemed edible despite being soggy (Woods Hole Oceanographic Institution, 2014).

The recovered food products (**Figure 2**) seemed well preserved when it comes to general appearance, texture, smell, taste, and biochemical and bacteriological assays, opening the possibility of store food products under pressure at RF temperatures to extend their shelf-lives. It was also observed that after keeping these materials at RF conditions (3 ºC) they spoiled in a few weeks, as normally observed (Jannasch et al., 1971).

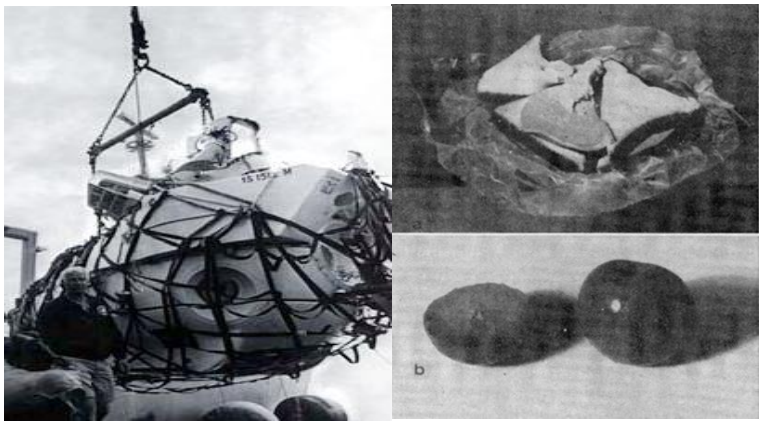


Figure 2: On the left, Alvin research submarine recovery in 1969. On the right, food products recovered after being almost one year at deep sea. Adapted from Woods Hole Oceanographic Institution (2014) and Smith (2012).

Two strategies to generate pressure within a container were employed on HS studies, using air or water (or another pressurization fluid – hydrostatic pressure, up to 220 MPa, which is the most used strategy). Pressure generated by gaseous systems will not be discussed, since hydrostatic pressure is the most used on the food industry, as well the equipment used on the aim of this thesis uses a pressurization fluid. Moreover, the pressure

levels used on gaseous systems are normally much lower when compared to hydrostatic pressure.

Several studies regarding HS at subzero (<0 ºC), refrigerated (1-5ºC), at and above RT were performed over the years. Since all the HS experiments on the scope of this thesis were performed at naturally variable/uncontrolled RT, a special focus will be given to the studies made over the years at such conditions, while HS below and above RT will be briefly described. Respecting the chronological arising of HS studies, the following sections will aim HS at RF temperatures (1-5 ºC), HS subzero (<0 ºC), HS at and above RT.

I.3.2. Hyperbaric storage at refrigeration temperatures (1 to 4 ºC)

In order to simulate the conditions at which the research submarine *Alvin* was sunken, Jannasch et al. (1971) performed an experiment wherein the same food products were kept under pressure (15 MPa at 3-4 ºC) for 10 months, demonstrating that the combination of pressure with low temperatures was more efficient than RF alone (at 0.1 MPa) retarding the degradation (microbial and enzymatic) of food products. Then, other studies were performed at RF temperatures, as summarized in the **Table 1**.

Table 1: Studies concerning HS performed at refrigerated temperatures. Adapted from Fernandes et al. (2014) with modifications.

Product	Conditions	Period	Outcomes	Reference
Apples, bouillon and sandwiches	15 MPa/3–4 ºC	10 months	Stable after 10 months under deep sea, when stored at 0.1 MPa at refrigerated conditions, they quickly spoiled	Jannasch et al. (1971)
Rice, wheat and soy beans	3.5 MPa/1 ºC	1 year	Stable for 1 year. Lower changes in seed moisture, fatty acids and reducing sugars. Improved germinative capacity	Mitsuda et al. (1972)
Dressed cod	24.12 MPa/1 ºC	21 days	Stable and consumable after 2 days, while samples stored at 0.1 MPa were unacceptable	Charm et al. (1977)
Pollock		12 days	Stable and consumable after 12 days with higher quality than those at 0.1 MPa	
Cape hake loins	50 MPa/5 ºC	7 days	Shelf-life extension, total volatile basic-nitrogen was kept stable. Drip loss, shear resistance and whiteness increment	Otero et al. (2017)

Despite HS feasibility at RF temperatures, another strategy of food preservation using hydrostatic pressure was used by means of temperature control, i.e. as pressure can depress the freezing point of water, allowing the maintenance of its liquid state at temperatures below 0 ºC, a new concept of HS arose, known as HS subzero.

I.3.3. Hyperbaric storage at subzero temperatures (<0 ºC)

As seen in the **Figure 3**, pressure can depress the water freezing point, keeping it on its liquid state when below 0 ºC.

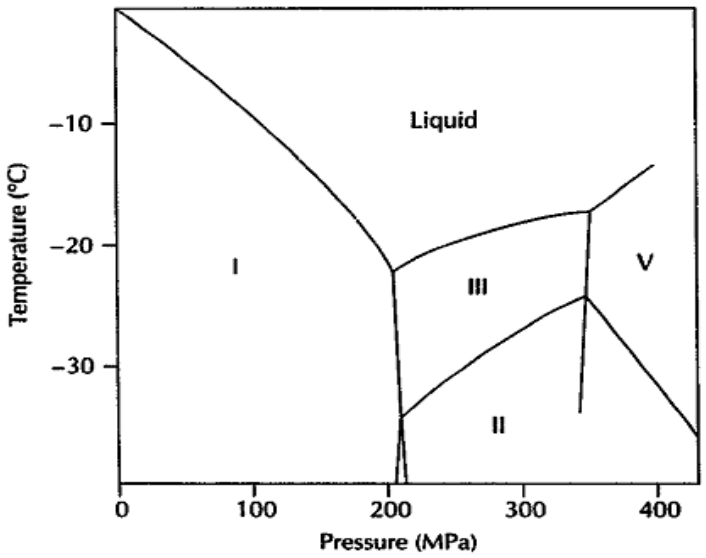


Figure 3: Water phase diagram according with pressure (MPa) and temperature (ºC). The Roman numbers (I, II, III, IV and V) represent different types of ice that are formed under pressure. Adapted from Kalichevsky et al. (1995).

Taking the aforementioned into account, it was expected that HS could be performed at freezing temperatures, without freezing food products, with advantages over the conventional freezing process, since texture damages caused by the formation of ice crystals (that disrupts the tissues of solid food products such as fish and meat) are not verified (Kalichevsky et al., 1995). Thus, it was important to ensure the microbial safety and physicochemical stability of foodstuff at HS subzero. Concerning the given examples in **Table 2**, it was proved that HS at subzero temperatures could be as efficient or even better than conventional freezing (at 0.1 MPa).

Table 2: Studies concerning HS subzero performed at freezing temperatures. Adapted from Fernandes et al. (2014).

Product	Conditions	Period	Outcomes	Reference
Cod fish fillets	22.8 MPa/–3 ºC	36 days	Stable and consumable for at least 36 days. Similar in quality to frozen samples at 0.1 MPa	Charm et al. (1977)
Beef	200 MPa/–20 ºC	(*)	Microbial load reduction and inactivation of yeasts and some bacteria	Deuchi and Hayashi (1990)
Strawberry and tomatoes	50–200 MPa/–5 and –20 ºC	(*)	Stable for a few more days/ weeks. Fresh flavor and colour preserved. Catalase, β-amylase, cathepsin and lactate dehydrogenase inhibition by pressure	Deuchi and Hayashi (1992)
Chicken and carp	170 MPa/–8 and –15 ºC	50 days	Stable for 50 days. Enzymatic activity associated to nucleic acids degradation reduced	Ooide et al. (1994)

(*) The authors did not precisely report the storage period, describing it as "a few days or weeks".

Despite subzero HS seems a reliable alternative to store food products at freezing and refrigerated temperatures, both techniques have as major disadvantage the highly energetic costs involved on the temperature control (Fernandes et al., 2014).

As aforesaid, RF is responsible for the consumption of about 35–50% of the total energy in super and hypermarkets, contributing for approximately 1% of the CO_2 emissions worldwide, being also the third major source of CO_2 emissions in food industry (Gilbert, 2012; James and James, 2010). Thus, it is convenient to invest on strategies that can decrease the energy spent to reduce the carbon footprint, without compromising food safety and quality. As HS can be feasible as a RF improvement, it was hypothesized if it could be performed at and above RT, as described and discussed on the next section.

I.3.4. Hyperbaric storage at and above RT

HS at and above RT seems to be a promising food preservation procedure due to the reduced energetic costs associated, as well by many other advantages brought by this technique as several studies have been performed in the last 4 to 5 years, which have revealed a positive impact of use HS at and above RT and showing outcomes such as improvement on the quality characteristics on food products along storage, as well shelf-life extensions over RF (Freitas et al., 2016; Lemos et al., 2017; Pinto et al., 2017, 2016), among others, as displayed in the **Table 3**. The major advantages of HS/RT are the

reduced energetic spends, since energy is only required on the compression and decompression phases (of the pressure vessel) and no temperature control is needed (Fidalgo et al., 2014; Moreira et al., 2015; Pinto et al., 2016; Queirós et al., 2014; Santos et al., 2015).

Table 3: Studies concerning HS at and above room temperature (RT) up to 220 MPa. Adapted from Fernandes et al. (2014) with modifications.

Product	Conditions	Period	Outcomes	Reference
Tilapia filets	203 MPa/25 ºC	12 h	Improved freshness than those stored at 0.1 MPa. Microbial count reduction of about 2.0 log CFU/g	Ko et al. (2006)
Sea cucumber guts	60 MPa/30 ºC	24 h	Reduction of the psychotrophic counts of about 0.9 log CFU/mL	Okazaki et al. (2007)
Watermelon juice	100 MPa/18–21 ºC	60 h	Inactivation plus inhibition of microbial growth up to 60 h. Extended shelf-life at 0.1 MPa after HS	Fidalgo et al. (2014)
	25–150 MPa/20–37 ºC	8 h	Microbial growth inhibition at 75 MPa and inactivation at 100 and 150 MPa. No significant changes on the physicochemical parameters	Santos et al. (2015)
	100 MPa/18–21 ºC	7 days	Shelf-life extension when compared to the juice kept at 4 ºC and 0.1 MPa	Pinto et al. (2016)
	50–100 MPa/18-23 ºC	10 days	Shelf-life expansion at 75 and 100 MPa, feasible inhibiting pathogenic surrogated microorganisms (*E. coli* and *L. innocua*) growth, minimal impact on enzymatic activities, mainly at 75 MPa	Pinto et al. (2017)
	50–75 MPa/10–25 ºC	58 days	Shelf-life extension at 50 MPa/10 ºC, 62.5 and 75 MPa. Colour parameters and pH less affected by HS than at 0.1 MPa (4 and 15 ºC)	Lemos et al. (2017)
Melon juice	25–150 MPa/20–37 ºC	8 h	Stability verified at all temperatures for pressures above 50 MPa. Microbial growth inhibition achieved at 50/75 MPa and inhibition plus inactivation at 100/150 MPa	Queirós et al. (2014)
Requeijão (Portuguese whey cheese)	100-150 MPa/25–37 ºC	8 h	Microbial load reduction after HS. Pressure retained the colour, pH and a_W of the whey cheese. Lipid oxidation levels stable when compared to RF at 0.1 MPa	Duarte et al. (2014)
Carrot soup	100-150 MPa/25-30 ºC	8 h	Microbial growth inhibition at 100 MPa and inactivation at 150 MPa after HS. General physicochemical parameter similar to RF	Moreira et al. (2015a)

Table 3: Studies concerning HS at and above room temperature (RT) up to 220 MPa. Adapted from Fernandes et al. (2014) with modifications (continued).

Product	Conditions	Period	Outcomes	Reference
Sliced cooked ham	25–150 MPa/23–37 ºC	8 h	HS was efficient to inhibit microbial growth at pressures above 50 MPa for similar levels of RF. Microbial inactivation at 100 and 150 MPa	Fernandes et al. (2015)
Caldo verde and *bacalhau com natas*	50–150 MPa/21 ºC	12 h	Microbial growth inhibition at 100 MPa and inactivation at 150 MPa. No significant changes on the physicochemical parameters evaluated	Sílvia A. Moreira et al. (2015)
Raw bovine meat	50–150 MPa/21 ºC	12 h	At 50 MPa it was faced a similar microbial development inhibition when compared to RF storage, while at 100 and 150 MPa it was verified an additional microbial inactivation effect	Freitas et al. (2016)
	100 MPa/21 ºC	10 days	The raw bovine meat shelf-life was extended over RF at 0.1 MPa and no significant differences were found on the quality parameters of the meat	
Strawberry juice	25- 220 MPa/20 ºC		Samples stable for 15 days during HS and for more 15 days at RF (PHS)*. Microbial loads (yeasts and moulds and total aerobic mesophiles) below the detection limit after HS at 100 and 220 MPa	Segovia-Bravo et al. (2012)
	50–200 MPa/20 ºC	15 days	Pressure avoided spoilage of samples stored at 20 ºC for 15 days and kept the volatile profile of the strawberry juice similar to the initial samples	Bermejo-Prada et al. (2015)
			Neither pectin-methylesterase (PME) catalytic activity was affected by pressure on strawberry extract, nor PME inactivation was found up to 200 MPa	
			Significant peroxidase inactivation on longer storage periods (5, 7 and 15 days) and lower percent of polymeric colour at the 5^{th}, 7^{th} and 10^{th} days at 200 MPa, compared to samples stored at 0.1 MPa	Bermejo-Prada and Otero (2016)
	25–200 MPa/20 ºC		At 25 MPa the microbial growth was retarded, while at 50 MPa a microbial growth reduction was observed while higher pressures resulted in higher microbial loads reductions	Bermejo-Prada et al. (2016)

*PHS – Post-hyperbaric storage assay consisting on store a food product at RF conditions for an additional period of time after being subjected to HS conditions.

I.3.5. Storage costs estimations

The potential energetic savings allowed by HS/RT were referred by several authors, such as Fernandes et al. (2014), Fidalgo et al. (2014), Freitas et al. (2016), Moreira et al. (2015), Pinto et al. (2016) and Segovia-Bravo et al. (2012), among others, with inherent economic and environmental gains, stating that energy would be only needed to compress and decompress the pressure vessel, since when the desire pressure was achieved, energy would not be needed to keep it during storage, along with the needless temperature control, thus, virtually, with no energetic costs. Although, until now this statement was only deeply investigated by Bermejo-Prada et al. (2017), who estimated that the energetic costs inherent to HS of 800 kg of strawberry juice at 25 MPa and RT (20 ºC) for 15 days was 0.001 €/kg against 0.026 €/kg of RF.

Although a great reduction of the energetic costs is associated to this new food preservation methodology, the equipment price can overlap that potential, mainly due to the costs of the pressure vessel, intensifiers and hydraulic pumps, which are considerably higher than the conventional RF facilities, which resulted, as estimated by Bermejo-Prada et al. (2017), in a total storage cost for HS of about 0.291 €/kg of strawberry juice, against 0.081 €/kg for conventional RF. These costs include equipment maintenance and amortization (a measure of the initial investment depreciation), as well the inherent energetic costs (this last parameter seems to be the trump of HS/RT against RF). In addition, this study estimated HS/RT costs considering a completely loaded vessel (maximum mass of 2,000 kg) so it could be moved to a warehouse with a forklift.

Moreover, as the storage pressure increases, the pressure vessel thickness required to keep it for long periods of time also increases, thus increasing the storage costs. As mentioned, despite the HPP equipment high initial cost, it did not stop its implementation in the food industry as a non-thermal pasteurization method, and as result, a decreasing trend in equipment costs was observed from 1996 until now. Innovations related to the HPP technology, such as HS, might lead to the arising of new manufacturers, which could also lower the price of these units (Mújica-Paz et al., 2011), even if specifically designed for HS, that would require less resistant units once the pressure levels employed on HS are considerably lower than in HPP.

I.3.6. Carbon footprint assessment

As mentioned before, RF is the third major source of CO_2 of all food industry (with 490 megatons of CO_2 released to the atmosphere in 2008), being even estimated that 35 to 50% of the energetic consumptions in super and hypermarkets is due to RF and freezing facilities, representing 1% of the CO_2 emissions worldwide (Gilbert, 2012; James and James, 2010). Thus, more environmentally friendlier food preservation methodologies are to be considered, in order to reduce the carbon foot-print related with RF, being HS a possible solution for this situation. Besides CO_2, RF facilities are also responsible for considerable emissions of greenhouse effect gases, which are used as refrigerant on these facilities, belonging to a class of compounds known as chlorofluorocarbons (CFC's) and hydrochlorofluorcarbons, which are responsible for ozone degradation (James and James, 2010).

The carbon footprint associated with HS of 800 kg of strawberry juice for 15 days was assessed by Bermejo-Prada et al. (2017) and compared with RF storage. The outcomes revealed that RF had a 26-fold higher carbon footprint when compared to HS (0.1085 kg CO_2/kg against 0.0042 kg CO_2/kg of strawberry juice, respectively). In what concerns RF, the two main sources of CO_2 were the energetic consumption and the refrigerant leakage, while for HS the main source of CO_2 emission was attributed to the hyperbaric chamber material, with an estimated emission of 0.0041 kg CO_2/kg of strawberry juice, while the CO_2 released by the energetic consumption was negligible ($3x10^{-5}$ kg CO_2/kg of strawberry juice), proving that HS is considerably less pollutant than the conventional RF processes.

From the social point of view, Bermejo-Prada et al. (2017) concluded that HS/RT could also be preferred over RF, since the pressure vessels could be shipped to foreign geographies where electricity is less available, thus providing safer food products, while contributing for a more sustainable food-chain industry.

I.4. Microbial spores – bacterial *vs.* fungal spores

The identification of dormant forms of life was almost simultaneously found out by Koch (1876), Tyndall (1877) and Cohn (1877), namely the possibility of certain bacteria species spend part of their lifecycle on a dormant cellular structure named (by that time) as spore, being later renamed as endospore. In fact, there are several bacteria and fungi able to form spores (in a process named sporulation), although by different pathways, since bacterial sporulation is seen as an adaptive survival mechanism that is triggered when these microbes (such as *Bacillus cereus* or *Clostridium botulinum*) find unfavourable conditions at a particular environment, such as lack of nutrients, abrupt changes on the culture media (pH…), salinity, radiation, among other stress sources (Black et al., 2007; Wells-Bennik et al., 2016), being the resultant structures named endospores (**Figure 4**).

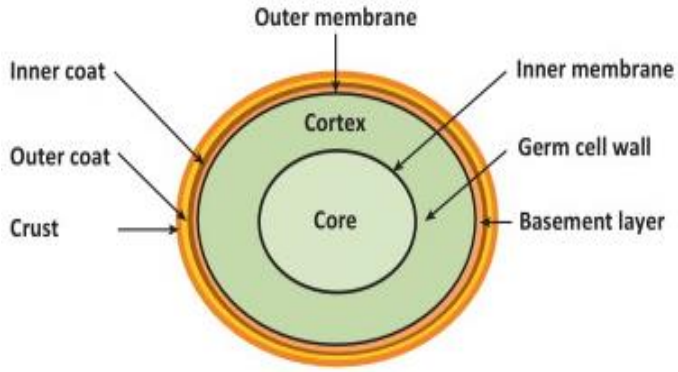


Figure 4: Schematic representation of a *Bacillus* spp. endospore. Adapted from Reineke, Mathys, et al. (2013).

Once the environmental conditions are more favourable for the bacterial survival, the endospore activates its germination pathways and originates a fully competent vegetative cell (Wells-Bennik et al., 2016). The formation of fungal spores is seen as an asexual reproduction mechanism, contrarily to the formation of bacterial endospores (Adams et al., 1998), being the resultant structure called ascospores.

In this section, a special focus will be given to the bacterial endospores since they play a major role on the food industry, being a constant preoccupation among food industrials and authorities when it concerns food safety, despite the importance of ascospores for food industry too.

I.4.1. Formation and characteristics of bacterial endospores

An extensive comprehension on the mechanisms that vegetative bacteria undergo to originate endospores is of upmost importance to understand how to avoid its formation and how to destroy or reduce its loads to minimal levels for food safety.

The formation of an endospore begins when a healthy vegetative bacteria receives and precepts diverse environmental stimulus such as nutrient exhaustion (normally carbon and nitrogen starvation), elevated mineral concentration, pH at neutral values, abrupt temperature changes and high cell densities, and undergoes the differentiation process onto endospores (Sella et al., 2014). Once the stimulus are perceived, the cellular mass increases due to the accumulation of peptides that are secreted and sensed by the cell surface receptors, and the sequential activation of the master regulator *Sp0A* is activated (Molle et al., 2003).

Then, the sporulation goes throughout seven stages (**Figure 5**), occurring as follows (Sella et al., 2014):

- **Stage I:** Axial deposition of the nuclear material into filaments;
- **Stage II:** Plasmatic membrane invagination (which occurs simultaneously with the segregation of the DNA molecule) in a non-symmetric position close to the cell extremity (pole), leading to the formation of a septum;
- **Stage III:** Septum curvature, the immature spore is engulfed by the double membrane of the mother cell (similarly to phagocytose), resulting in a structure (forespore) entirely involved on the mother cell;
- **Stage IV:** Development of the spore (from the forespore) in a process mediated by the mother cell. Assembly of both inner and outer proteic layers and synthesis of the spore cortex, which consists of a bushy peptidoglycan layer present between the inner and the outer membranes. Accumulation of calcium dipicolinate (Ca-DPA) within the spore nucleus;
- **Stage V:** Synthesis of the spore coat resulting from the deposition of approximately 80 proteins from the mother cell that are to be disposed on the inner and outer layers of the spore;
- **Stage VI:** Maturation of the new spore. During this stage, the spore becomes heat and organic solvent resistant;

- **Stage VII:** Release of the mature spore by disruption the mother cell by the action of lytic enzymes.

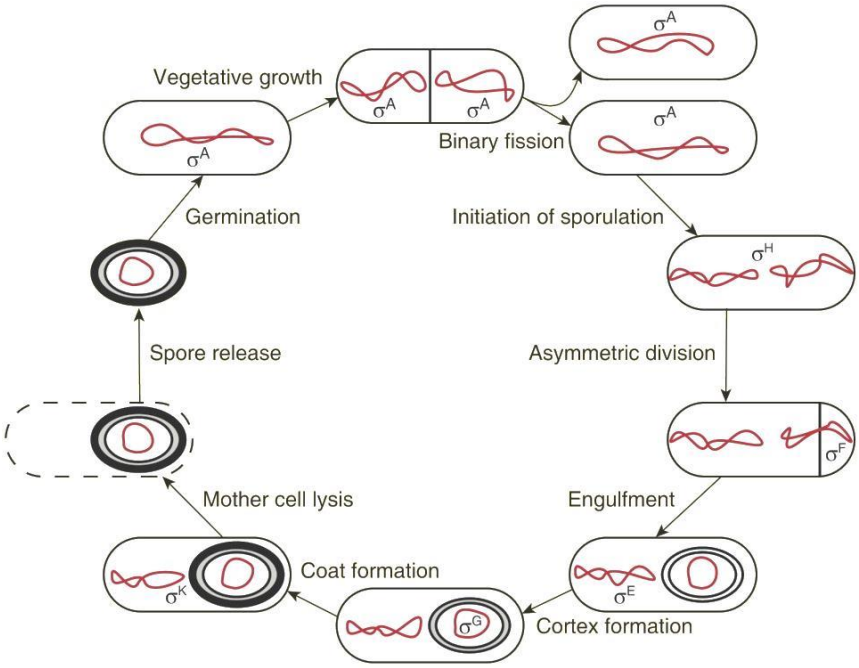


Figure 5: Mechanism of *Bacillus* spp. endospore's formation from a vegetative cell. The sigma (σ) factors refer to specific transcription factors that are involved on the sporulation process. Adapted from De Hoon, Eichenberger, and Vitkup, (2010).

Right after their formation, the spores can remain dormant for long periods of time, even for thousands or millions of years (Kort et al., 2005) since these structures show incredible resistance against physical, chemical, and environmental damages (Setlow, 2006).

Regarding food industry, endospores are resistant to the majority of processing procedures (whose defence strategies are summarized in the **Table 4**), including thermal food processing (except commercial sterilization) such as pasteurization, as well to non-thermal technologies as HPP (Sarker et al., 2015), ultraviolet (UV) light (Gayán et al., 2013), pulsed light (Levy et al., 2012), ultrasounds (Chemat et al., 2011), pulsed electric field (PEF) (Pillet et al., 2016) and cold plasma (Schlüter and Fröhling, 2014). Despite irradiation seems to be an efficient non-thermal technology to achieve high rates of endospore inactivation, its use among foodstuff is still very limited due to safety issues and consumers reluctant acceptance (Li and Farid, 2016; Odueke et al., 2016).

Table 4: Mechanisms of endospore survival against physical and chemical treatments. Adapted from Reineke et al. (2013a).

Sporicidal treatment	Defence mechanism/ factors affecting the resistance
Wet-heat	Sporulation temperature Core level of Ca^{2+} α- and β-type small acid soluble proteins (SASPs) Low water content in the spore core
Dry-heat*	DNA protection by α- and β-type SASPs DNA repair enzymes ExoA and Nfo (active during germination)
Desiccation*	DNA protection by α- and β-type SASPs
Chemical resistance*	Sodium chloride/ hypochlorite: Spore coat – coat proteins react and detoxify Hydrogen peroxide: DNA protection by α- and β-type SASPs
Ionizing radiation*	DNA repair enzymes ExoA and Nfo (active during germination) Decreased level of core water Sulfur-rich spore coat proteins and DPA Increased levels of Mn^{2+} and other divalent cations
UV radiation*	UV photochemistry of DPA DNA – formation of 'spore photoproduct' Error-free repair of spore photoproducts DNA protection by a- and b-type SASPs DNA repair enzymes *ExoA* (active during germination) Specific DNA repair system for spore photoproduct
HPP	Sporulation temperature Demineralization of the spore core Ability to retain DPA

***Note:** The marked sporicidal treatments are not of the aim of this thesis, thus the marked defence mechanisms will not be discussed.

I.4.2. Germination mechanisms

When the environmental conditions are favourable for the spore germination and outgrowth, the spore dormancy is surpassed and the germination process occurs in three fundamental stages (**Figure 6A-B**) (Sella et al., 2014), as follows:

Stage I: Activation of the dormant spore induced by the presence of nutrients (germination inducers) such as low-molecular-weight amino acids, glucose, fructose and purine nucleosides that are detected by germination receptors (GR's) present in the inner membrane of the spore (Paredes-Sabja et al., 2011). According with Yi and Setlow, (2010), the germination of *B. subtilis* spores is triggered by L-alanine (that binds to the germinant receptor *gerA*), L-valine or by a combination of L-asparagine, D-glucose, D-fructose and K^+ (AGFK) that binds to the GR's (namely on the receptors *gerB* and *gerK*). This results on the release of H^+, K^+, Na^+ and Ca^{2+}, leading to an increase of the spore core pH from 6.5 to 7.7 (Sella et al., 2014). In addition, according with Paredes-Sabja et al. (2011) the

spore peptidoglycan layer is degraded by cortex lytic enzymes that are activated by the release of pyridine-2,6-dicarboxylic acid, commonly designated as dipicolinic acid (DPA) (whose concentration can reach 1 M on the spore core, representing 10 to 20% of the spore dry weight (Black et al., 2007)). The activation process do not necessarily implies germination and outgrowth of the spores, being considered a reversible process (Setlow, 2006);

Stage II: In accordance with Reineke, Mathys, and Knorr (2011), Setlow (2003), and Zhang et al. (2010), the second germination stage is characterized by the completion of cortex hydrolysis, and a rehydration of the spore core takes place, leading to the reactivation of biochemical pathways (since protein motility increases with rehydration) and loss of resistance mechanisms;

Stage III: Hydrolysis of the spore coat for further outgrowth, originating a competent vegetative cell. During this stage, the SASPs that are bound to the spore DNA (protecting it from several stress sources) are degraded.

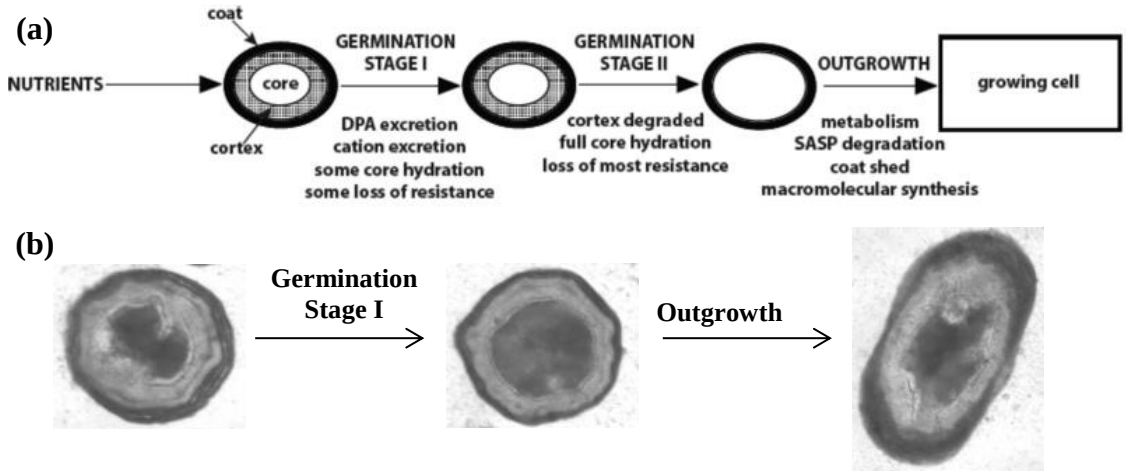


Figure 6: Schematic representation of the nutrient-induced germination (a) and transmission electron micrograph during the successive germination stages (b) of *Bacillus* spp. endospores. Adapted from Black et al. (2007).
Note: The **Figure 6b** omits the intermediate state between the germination stage II and the beginning of the outgrowth stage that is represented in **Figure 6a**.

According with Sella et al. (2014) and Zhang et al. (2010), outgrowth consists of a transition state between the germinated spore and the growing cell, being observed cellular division. In this step, the spore uses its own reserve molecules (namely 3-phosphoglycerate that is laid up on the spore core) to generate adenosine triphosphate (ATP). On the latter stages of the outgrowth process, exogenous molecules (extracellular nutrients) are mobilized for the bacterial metabolism.

Additionally, the germination process can also be triggered by non-nutrient agents such as cationic surfactants (dodecylamine), exogenous Ca-DPA, elevated pressures, specific peptidoglycan fragments, lysozyme and bryostatin (that activates *Ser*/*Thr* protein kinases) (Setlow, 2003; Shah et al., 2008; Wei et al., 2010).

I.4.3. Occurrence of endospores on foodstuff

I.4.3.1. *Bacillus* spp.

These genus of bacteria are gram-positive, aerobic (or facultative aerobes in some cases) and spore-forming microorganisms, with a rod-shaped form (Turnbull et al., 1992). As above-mentioned, *Bacillus* spp. are able to produce endospores that are ubiquitous on the biosphere, although related with food poisoning illness or spoilage when those structures germinate and outgrows, leading to the production of enzymes and toxins, resulting in economic losses and/or health issues (Witthuhn et al., 2011).

Endospores formed by the pathogenic *B. cereus* can be found in meat (Soni et al., 2016), raw (Christiansson et al., 1999), and pasteurized (Eneroth et al., 2001) milk, dairy and in farinaceous products, being its prevalence on such low acidic products an health-threat, since some strains of *B. cereus* are able to produce cereulide, an emetic toxin that destroys mitochondria, resulting in food poisoning illness (such as vomits and nausea) (Agata et al., 2002). This toxin is not destroyed by thermal pasteurization or sterilization processes, even at low (Corthouts and Michiels, 2016) and elevated pH values (Rajkovic et al., 2008), and the endospores that lead to its formation are known to be more heat-resistant than those that do not produce the toxin (Carlin et al., 2006).

Furthermore, *B. amyloliquefaciens* is a non-pathogenic spore-former bacteria commonly found on milk and dairy products, being also heat-resistant to pasteurization or even ultra-high temperature (UHT) treatments (Scheldeman et al., 2006). In fact, Huemer, Klijn, Vogelsang, and Langeveld (1998) reported that spores of *B. amyloliquefaciens* were able to survive an UHT treatment at 130 ºC for 4 s, which means that more intense processes are needed to destroy these endospores, although, by increasing the temperature and/or the time of thermal exposure of the milk would result in quality loss and, consequently, consumers acceptance due to the compounds formed as a consequence of the *Maillard* reactions, among others (Van Boekel, 1998).

I.4.3.2. *Alicyclobacillus* spp.

Alicyclobacillus acidoterrestris is a non-pathogenic, moderately thermophilic, acidophilic and spore-forming bacteria, whose occurrence in 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 bacteria, whose endospores are quite heat resistant, presenting D-values of 65.6 min at 85 ºC and 11.9 min at 91 ºC in acidic 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 et al., 1999), which are higher time/temperature binomials than those commonly employed on juice pasteurization processes by the industry before the discovery of this spore-forming bacteria on acidic juices in Germany, in 1984 (Walker and Phillips, 2008). These endospores are able to germinate and outgrowth after a heat shock (typically 86 ºC to 96 ºC for 15 seconds to 2 min (Lee et al., 2002)), 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 fruit juices (Heyndrickx, 2011), causing production of guaiacol (2-methoxyphenol) that is responsible for antiseptic off-flavours and odours (Corli Witthuhn et al., 2013; Walker and Phillips, 2008), as well as sediment deposition, cloudiness increase and discoloration in some juices (Tianli et al., 2014).

The heat-resistance of *A. acidoterrestris* endospores is intimately related with the temperature at which the sporulation process takes place, whereas lower sporulation temperatures confer lower heat-resistance. For example, Goto, Tanaka, Yamamoto, Suzuki, and Tokuda, (2007) reported that endospores prepared at 45 ºC presented D-values of 0.48 min at 110 ºC, contrarily to those sporulated at 65 ºC, whose D-value for the same temperature was 3.9 min.

There are known at least eighteen species of the genus *Alicyclobacillus* spp. that are commonly found in the beverage industry, such as *A. fastidious*, *A. acidiphilus*, and *A. herbarius*, among others (Smit et al., 2011), being all able to form endospores and whose thermal resistance is quite different from each other. For example, *A. acidocaldarius* endospores are more heat-sensible than *A. acidoterrestris*, inasmuch the first one presents D-values of 10–12 min at 86 ºC (while for *A. acidoterrestris* the D-value is 65.6 min at 85 ºC) (Wisotzkey et al., 1992).

Intrinsic parameters of the food products such as pH, water activity, brix degree, or characteristics regarding the bacterial species and strain are known to play a key-role on the thermal resistance of *A. acidoterrestris* endospores (Sinigaglia et al., 2003; Vieira et al., 2002; Walker and Phillips, 2008), since the heat-resistance decreases at lower pH and increases at higher water activities (Goto et al., 2007).

The most affected food products by *A. acidoterrestris* spoilage are pasteurized apple and orange juices, although Silva et al. (1999) reported its presence on high-acidic carbonated drinks and vegetable products, even after pasteurization, canning (sterilization) and UHT.

I.4.3.3. *Clostridium* spp.

The genus *Clostridium* is characterized by a variety of gram-positive, anaerobic spore-forming bacteria that includes both pathogenic and non-pathogenic species. A classic example of food poisoning illness is botulism, which is caused by the pathogenic microorganism *C. botulinum* that, under anaerobic conditions, produces an enterotoxin named botulinic toxin that causes muscular paralysis. This microorganism is commonly found in poorly sanitized slaughterhouse's, leading to the contamination of meat products such as chorizos, hams and other meat products (Reddy et al., 2010).

Under aerobic conditions, *C. botulinum* triggers the required sporulation mechanisms to form highly resistant endospores and when it founds once again anaerobic conditions (similarly to those found on the inside of chorizos, ham pieces or in cans) it starts to germinate and outgrows, with consequence production of the botulinic toxin (Olguín-Araneda et al., 2015).

Moreover, *C. perfringens* is commonly found on soil, sewage, food products, feces, etc. being known to be the cause of several histotoxic infections such as clostridial myonecrosis, anaerobic cellulitis, wound infections and other infection originated on both human and animal intestine (Olguín-Araneda et al., 2015).

I.4.4. Superdormant endospores

Besides the germination occurs quickly by nutrient-induction, there is a small fraction of endospores that do not undergo the germination process, being considered superdormant spores, since they require longer periods of time to germinate, or instead a heat-shock is necessary to activate its germination (Gould, 1970; Wei et al., 2010).

These superdormant endospores are a threat to the food industry, due to their resistance to the thermal processing, being able to germinate, outgrowth and originate a vegetative cell leading to food poisoning illness or food spoilage (Markland et al., 2013). According with Setlow et al. (2012), the amount of superdormant endospores can be reduced by heat activation, higher quantities of nutrient germinants targeted to a specific GR, mixtures of nutrient germinants targeting multiple GR, or by increased numbers of GR's per spore.

I.4.5. Effect of pressure on bacterial endospores

I.4.5.1. Resistance of endospores to hydrostatic pressure

The sporulation temperature and culture media where the sporulation takes place were found to play a key-role on the pressure-resistance of both *Bacillus* spp. (Checinska et al., 2015; Margosch et al., 2004; Raso et al., 1998) and *Clostridium* spp. (Lenz and Vogel, 2015; Olguín-Araneda et al., 2015) endospores, as well the mineral content and the pH of the sporulation media (Atrih and Foster, 2002; Reineke et al., 2013a). The characteristics of the food product (pH, a_W, mineral content) whereas the endospores are suspended are also to be considered when it comes to endospore resistance to HPP (Lenz and Vogel, 2015, 2014; Paredes-Sabja et al., 2007; Reddy et al., 2010).

I.4.5.2. Composition and features of the food matrix

The intrinsic characteristics of food products or matrices wherein the endospores are suspended, such as a_W and pH, are to be taking into account when it comes to endospore resistance to HPP. In fact, the pH shifts (normally to lower values than the initial one) that occurs under pressure and high temperatures seem to influence the inactivation rates of endospores (Mathys et al., 2008).

According with Black et al. (2007), acidic food products processed by HPP tend to evidence lower endospore loads, which means that such structures are more sensible to low pH when submitted to HPP, contrary to the a_W, i.e., food products with lower a_W offer a protection to endospores against HPP. Moreover, natural antimicrobials on food products can enhance the thermal inactivation of endospores (or inhibit it, as in the case of HPP as will be furtherly discussed).

I.4.5.3. Sporulation temperature

The temperature at which the sporulation takes place is a key-factor among the resistance of endospores to the inactivation procedures. It was found for *B. subtilis* endospores by Igura et al. (2003) that by decreasing the sporulation temperature, the pressure resistance increased, since the spores obtained at 30 ºC presented the highest resistance to HPP among the endospores produced at higher temperatures (37 and 44 ºC) to a treatment in a pressure range between 100 and 300 MPa at 55 ºC for 30 min. The same resistance was observed by Olivier et al. (2012) for *B. amyloliquefaciens*, *B. coagulans* and *B. sporothermodurans*, concluding that there was a significant increase on *B. amyloliquefaciens* endospores resistance to HPP inasmuch the sporulation temperature was reduced from 37 to 30 ºC, which resulted on an increase on the D-values (110 ºC, 600 MPa) from 0.58 to 4.0 min.

This behaviour is precisely the opposite regarding thermal inactivation of endospores, since higher sporulation temperatures increase their heat-resistance (Igura et al., 2003), which might be related with the increase on the number of heat shock proteins as the sporulation temperature rises (Heredia et al., 1997).

Lenz and Vogel (2015) proved that the sporulation temperature plays a key-role on the resistance of *C. botulinum* type E (a non-proteolytic, psychrophilic and a toxin producer that undergoes sporulation in certain conditions) to HPP, since when sporulation took place at low temperatures (13 ºC), there was an increased pressure resistance than those sporulated at higher temperatures (38 ºC), whereas an almost-complete inactivation of the *C. botulinum* type E strain after a pressure/temperature treatment of 800 MPa/80 ºC for 10 min, against almost 2 log CFU/mL of spore counts sporulated at 13 ºC.

I.4.5.4. Mineral content and pH of the sporulation media

Igura et al. (2003) studied the effect of the mineral content on the pressure resistance of *B. subtilis* endospores. The outcome was that after demineralization of the endospores, the pressure resistance increased. This process was reversible by the addition of Ca^{2+} and Mg^{2+} to the endospores, nevertheless, the pressure resistance did not decrease by adding Mn^{2+} or K^{+}, what, according with Reineke et al. (2013a), might be related with the activation of the endospore's cortex lytic enzymes (CLE's) by Ca^{2+} and Mg^{2+} during the germination induced by pressure.

A similar behaviour was noticed by Olivier et al. (2012) for *B. coagulans* and *B. sporothermodurans* endospores, notwithstanding it was reported that the pressure resistance of *B. amyloliquefaciens* decayed after the addition of minerals to the culture media. In contrast, the heat-resistance of endospores is known to increase with the addition of minerals to the endospore's culture media (Lenz and Vogel, 2014).

Oh and Moon (2003) evaluated the resistance of *B. cereus* to HPP as a function of the sporulation pH. The results showed that endospores prepared at pH 6.0 were more pressure-resistant than those obtained at pH 8.0 at pressures of 600 MPa (20 to 60 ºC, 15 min), unlike the endospores of *B. subtilis* that, according with Nguyen et al. (2011), when sporulated at pH 10 evidenced increased pressure-resistance when compared to those sporulated at pH 6 after treatment at 350 MPa at 40 ºC for 60 min.

I.4.5.5. Endospore strain

The resistance of several *Clostridium* spp. (such as the opportunistic pathogen *C. perfringens*) to HPP is attributed to the lack of inner membrane GR's, which represents a threat to the food industry (and, consequently, to the consumers themselves), while those species that endue the inner membrane GR's are suitable to be inactivated by HPP, although by different mechanisms, depending on the applied pressure (Doona et al., 2016). Moreover, according with Reineke et al. (2013a), as high pressure decreases the fluidity of microbial membranes, the inner spore membrane (of endospores) might keep its fluidity even under high pressure, acting as a barrier against HPP, thus resisting to such treatment.

I.4.5.6. Antimicrobials (chemicals and enzymes)

Certain compounds as carvacrol were reported to inhibit the inactivation of endospores by HPP (combined with high temperatures), while it stimulates the germination at high temperatures (50 to 60 ºC) at atmospheric pressure (AP), suggesting that carvacrol strongly suppressed the physiological germination during HPP (at temperatures lower than 65 ºC) (Luu-Thi et al., 2015), as well chemical compounds containing Hg^{2+} are known to inhibit the release of DPA of the endospore core (both pressure-induced and nutrient-induced germinations).

Regardless of the use of mild heat, or even high temperatures (such as in pressure-assisted thermal sterilization) to destroy endospores, several studies reported the possibility of using chemical agents and/or to enhance its inactivation by HPP (Sarker et al., 2015;

Soni et al., 2016), while the same effect might not be verified when enzymes are used as antimicrobials. For example, Sokołowska et al. (2012) reported that the inactivation ratio of *A. acidoterrestris* by HPP at 300 MPa at 50 ºC for 30 min in apple juice supplemented with lysozyme was practically the same for the same treatment without lysozyme supplementation (independently of the lysozyme concentration). López-Pedemonte et al. (2003) reported that the inactivation of *B. cereus* endospores by HPP was not enhanced by the addition of lysozyme, suggesting that the feasibility of lysozyme against endospores is not as successful as for vegetative microorganisms.

I.4.6. Pressure-induced germination of endospores

According with Reineke et al. (2012), pressure is a suitable stimulus to trigger the germination and/or inactivation of endospores by different pathways, varying with the pressure and temperature employed on the matrix. Reineke et al. (2013a) and Black et al. (2007) reviewed how different pressure ranges are able to induce non-nutrient germination, that can be divided in two kinds of germination: (1) nutrient-like physiological germination, whereas the germination is induced not by presence of nutrients but by the fact that pressure triggers the spore nutrient receptors; (2) non-physiological germination process, which occurs at elevated pressures than in nutrient-like physiological germination, where a release of DPA from the spore core takes place by direct opening of the spore DPA-channels, as a result of structural changes on the spore membrane proteins enhanced by high pressure.

At low pressures (<150 MPa), the nutrient-like physiological germination pathway seems to be predominant, while it is stipulated that the germination of spores between 100 and 200 MPa is more likely to be a non-physiological germination, being the germination rates inversely proportional to the pressure applied (the higher the pressure level, the lower the nutrient-like physiological germination rate and vice-versa). Moreover, it seems that the endospores have different sensibilities and respond (according with the species) to the same pressure level and an overlap of the germination mechanisms is common for different species subjected at the same pressure level (Reineke et al., 2012).

Thus, Reineke et al. (2013a) also stated how germination can be induced differently according with the pressure at low (up to 400 MPa), moderate (up to 600 MPa) and elevated (>600 MPa) pressures. Thus, the following sections aim the compilation of the

studies concerning the effect of different pressure levels on bacterial spores, both solely germinated under pressure and pressure-induced germination with further processing. It is opportune to point out that the concept of low, moderate, high and very high pressures varies between the authors, thus it was defined on the aim of this thesis that low pressures are up to 300 MPa, high pressures at 600 MPa and very high pressures are to be higher than 600 MPa.

I.4.6.1. Germination induced by low hydrostatic pressure (<300 MPa)

As reviewed by Black et al. (2007), HPP is suitable to trigger the germination of endospores, stating that is the main reason why HPP can result on endospores inactivation. In fact, several authors reported that low pressures (up to 300 MPa) are able to induce germination of endospores, making them more sensitive to subsequent high pressure treatments combined (or not) with temperature. A special focus will be given to the germination triggered by low hydrostatic pressure (also known as nutrient-like induced germination) since it includes the range of pressures employed in HS studies (up to 220 MPa).

According with Reineke et al. (2011), pressures below 300 MPa are suitable to induce the germination of *Bacillus* spp. due to the activation of some inner membrane GR's, depending on the pressure-holding time and the applied temperature, even on the absence of nutrients. For instance, Paidhungat et al. (2002) studied the germination mechanisms of *B. subtilis* at 100 and 550 MPa, and found out that after 30 min at 100 MPa and 23 ºC, the germination was induced by the activation of the GR's on the inner membrane.

A structural analysis of *B. subtilis* endospores suspended on N-(2-acetamido)-2-aminoethanesulfonic acid (ACES) buffer, submitted to a pressure level of 150 MPa for 90 min at 30 ºC resulted on the release of DPA and further activation of the CLE's that were responsible for the reduction on the thickness of the peptidoglycan layer of the endospores (**Figure 7**), proving that nutrient-like physiological germination shares common steps with the nutrient-induced germination (Reineke et al., 2013).

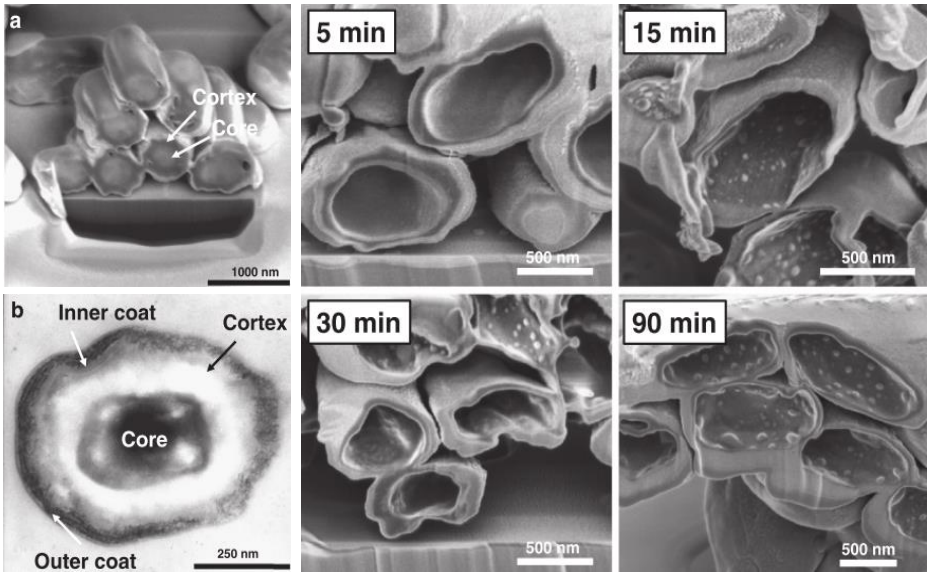


Figure 7: On the left (a-b), *B. subtilis* endospores aspect before the experiments. On the right, focused ion beam sectioned *B. subtilis* endospores after a pressure treatment at 150 MPa at 37 ºC for 5, 15, 30 and 90 min. Adapted from Reineke et al. (2013).

When it comes to *Clostridium* spp., the same phenomenon might not be verified, since Doona et al. (2016) reported that at 150 MPa and 37 ºC for 20 min resulted in less than 4% of *C. difficile* becoming dark on phase contrast microscope, meaning that germination was slightly induced but not completed, whereas there was only a small release of dipicolinic acid from the spore core, although, Reineke et al. (2011) and Sokołowska et al. (2015) suggested that the germination rate induced by hydrostatic pressure is inversely proportional to the employed pressure level, i.e., lower pressure levels tend to stimulate more the germination than elevated pressure levels, as aforesaid.

A compilation of studies regarding the induction of germination at low pressures is presented in the **Table 5** for several *Bacillus* and *Clostridium* spp, describing the outcomes of each pressure/temperature/time treatment.

In coherence with Shigeta et al. (2007), the highest germination rates were found at a temperature of 40 ºC for all the studied microorganisms (*B. subtilis, B. cereus* and *B. polymyxa*), even though this study was performed at a range of temperatures between 30 and 80 ºC, suggesting that, from a certain temperature (above 40 ºC – thought to be the optimal temperature of CLE's), the germination does not occur, or is less extended than at lower temperatures, even when the endospores are subjected to low pressures to induce germination, which means that low pressure-induced germination can be overcome at temperatures higher than the optimal temperature of the CLE's, what was also found by Aoyama et al. (2005a) for several *Bacillus* spp. endospores.

One of the strategies to attempt higher spore counts reductions relies on oscillatory pressure treatments, consisting on the application of mild pressure levels and temperatures for short periods of time to trigger the germination process, followed by intense pressure exposure (Black et al., 2007). Gola et al. (1996) reported 4 log CFU/mL of *B. stearothermophilus* in phosphate buffer after a double-pulse treatment of 200 MPa at 20 ºC for 1 min and then at 900 MPa at 20 ºC for 1 min, while for *B. cereus*, an inactivation strategy consisting on a pressure exposure of 60 MPa at 20 ºC for 230 min and then a pressure increment to 300 and 400 MPa at 30 ºC for 15 min, resulted in 2.4 log CFU/mL of spores inactivation (López-Pedemonte et al., 2003).

Table 5: Compilation of studies regarding the induction of the germination of endospores by hydrostatic pressure up to 300 MPa.

Microorganism	Optimum germination conditions[1]		Matrix	Reference
	Conditions	Outcomes		
B. stearothermophilus	60 MPa/40 ºC/12 h	Reduction of about 1 log CFU/mL on the spore counts	Phosphate buffer	Furukawa et al. (2001)
	40 and 50 MPa/55 ºC/4 h	Reduction of about 1.72 and 2.1 log CFU/mL on the spore counts at 40 and 50 MPa, respectively	Gifu anaerobic broth (GAM broth)	Aoyama et al. (2004)
	50 MPa/55 ºC/ 48 h	Reduction of about 2.7 log CFU/mL on the total spore counts	Minced anchovies	
B. coagulans	40 and 50 MPa/55 ºC/4 h	Reduction of about 1.0 log CFU/mL of the total spore counts at 50 MPa	GAM broth	Aoyama et al. (2004)
	60 MPa/55 ºC/48 h	Reduction of about 1.0 log CFU/mL on the spore counts	Minced anchovies	
	100-300 MPa/40 ºC/10 min	Spore germination up to 2 log CFU/mL observed on both matrixes	Citric acid and phosphate buffers	Vercammen et al. (2012)
	60 MPa/40 ºC/24 h	Reduction of about 5.8 log CFU/mL	GAM broth	Aoyama et al. (2005a)
B. cereus	60 MPa/30 ºC/3.5 h	Lethality of about 1.7 log (N_0/N)	Curd cheese	López-Pedemonte et al. (2003)
	20-100 MPa/40 ºC/1 h	Germination ratio up to 5 log CFU/mL and inactivation ratio of almost 2 log CFU/mL at 100 MPa	Glucose broth	Shigeta et al. (2007)
	20-100 MPa/40 ºC/1 h	Germination ratio up to 4 log CFU/mL and inactivation of about 2 log CFU/mL at 100 MPa	Phosphate buffer	
	60 MPa/40 ºC/24 h	Reduction of about 5.4 log CFU/mL	GAM broth	Aoyama et al. (2005a)

[1] In each case, optimum germination conditions refer to the cases whereas the maximal germination and/or inactivation was found at pressure levels up to 300 MPa.

Table 5: Compilation of studies regarding the induction of the germination of endospores by hydrostatic pressure up to 300 MPa (continued).

Microorganism	Optimum germination conditions[1]		Matrix	Reference
	Conditions	Outcomes		
B. subtilis	100-600 MPa/40 ºC/30 min	Fraction of survivors was minimal after 200 MPa treatment	Potassium phosphate buffer	Wuytack et al. (1998)
	40-60 MPa/40 ºC/4 h	Pronounced reduction at 60 MPa (≈ 5.7 log CFU/mL) of the total spore count	GAM broth	Aoyama et al. (2004)
	60 MPa/40 ºC/48 h	Reduction >4.7 log CFU/mL of the total spore counts	Minced anchovies	
	60 MPa/40 ºC/24 h	Reduction of about 5.0 log CFU/mL of spore counts	GAM broth	Aoyama et al. 2005b)
	80 MPa/60 ºC/2 h	Reduction of about 1.0 log-cycle on the spore counts	Phosphate buffer	
		Three log-cycle reduction of spore counts, almost all spores were phase-dark under phase contrast microscopy	Glucose broth	
	100 MPa/40–60 ºC/1 h	Spores germinated approximately 4 and 1.5 log-cycles at 40 and 60 ºC, respectively	Glucose broth	
	50–300 MPa/40-60 ºC/30 min	Germination ratio of 0.95 log-cycles at 100 MPa and 40 ºC and 0.85 log-cycles at 100 MPa and 60 ºC		
	50–300 MPa/40-60 ºC/30 min	Germination ratio of 1.7 log-cycles/100 MPa at 40 ºC and 1.4 log-cycles/100 MPa at 60 ºC	Phosphate buffer	
	20–100 MPa/40 ºC/60 min	Germination ratio up to 5 log CFU/mL and no significant inactivation of spores	Glucose broth	Shigeta et al. (2007)
	20–100 MPa/40 ºC/60 min	Germination ratio up to 4 log CFU/mL and no significant inactivation of spores	Phosphate buffer	
	80 MPa/38 ºC/5 h	Reduction of almost 5 log CFU/mL on spore counts	McIlvaine buffer	Obaidat et al. (2015)

[1] In each case, optimum germination conditions refer to the cases whereas the maximal germination and/or inactivation was found at pressure levels up to 300 MPa.

Table 5: Compilation of studies regarding the induction of the germination of endospores by hydrostatic pressure up to 300 MPa (continued).

Microorganism	Optimum germination conditions[1]		Matrix	Reference
	Conditions	Outcomes		
B. lincheformis	60 MPa/40 ºC/24 h	Reduction of about 5.9 log CFU/mL on the total spore counts	GAM broth	Aoyama et al. (2005a)
A. acidoterrestris	100–500 MPa/50 ºC/20 min	Germination and inactivation levels reached 3.75 and 2.02 log CFU/mL at 200 MPa (similarly to 300 MPa)	McIlvaine buffer	Sokołowska et al. (2015)
		Pronounced germination at a pressure range of 100-300 MPa, no significant inactivation on such pressure range		
		Maximum germination and inactivation at 200 MPa (of about 3.59 and 1.95 log CFU/mL, respectively)		
	200 MPa/20–50 ºC/30 min	Germination and inactivation of about 2.04 and 0.65 log CFU/mL, respectively at 20 ºC and 4.06 and 2.76 log CFU/mL, respectively at 50 ºC	Commercial apple juice	
C. sporogenes	40/50 MPa/30 ºC/4 h	Reduction of about 0.50 log CFU/mL on the total spore count	GAM broth	Aoyama et al. (2004)
	50 MPa/30 ºC/48 h	Decrease of about 0.60 log CFU/mL on the total spore count	Minced anchovies	

[1] In each case, optimum germination conditions refer to the cases whereas the maximal germination and/or inactivation was found at pressure levels up to 300 MPa.

I.4.6.2. Germination induced by high hydrostatic pressure (up to 600 MPa)

Also known as non-physiological germination, the germination triggered by elevated hydrostatic pressures seems to be quite different from nutrient- and nutrient-like physiological induced germinations, since non-physiological germination induces the endospore germination by bypassing the individual germination steps aforementioned (Reineke et al., 2013a).

The increase of the processing temperature at high pressures enhances the inactivation rates of endospores on food products when compared to the same processing conditions at RT. For example, Moerman, Mertens, Demey, and Huyghebaert (2001) found out that a temperature increment of 30 ºC on the initial processing conditions (from 20 to 50 ºC and 400 MPa for 30 min) led to an inactivation of almost 4 log CFU/mL of two *Bacillus* spp. spores in minced pork, similarly to *C. sporogenes*, in which a temperature increase on the processing conditions (from 20 to 60 ºC and 400 MPa for 30 min) resulted in a reduction of about 3 log CFU/mL in distilled water.

A structural analysis of *B. subtilis* endospores performed by Reineke et al. (2013) revealed a porous network in the spore core (**Figure 8**) after a treatment at 550 MPa at 37 ºC for 60 min, suggesting that the spores submitted at such pressure levels and moderate temperatures were able to release DPA and degrade their cortex, but not their SASPs (that are degraded during nutrient-induced germination), which is attributed to an inactivation of the germination protease (Gpr), being essential for a rapid degradation of SASP.

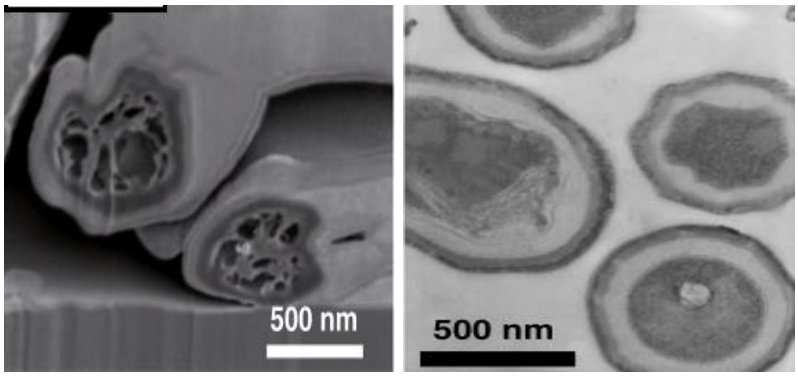


Figure 8: Focused ion beam sectioned scanning electron microscopy and transmission electron microscopy images of *B. subtilis* endospores after a pressure treatment at 550 MPa and 37 ºC for 60 min. Adapted from Reineke et al. (2013).

At high hydrostatic pressures, it was also proved by Paidhungat et al. (2002) that the germination of *B. subtilis* endospores lacking the majority of the GR's submitted to 550

MPa and 40 ºC for 30 min was due to the direct opening of the DPA-channels located on the spore's inner membrane, with DPA release that led to the later stages of the germination steps, similarly to a germination assay with a mutant strain of *B. subtilis* endospores lacking the nutrient germination receptors performed at 200 MPa at temperatures lower than 50 ºC for 30 min, resulting in the release of DPA (Reineke et al., 2011). This means that high hydrostatic pressure is more likely to trigger the cortex lysis (Black et al., 2007), once DPA activates the CLE's (namely CwlJ, which is more responsive to DPA) (Moir, 2006).

In short, non-physiological germination is retarded at pressures below 200 MPa (with few exceptions) and it is dominant in a pressure range of 300 to 600 MPa at temperatures below 60 ºC. At these conditions, non-physiological germination result in Ca-DPA complex release from the spore core, with partial hydration of such compartment. A full and complete core hydration though is retarded in a pressure range of 400 to 600 MPa and temperatures below 50 ºC, possibly to the inactivation of the Gpr. This intermediate state of partial core hydration can be surpassed and an extended inactivation can be achieved if the employed temperature increases, leading to spore inactivation (Black et al., 2007; Reineke et al., 2013a).

I.4.6.3. Germination induced by very high pressure (>600 MPa)

When performed at moderate temperatures (<60 ºC) and pressures higher than 600 MPa, the endospore response seem to share common steps with the non-physiological germination performed at 400-600 MPa at temperatures below 60 ºC.

Attempts to destroy *B. coagulans* endospores in tomato sauce (pH 4.2 and 5.0, at pressures up to 800 MPa at temperatures of 25, 40 and 60 ºC for 10 min) were made by Vercammen et al. (2012). After processing at 800 MPa at both 25 and 40 ºC, the microbial counts reached similar levels to those submitted at lower pressures (100 to 700 MPa) for both pH's, being only verified higher inactivation rates at 60 ºC for pressures higher than 600 MPa, also for both pHs.

As regards to *Clostridium* spp. endospore's response under very high pressures, Reddy et al. (1999) revealed that no germination was observed for *C. botulinum* type E (Alaska and Beluga strains) in phosphate buffer (0.067 M at pH 7.0) after a pressure treatment of 827 MPa for 5 min at temperatures below 35 ºC, although, by increasing the

processing pressure and temperature, it was achieved Alaska's spore count reductions of about 5 log CFU/mL after 10 min of processing at 40 ºC and 5 log CFU/mL of reduction for both Alaska and Beluga spore counts at 827 MPa for 5 min by increasing the processing temperature from 35 ºC to 50 and 55 ºC, respectively. The results proved that the processing time and temperature have an amplified effect when it comes to endospore germination and inactivation at very high pressures, which is relevant for processing design and modelling to reach commercial sterilization or, at least, acceptable microbial levels to ensure food safety.

I.5. Objectives

As aforesaid, the conventional RF processes are known to have an inhibitory effect on the germination and outgrowth of endospores for a certain period of time, being the product shelf-life determined by the processing technique, as well by its intrinsic pH and $a_{W,}$ along with other factors. As HS/RT has been proposed as a new preservation methodology to replace the RF processes, it is of upmost importance its feasibility evaluation on endospores to validate it is a safe preservation technique.

Thus, this thesis aims the study of HS/RT as a safe alternative to the cold (1-7 ºC) storage techniques for food preservation when it comes to endospores, using *B. subtilis* and *A. acidoterrestris*, which were inoculated on three nutritionally different matrices (McIlvaine buffer, carrot and commercial apple juices, and BHI-broth), due to the scarcity of data on the literature concerning the behaviour of such biological structures at HS conditions, as well to give a first insight on the matrix influence on the endospore behaviour at such conditions.

To fulfil the aforementioned goals, microbiological analyses were performed for all the matrices inoculated with both endospores.

I.6. Chosen case-studies

I.6.1. Endospores

The *B. subtilis* endospores were chosen since they are the most studied endospores and information regarding their behaviour and response under pressure is available on the literature, being also associated with food spoilage. *A. acidoterrestris* endospores were chosen due to fact of being related with spoilage of acidic fruit juices, resulting in losses for the food industry.

I.6.2. Matrices

Nutritionally distinguishable inoculation matrices were chosen for each endospore, as summarized in the **Table 6**.

Table 6: Chosen inoculation matrix/matrices for each studied endospores.

Microorganism	Inoculation matrix
B. subtilis ATCC 6633	McIlvaine buffer
	Carrot juice
	Brain-heart infusion broth
A. acidoterrestris ATCC 49025	Commercial apple juice

Citrate-phosphate buffer, also known as McIlvaine buffer, consists of different proportions, according with the desired final pH, of stock solutions of citric acid and disodium hydrogen phosphate, having a large range of pH, from 2.2 to 8.0 (McIlvaine, 1921). In fact, that was the main reason why this buffer was chosen to be inoculated with *B. subtilis* endospores, along with the availability of literature regarding the behaviour of this endospore under high hydrostatic pressure, namely on inactivation studies (Obaidat et al., 2015). The fact that McIlvaine buffer is a nutrient-free matrix that cannot unleash the nutrient-induced germination pathways of endospores (Reineke et al., 2013a), allowed to evaluate the effect of the low pressures (the ones used on HS) on such biological structures, in order to infer if the composition of the inoculation matrix could influence the endospore behaviour at different storage conditions.

Raw carrot (*Daucus carota* subsp. *Sativus*) juice is a common example of a highly perishable juice (due to its high water content and pH close to neutral values) that does not hurdle the microbial development, resulting in quick spoilage, even at RF conditions, representing a worst-case scenario on food preservation (Aneja et al., 2014). These were the main reasons for this product selection as a case-study to evaluate the HS effect on *B. subtilis* endospores. The common nutritional composition of raw carrot is summarized in the **Table A1** (**Appendix A**).

BHI-broth is a general, non-specific liquid culture media containing brains and hearts infusions of cow and porcine that, when supplemented with yeast extract or sodium chloride, becomes even richer for bacteria cultivation. In fact, the brain and heart infusions,

along with the peptones, are important carbon and nitrogen sources (Merck, 1996). Due to its very rich nutritional content, it was chosen as an inoculation matrix to evaluate if the matrix composition influenced the endospore behaviour under HS conditions. The BHI-broth composition used in this master thesis is summarized in the **Table A2** (**Appendix A**).

Apple (*Malus domestica*) juice is a typical example of acidic fruit juice, widely consumed worldwide. As *A. acidoterrestris* are frequently found on acidic fruit juices (Tianli et al., 2014) such as apple juice, this product was chosen as a case-study in order to understand the behaviour of this endospore under HS conditions and acidic pH (3.50 ± 0.01). The nutritional content of the apple juice is summarized on **Table A3**, at the **Appendix A**).

I.7. Schedule

The development of this thesis consisted, generally, on three distinguishable stages, as illustrated in **Figure 9**.

- **September to October of 2016:** Literature review regarding the concept of HS and endospores was performed in the aim of the curricular unit "Seminar in Biotechnology";

- **October of 2016 to February of 2017:** Experimental work regarding *B. subtilis* endospores were made, namely microbiological analyses and attempts to quantify dipicolinic acid for each chosen matrix;

- **March to July of 2017:** Experimental work aiming *A. acidoterrestris* endospores took place, namely microbiological analyses. Elaboration of a research note regarding the results obtained with the *B. subtilis* endospores and collaboration on a review article concerning the HS concept.

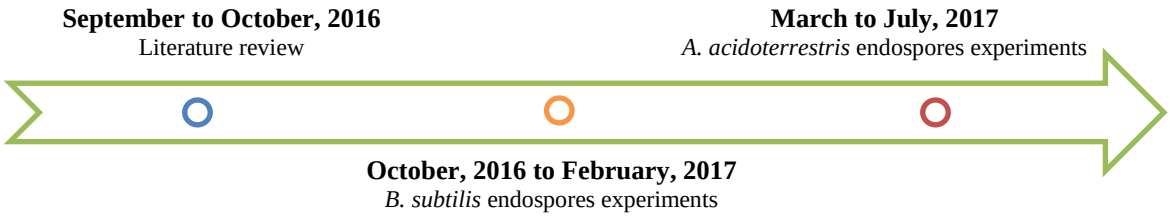


Figure 9: Used schedule on the organization of the thesis inherent work (timeline not on scale).

Chapter II – Materials and methods

THIS SECTION COMPRISES A DETAILED DESCRIPTION OF ALL THE METHODOLOGIES EMPLOYED ON THIS WORK, SUCH AS MICROBIOLOGICAL AND PHYSICOCHEMICAL ANALYSES.

I.1. Culture media and reagents

Physiological solution (0.9% NaCl), citric acid, *Bacillus acidophilus* agar (BAT-agar), potato dextrose broth (PDB), potato dextrose agar (PDA) and plate count agar (PCA) were purchased from Applichem Panreac (Darmstadt, Germany), brain-heart infusion (BHI) broth and BHI agar were obtained from Oxoid (Cheshire, United Kingdom), sodium phosphate dibasic was purchased from Riedel-de Haën (Seelze, Germany), dipicolinic acid (DPA), terbium-chloride hexahydrate and sulphuric acid was bought from Sigma-Aldrich (Seelze, Germany), and N-(2-acetamido)-2-aminoethanesulfonic acid (ACES) buffer was purchased from Biochem Chemopharma (Cosne-Cours-sur-Loire, France).

I.2. Matrices preparation

I.2.1. McIlvaine buffer

The McIlvaine citrate-phosphate buffer (0.2 M of Na_2HPO_4 and 0.1 M of citric acid) at pH 6.00 was prepared as proposed by McIlvaine (1921).

I.2.2. Carrot juice

Fresh carrots (*Daucus carota* subsp. *Sativus*) were purchased at a local supermarket. Then, the carrots were washed with distilled water to remove dust and other adhered particles and cut in small pieces that were crushed with a blender (for each 150 g of carrots, 300 mL of distilled water were added). The juice was then filtered with a cotton filter to remove coarse particles, stored in plastic containers and then frozen at -45 ºC until use. Afterwards, the juice was heat-sterilized on the autoclave at 121.1 ºC for 15 min.

I.2.3. Brain-heart infusion broth

BHI-broth was prepared according to the instructions provided by the supplier. For each 37 g of BHI-broth powder, 1 L of distilled water was added. Then, it was heat-sterilized on the autoclave at 121.1 ºC for 15 min.

I.2.4. Commercial apple juice

Commercial apple juice (UHT, **Figure A1** – **Appendix A**) was purchased at a local supermarket (Jumbo, Auchan Portugal Hipermercados, S.A), whose nutritional specifications are described in the **Table A3** (**Appendix A**)

The commercial apple juice was not sterilized before the inoculations, since it is an UHT acidic fruit juice, and preliminary microbiological analyses on general (PCA and BHI-agar) and specific (BAT-agar) culture media (for *A. acidoterrestris*) were unable to detect microbial counts.

I.3. pH and water activity

As the main purpose of this study concerns the HS evaluation on endospores, and as both a_W (Sevenich et al., 2015) and pH (Black et al., 2007; Reineke et al., 2013a) are known to influence endospore behaviour under pressure**,** the pH of both carrot juice and BHI-broth were adjusted to 6.00 with filter-sterilized citric acid (0.1 M), while the a_W was just measured with a hygrometer (Lab Swift – a_W, Novasina AG, Switzerland) and the values are displayed in the **Appendix A**. The pH of the commercial apple juice was not adjusted once *A. acidoterrestris* endospores are common on such matrices on the range of acidic pH. The pH of all matrices was measured at 25 ºC with a properly calibrated glass electrode (pH electrode 50 14, Crison Instruments, S.A., Spain).

I.4. Endospore preparation

I.4.1. *B. subtilis* endospores

The *B. subtilis* endospores were prepared as proposed by Reineke et al. (2013), with minor modifications. *B. subtilis* ATCC 6633 (DSM-347), purchased from *Deutsche Sammlung von Mikroorganismen und Zellkulturen* (DSMZ, Braunschweig, Germany), was grown in BHI-agar at 30 ºC for 24 h. Then, a single colony was isolated to obtain an overnight liquid culture (in BHI-broth, kept at 30 ºC with shaking at 150 rpm). Hereafter, the liquid culture was aseptically spread-plated (0.1 mL) onto BHI-agar plates and incubated at 30 ºC for 24 h. The sporulation process was verified by phase-contrast microscopy, and it took 15 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, following washed three times with cold, sterile distilled water by centrifugation (10 min at 5,000 ×*g* at 4 ºC). The washed endospores were stored in distilled water and kept in the dark at 4 ºC until use.

I.4.2. *A. acidoterrestris* endospores

A. acidoterrestris ATCC 49025 (DSM-3922) was obtained from DSMZ. This microorganism was grown in *Bacillus acidoterrestris* agar (BAT-agar) at 45 ºC for 5 days, 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 routinely checking of the sporulation state of the bacteria, taking 20 days to achieve more than 95% of bright-phase endospores. Then, the endospores were harvested as described for the *B. subtilis* endospores. The washed endospores were kept in the dark at 4 ºC in sterile distilled water until use.

I.5. Endospore inoculations

After sterilization, 2.7 mL of each matrix were aseptically placed in UV-light sterilized, low permeability polyamide–polyethylene, bags (PA/PE-90, Plásticos Macar – Indústria de plásticos Lda., Palmeiras, Portugal) (8.0 x 2.5 cm), using a laminar flow cabinet (BioSafety Cabinet Telstar Bio II Advance, Terrassa, Spain) to avoid contaminations. Then, 300 µL of *B. subtilis* endospore suspension was inoculated in McIlvaine buffer, carrot juice and BHI-broth , at a concentration of about 10^6-10^7 cells/mL, while the *A. acidoterrestris* endospores were inoculated in commercial apple juice, at a concentration of about 10^4 cells/mL

The endospores used in this thesis were not heat-treated to avoid changes on its pressure resistance in order to simulate the worst-case scenario on food preservation (low acidic and elevated a_W matrices containing non-heat-treated endospores that are known to be more pressure-resistant than those heat-treated) (Vercammen et al., 2012).

I.6. Storage conditions

The storage experiments were carried out at 25, 50 100 MPa for 60 days at naturally variable/uncontrolled RT (18-23 ºC), as summarized in the **Table 7**, using a high pressure equipment, using a mixture of propylene glycol and water (40:60 v/v) as the pressurization fluid. Simultaneously, two control samples were kept at AP and RT (AP/RT) and at RF (4 ºC), submersed in the same pressurization fluid and kept in the dark.

Table 7: Schematization of the storage conditions according with the typology of matrix.

	Matrix	Storage conditions	Storage period (up to)
Low acidic matrices (pH 6.00)	Carrot juice McIlvaine buffer BHI-broth	AP: 4 and 18-23 ºC (RT) HS: 25*, 50 and 100 MPa	60 days
Acidic matrix (pH 3.50)	Commercial apple juice	AP: 4 and 18-23 ºC (RT) HS: 25, 50 and 100 MPa	30 days

(*) The HS/RT experiments at 25 MPa were only performed in carrot juice

I.7. Determination of endospore germination and inactivation

To assess both germinated and ungerminated endospores after each storage condition, an aliquot of each matrix (non-heated samples, containing both vegetative and endospores, since germination and outgrowth might have occurred) was heated at 80 ºC for 20 min to inactivate the vegetative bacteria. Then, decimal dilutions were performed (1.0 mL of each sample for 9.0 mL of physiological solution, 0.9% NaCl) that were plated in BHI-agar and incubated at 30 ºC for 24 h.

As regards to *A. acidoterrestris* endospores, the procedure used to infer the endospore germination and inactivation was similar to that reported by Porębska et al. (2016), with minor modifications. An aliquot of each matrix was heat-treated at 80 ºC for 20 min to inactivate the vegetative bacteria, and then 100 µL were spread-plated onto BAT-agar and incubated at 45 ºC for 5 days. The preparation of each culture media is summarized in the **Table C1** (**Appendix C**).

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 quantification limit of 2.00 log CFU/mL was established. In BHI-agar media, the plates were considered as countable in a range of 10-300 colonies, while in BAT-agar media were in a range of 1-150 colonies. The microbial counts were calculated according with the **Equation 1**, as described on the ISO 4833, (2003):

$$N = \frac{\sum characteristic\ colonies}{V[(n_1+0.1\times n_2)\times d]} \qquad \textbf{(Equation 1)}$$

wherein *N* represents the number of colonies, *V* represents the volume of sample (1 mL on BHI-agar and 100 μL on BAT-agar), n_1 and n_2 refer to the number of plates countable on the first and on the second dilutions and *d* refers to the first countable dilution.

I.8. Dipicolinic acid release from the endospore core

In order to quantify the amount of dipicolinic acid released from the endospore core (as a measure of the amount of germinated endospores), the fluorimetric-based procedures proposed by Rosen et al. (1997) and Pellegrino et al. (1998) were employed. These methodologies state that the dipicolinic acid can be detected and quantified by the complex formed between Tb^{3+} and dipicolinic acid, since when this complex is excited at 270 nm, it emits fluorescence that is measured at 545 nm.

Thus, after each storage condition, 1.0 mL of each sample containing endospores was added to 9.0 mL of a solution consisting of TRIS-HCl-$TbCl_3$ previously filtered-sterilized (with a 0.20 μm cellulose-acetate membrane filter). This solution consisted of 50 mM of Tris-HCl buffer (pH 7.0) and 30 μM of $TbCl_3$. The fluorescent intensities were measured with a fluorescence spectrophotometer (Hitachi F-2000, Hitachi, San Jose, CA, USA) at an excitation wavelength of 270 nm (which is where the maximum absorption is observed) and the emission wavelength was scanned with a width of 2 nm, at a scan speed of 60 nm/min. The maximal emission intensity was measured at a wavelength of 545 nm. The amount of DPA released from the endospore core was assayed from stock solutions of DPA (in a range of concentrations from 0 to 60 μM) prepared in ACES buffer (0.05 M, pH 7.00) that allowed to obtain a standard curve (DPA concentration *vs.* fluorescence intensity), as shown in the **Figure D1** (**Appendix D**). Ultra-pure water was used as solvent on all the prepared solutions.

I.9. 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.

Chapter III – Results and discussion (*B. subtilis*)

THIS SECTION REPORTS ALL THE OBTAINED RESULTS REGARDING *B. SUBTILIS* ENDOSPORES INOCULATED IN THREE NUTRITIONALLY DIFFERENT MATRICES.

III.1. Microbiological analyses

The main focus of this thesis was to evaluate the effect of HS/RT on *B. subtilis* endospores (and on *A. acidoterrestris*, which will be discussed in the **Chapter V**) and to infer the inoculation matrix (low acidic matrices) influence on the behaviour of *B. subtilis* endospores at HS/RT conditions.

Thus, in this section, the results will be presented and discussed in increasing order of nutritional richness of the inoculation matrices, as follows: McIlvaine buffer; carrot juice; BHI-broth.

III.1.1. McIlvaine buffer

As the McIlvaine buffer is a nutrient-free matrix, the endospore germination and outgrowth processes induced by nutrients is less likely to occur, for so, in this section, the microbial counts on unheated and heated samples will be referred as total microbial load (TML) and total endospore load (TEL), respectively, despite the statistical similarities ($p>0.05$) found between unheated (TML) and heated (TEL) samples (for the same storage period) suggesting that in both cases there were only endospores on this matrix.

At large, samples kept at AP/RT did not undergo statistically significant ($p>0.05$) changes on both heated and unheated samples along the 9 days of storage (**Figure 10**), when compared to the initial load. Further analyses regarding AP/RT storage conditions did not take place, since McIlvaine buffer is a nutrient-free matrix, in which endospore germination (and further outgrowth) induced by nutrients is less likely to occur, as observed during the 9 days of storage experiments at the aforesaid condition.

Regarding AP/RF samples, both TML and TEL loads slightly evidenced an undefined behaviour along storage. Although, globally, there were no significant differences ($p>0.05$) between unheated and heated samples, with few exceptions (**Figure 10**), as expected, due to the lack of nutrients, as aforesaid.

HS/RT at 50 MPa performed similarly to AP/RF maintaining both TML and TEL loads, at least at the 2^{nd} day of storage experiments, wherein statistical similarities ($p>0.05$) were observed between conditions and storage periods. An extension of the storage experiments to 9 days resulted in TEL loads increment ($p>0.05$) of about 0.14 log units. Then, the TML load decreased ($p<0.05$) more pronouncedly (of about 1.76 log units), and fitted a zero order inactivation kinetics from the 5^{th} to the 60^{th} day of HS ($\text{Log } (N/N_0) =$

0.0359*(storage period (days) + 0.4577, R^2 = 0.9859), as seen in **Figure E1 (a-b)** (**Appendix E**).

In what concerns heated samples, the TEL loads did not varied (with few exceptions), at least up to the 9th day at 50 MPa. From that day forward, the TEL load on heated samples consecutively decreased ($p<0.05$) and followed a zero order kinetics (Log (N/N_0) = -0.0325*(storage period (days)) + 0.2852, R^2 = 0.9928), being reduced of about 1.64 log units by the 60th day.

HS/RT at 100 MPa yielded a remarkable TML and TEL load reductions along storage, which were more pronounced than at 50 MPa. One day of HS/RT resulted in a similar ($p>0.05$) TML and TEL load reduction of 0.70 and 0.84 log units, respectively, when compared to the initial values ($p<0.05$), which fitted a linear trend, up to at least the 5th day of storage, for both unheated (TML) (Log (N/N_0) = -0.3134*(storage period (days)) + 0.2128, R^2 = 0.9333) and heated (TEL) samples ((Log (N/N_0) = -0.3276*(storage period (days)) + 0.2852, R^2 = 0.8632), respectively, as seen in the **Figure E (c-d)** (**Appendix E**). The inactivation rates observed at 100 MPa slowed down from the 5th to the 9th day of storage, presenting a TML and TEL loads decreases of 1.69 and 1.81 log units at the 5th day, and 1.76 and 1.68 log units at the 9th day, respectively, which are statistically similar ($p>0.05$).

On the subsequent days, the inactivation rate slowed down even more, being the TML and TEL loads on both unheated and heated samples practically the same ($p>0.05$) on the remaining days of storage (except at the 60th day for unheated samples, whose TML was statistically different ($p<0.05$) from those observed on the 20th and 30th days).

Contrarily to HS/RT at 100 MPa, at 50 MPa the endospore loads were less affected by hydrostatic pressure, presenting a quite similar evolution throughout storage comparable with the conventional AP/RF storage, while at 100 MPa was more evident endospore inactivation throughout storage, which means that, for a nutrient-matrix as buffers, a storage pressure of at least 50 MPa should be set to perform HS/RT instead of AP/RF, although, more research is needed in this field to set the minimal pressure level required to preserve nutrient-free matrices.

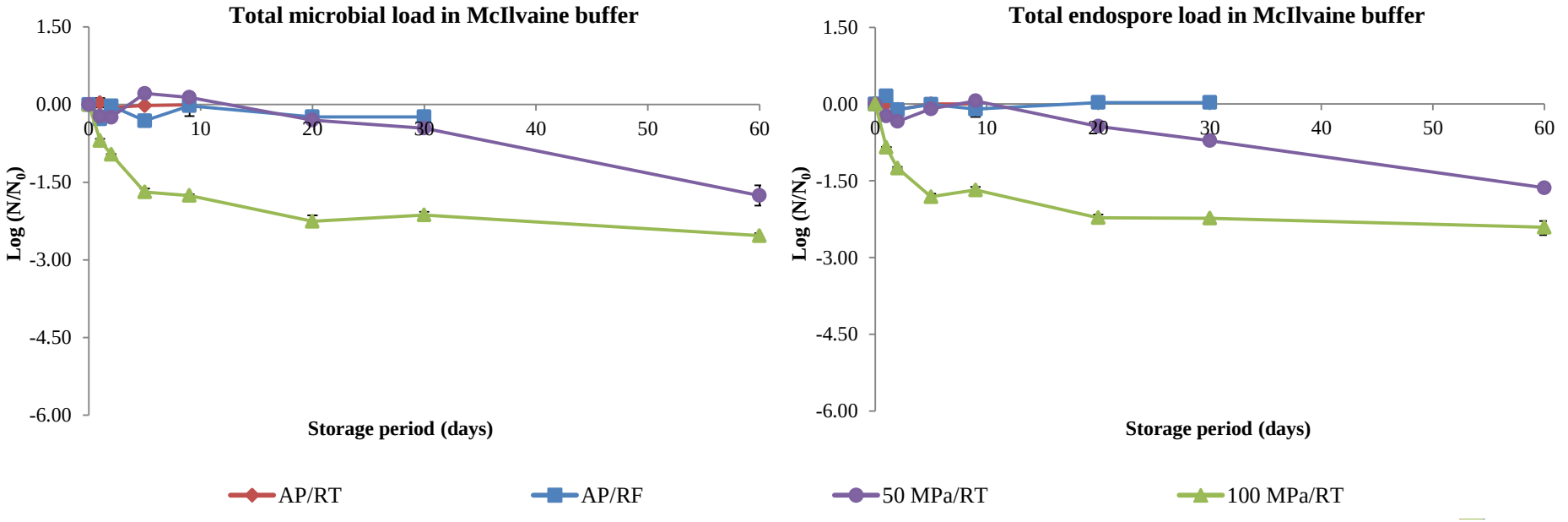
The slightly higher endospore load observed on some heated samples (**Table E1**, **Appendix E**) could be related with the presence of a population of endospores that is only activated by heat-shock (described as superdormant endospores that lack the majority of

the GR's on the inner membrane), thus not allowing a nutrient-like physiological germination induced by low pressure levels (Reineke et al., 2013a; Setlow et al., 2012; Wei et al., 2010), and, consequently, lower endospore loads on non-heated samples.

The statistical similarities observed between unheated and heated samples (**Figure 11 a-b**) suggest that not only a small fraction of TEL loose their defense mechanisms against hydrostatic pressure (as well their thermo-resistance), in a nutrient-free matrix, but also that nutrient-like physiological germination is slow at 100 MPa/RT (and even slower at 50 MPa/RT), due to the inactivation rate slowdown observed throughout storage.

These results are in agreement with those reported by Obaidat et al. (2015), who found negligible inactivation rates of *B. subtilis* endospores in McIlvaine buffer (pH 6.00) after being kept under pressure (80 MPa) for 1 h at 25 and 30 ºC. More pronounced reductions were also reported when the temperature increased above 33 ºC, which is closer to the optimal temperature of the cortex lytic enzymes that are known to have a fundamental role on the TEL germination and inactivation (Aoyama et al., 2005; Shigeta et al., 2007).

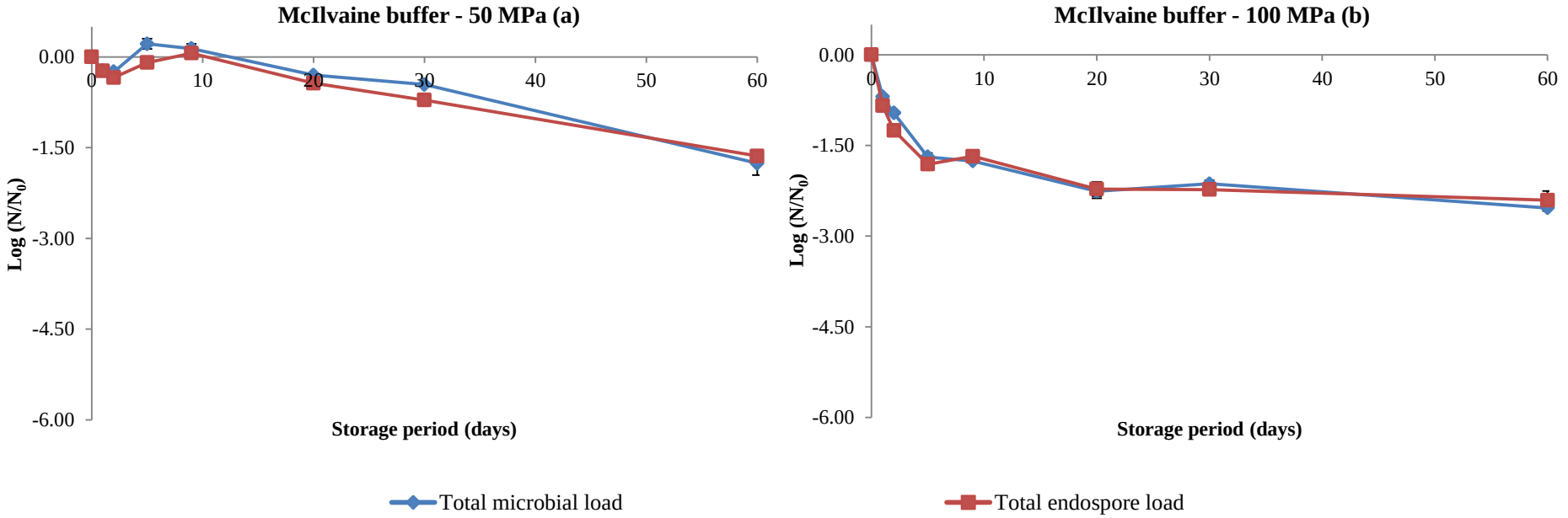
Moreover, as the McIlvaine buffer is a nutrient-free matrix, the TEL germination could only be unleashed by hydrostatic pressure, and not by nutrients (or a combination of germination pathways) due to the lack of nutrients in this matrix, which did not allow endospore outgrowth, despite their germination (observed by the TEL load reduction on heated samples along storage), contrarily to that observed on carrot juice and BHI-broth, as will be furtherly discussed.



Condition/Storage period (days)	1	2	5	9	20	30	60
AP/RT	aCε	aBε	aCε	aBε	-	-	-
AP/RF	aB	bBε	aB	bBε	abB	abB	-
50 MPa/RT	bBε	bB	cDε	cBε	bB	bB	aB
100 MPa/RT	eA	dA	cA	cA	bA	bA	aA

Condition/Storage period (days)	1	2	5	9	20	30	60
AP/RT	aC	aCε	aBε	aBε	-	-	-
AP/RF	bCε	aCε	abB	aBε	abCε	abCε	-
50 MPa/RT	cdB	cB	dB ε	eB ε	cB	bB	aB
100 MPa/RT	dA	cA	bA	bA	aA	aA	aA

Figure 10: Total microbial load (unheated samples) and total endospore load (heated samples) evolution in McIlvaine buffer (pH 6.00) kept at atmospheric pressure (AP) and naturally variable/uncontrolled room temperature (18-23 ºC, AP/RT), AP and refrigeration (4 ºC, AP/RF) and hyperbaric storage (50 and 100 MPa, HS) at naturally variable/uncontrolled RT. In the table, different upper/lower case letters (A-D)/(a-e) indicate significant differences ($p<0.05$) between different storage conditions/storage periods. The Greek letter ε indicates values that are not statistically different ($p>0.05$) from the initial value.



Case/Storage period (days)	1	2	5	9	20	30	60
Total microbial load	cA	cA	dBε	dAε	bcA	bB	aA
Endospore load	dA	cdA	deAε	eAε	cA	bA	aA

Case/Storage period (days)	1	2	5	9	20	30	60
Total microbial load	eA	dB	cA	cA	abA	bA	aA
Endospore load	dA	cA	bA	bA	aA	aA	aA

Figure 11: Comparison between the total microbial load (unheated samples, TML) and total endospore load (heated samples, TEL) in McIlvaine buffer (pH 6.00) kept at 50 and 100 MPa (a and b, respectively) for 60 days, at naturally variable, uncontrolled RT. In the table, different upper/lower case letters (A-B)/(a-d) indicate significant differences ($p<0.05$) between different cases/storage periods. In the figure, a protective effect conferred by the matrix is not noticeable, due to the statistical similarities ($p>0.05$) between storage periods. This figure shows the same data shown on the previous figure, in order to facilitate the comparison between TML and TEL for each storage pressure.

The composition of the food matrix (or food-like matrix) is known to play a key-role on endospore germination and inactivation rates under mild pressures. Few authors have studied the influence of the matrix composition on *B. subtilis* endospores while under pressure. For instance, Aoyama et al. (2005b) reported a *B. subtilis* endospore load reductions of 1.0 and 3.0 log-cycles in phosphate buffer and glucose broth, respectively, after a combined pressure/temperature treatment at 80 MPa/60 ºC/24 h, stating that the main reason for this difference might be on the composition of the inoculation system. In another study, Shigeta et al. (2007) induced the *B. subtilis* germination process at mild conditions, showing that in a range of pressures (20-100 MPa, and 40 ºC/60 min), the endospores reached a germination rate of ≈ 5 log cycles in glucose broth at 40 MPa (and forward), while in phosphate buffer, the maximal germination rate was ≈ 4 log cycles at 100 MPa, being considerably lower at inferior pressures.

These differences might be related, as reviewed by Black et al. (2007), with a combined effect of nutrient-induced and hydrostatic pressure-induced germination process, which means that nutrient-rich matrices are more likely to evidence higher endospore germination rates under hydrostatic pressure than nutrient-poor matrixes. The same author also reported that, if the pressure level is enoughly elevated, the endospore germination cannot be fully completed, since pressure acts as a hurdle on the endospore development, thus reducing its loads.

It is important to point out that the aforementioned studies aimed a different goal from the concept of HS, since the aforesaid authors intent to induce the germination processes by combining mild pressures and temperatures (in the range of 40-80 ºC) for short periods of time, followed by endospore destruction, while the present work (in the aim of this thesis) meant to study, for the first time, the efficacy of HS/RT.

III.1.2. Carrot juice

As the carrot juice is a nutrient-rich matrix, a nutrient-induced germination and outgrowth of the endospores was expected, thus, on this section, the unheated samples are expected to contain both vegetative and endospore forms of *B. subtilis* that will be termed as total microbial load (TML), while heated samples as TEL, similarly to the terminology previously used. The initial TML and TEL loads in carrot juice are presented in the **Table F1** (**Appendix F**).

Samples kept at AP/RT conditions quickly underwent a pronounced ($p<0.05$) TML and TEL growth (1.0 and 1.2 log units, respectively), thus causing severe juice spoilage, which was the reason why further microbiological analyses to samples kept at AP/RT did not take place.

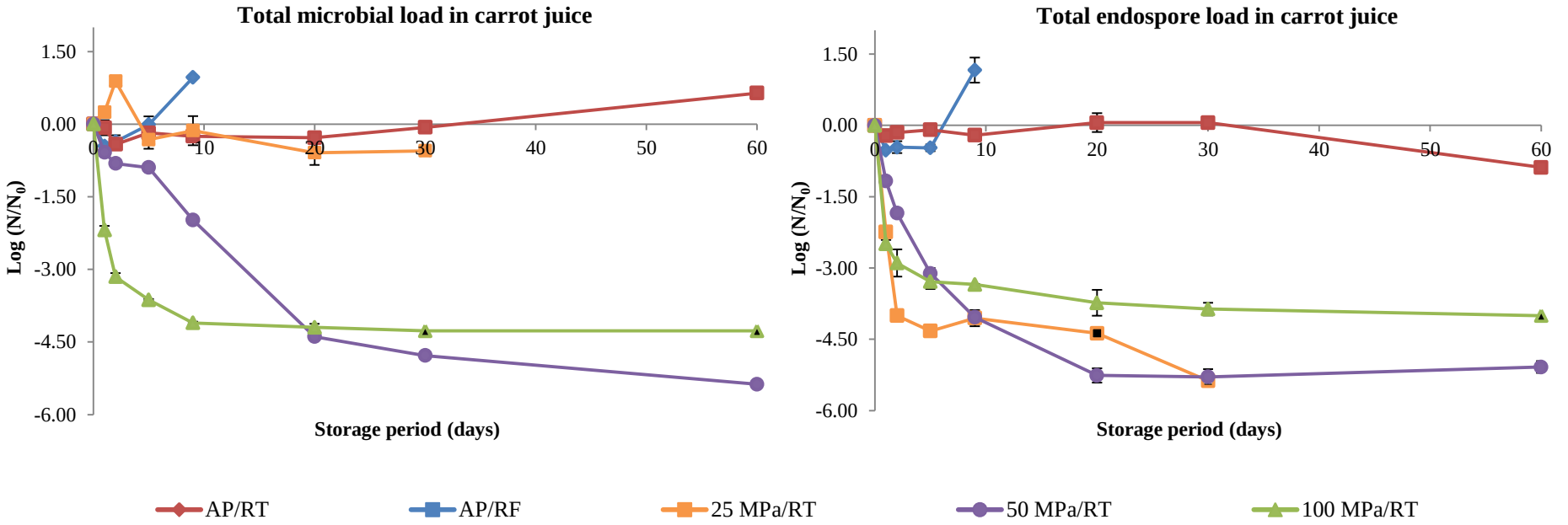
The AP/RF storage allowed to maintain both TML and TEL loads to similar levels ($p>0.05$) when compared to the initial values until the 30th day of storage (**Figure 12**), inclusive. Then, at the 60th day, the TML increased ($p<0.05$) about 0.64 log units, which was accompanied by a significant TEL load reduction ($p<0.05$) of 0.90 log units on heated samples, attributed to the germination and outgrowth of TEL, becoming, once again, heat-sensitive (thus reducing the endospore load) (Abel-Santos, 2014).

At 25 MPa, HS/RT yielded a significant increase ($p<0.05$) of the TML ($\approx$ 0.89 log units) right after 2 days of storage, which was accompanied by an accentuated TEL load reduction ($p<0.05$) of about 4.00 log units. This remarkable reduction on the TEL loads might be related to a combined effect of nutrient and nutrient-like physiological germination, loss of defence mechanisms (such as heat resistance, thus becoming more susceptible to the heat treatment) (Reineke et al., 2013a) with this pressure level (25 MPa) not hurdling the microbial development. Then, the TML remained stable ($p>0.05$) until the end of the storage experiments, contrarily to the TEL loads, which reached the detection limit at the 30th day of HS/RT, suggesting that, after nutrient exhaustion, the vegetative form of *B. subtilis* were not able to sporulate under pressure, as suggested by the continuous decrease of the TEL loads along storage, as seen in **Figure 12**. Nonetheless, further experiments at lower pressures are needed to confirm the aforesaid.

By increasing the storage pressure to 50 MPa, the TML were reduced along storage, although at a lower rate when compared to samples kept at 100 MPa, which was more evident until the 9th day of storage, wherein a TML and a TEL loads reductions ($p<0.05$) of about 2.0 and 4.0 log units were observed, respectively, which means that pressure might be triggering the endospore germination, but a pressure level of 50 MPa is less likely to affect the TML (on unheated samples), as seen in the **Figure 12**.

The TEL loads followed a linear reduction trend until the 5th day ($\text{Log } (N/N_0) = -0.5836*(\text{storage period (days)}) - 1.046$, $R^2 = 0.9391$), then, another linear trend was observed until the 20th day of storage ($\text{Log } (N/N_0) = -0.1363*(\text{storage period (days)}) - 2.5917$, $R^2=0.9670$), as seen in **Figure F1 (a)** (**Appendix F**). At the 60th day of storage, the

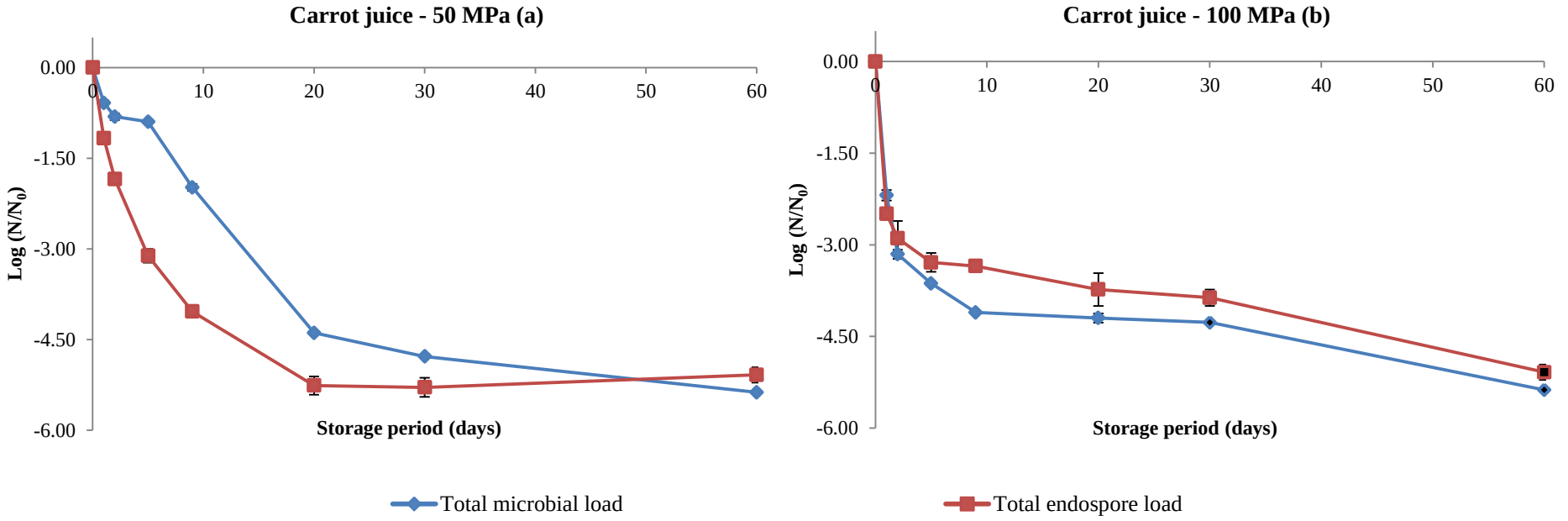
TML was reduced ($p<0.05$) of about 5.4 log units, comparatively to the initial load, against a reduction ($p<0.05$) of 5.1 log units on the TEL loads.



Condition/Storage period (days)	1	2	5	9	20	30	60
AP/RT	aB	aC	bDε	cD	-	-	-
AP/RF	bCε	aC	abCε	abCε	abC	bDε	cC
25 MPa/RT	cD ε	dD	abC	bC ε	aB	aC	-
50 MPa/RT	fB	Be	eB	dB	cA	bA	aB
100 MPa/RT	dA	Ac	bA	aA	aA	aB	aA

Condition/Storage period (days)	1	2	5	9	20	30	60
AP/RT	aC	aD	aC	bD	-	-	-
AP/RF	bCε	bDε	bCε	bCε	bDε	bCε	aC
25 MPa/RT	cA	bA	bA	bA	bB	aA	-
50 MPa/RT	eB	dC	cB	bA	aA	aA	aA
100 MPa/RT	dA	cB	bB	bB	abC	aB	aB

Figure 12: Total microbial load (unheated samples) and total endospore load (heated samples) evolution in carrot juice (pH 6.00) kept at atmospheric pressure (AP) and naturally variable/uncontrolled room temperature (18-23 ºC, AP/RT), AP and refrigeration (4 ºC, AP/RF) and hyperbaric storage (25, 50 and 100 MPa, HS) at naturally variable/uncontrolled RT. Empty/black filled symbols mean that the detection/quantification limit (1.00 and 2.00 log CFU/mL, respectively) was reached. Different upper/lower case letters (A-D)/(a-d) indicate significant differences ($p<0.05$) between different storage conditions/storage periods. The Greek letter ε indicates values that are not statistically different ($p>0.05$) from the initial value.



Case/Storage period (days)	1	2	5	9	20	30	60
Total microbial load	fB	efB	eB	dB	cB	bB	aA
Endospore load	eA	dA	cA	bA	aA	aA	aB

Case/Storage period (days)	1	2	5	9	20	30	60
Total microbial load	dA	cA	bA	aA	aA	aA	aA
Endospore load	eA	dA	cA	cB	bB	abB	aA

Figure 13: Comparison between the total microbial load (unheated samples, TML) and total endospore load (heated samples, TEL) in carrot juice (pH 6.00) kept at 50 and 100 MPa (a and b, respectively) for 60 days, at naturally variable, uncontrolled RT, evidencing the differences between TML and TEL loads while the carrot juice was under pressure. Black filled symbols mean that the quantification limit (2.00 log CFU/mL) was reached. In the table, different upper/lower case letters (A-B)/(a-d) indicate significant differences ($p<0.05$) between different cases/storage periods. This figure shows, partially, the same data shown on the previous figure, in order to facilitate the comparison between TML and TEL for each storage pressure.

A storage pressure of 50 MPa seems to unleash the endospore germination, given the more pronounced reduction of the TEL loads when compared to the TML (**Figure 13 a**), although, outgrowth is not fulfilled, probably due to the pressure hurdle.

Figure 12 evidences that, at 100 MPa, there were accentuated reductions ($p<0.05$) on the TML and TEL loads along storage, which were more pronounced than those found at 50 MPa. By the 20th day of HS/RT, a TML and TEL load reductions ($p<0.05$) of 4.2 and 3.7 log units, respectively. At the 30th day of HS/RT at 100 MPa, the total microbial load reached the quantification limit (of 2.00 log CFU/mL), which was maintained until the 60th day for unheated samples. In addition, the TEL loads generally decreased, following a linear trend ((Log (N/N_0) = -0.0313*(storage period (days)) -3.0128, R^2 = 0.8891 - **Figure F1 (b) – Appendix F**) from the 2nd to the 30th day of HS/RT, until reaching the quantification limit.

The higher endospore loads on heated samples (when compared to unheated samples, namely at 100 MPa), in some cases (**Figure 13 b**), might be related with the presence of superdormant endospores that are only activated by heat-shock, since they lack the majority of the GR's required to trigger the germination process on both nutrient and nutrient-like germination processes (Reineke et al., 2013a; Setlow et al., 2012; Wei et al., 2010), as aforementioned.

The more pronounced TML and TEL loads reductions along storage during HS/RT in carrot juice than in McIlvaine buffer, might be related with a combined effect of nutrient-induced endospore germination and of nutrient-like physiological germination, and then pressure acts as a hurdle that precludes endospore outgrowth (Black et al., 2007; Reineke et al., 2013a), which seems to be more feasible at higher pressures (100 MPa) due to the differences between the total microbial loads at 50 and 100 MPa, as seen in the **Figure H1** and **Figure H2** (**Appendix H**).

III.1.3. Brain-heart infusion broth

The initial TEL loads in BHI-broth are displayed in the **Table G1** (**Appendix G**). There were no significant differences ($p>0.05$) between the TEL loads on the initial unheated and heated samples in all cases.

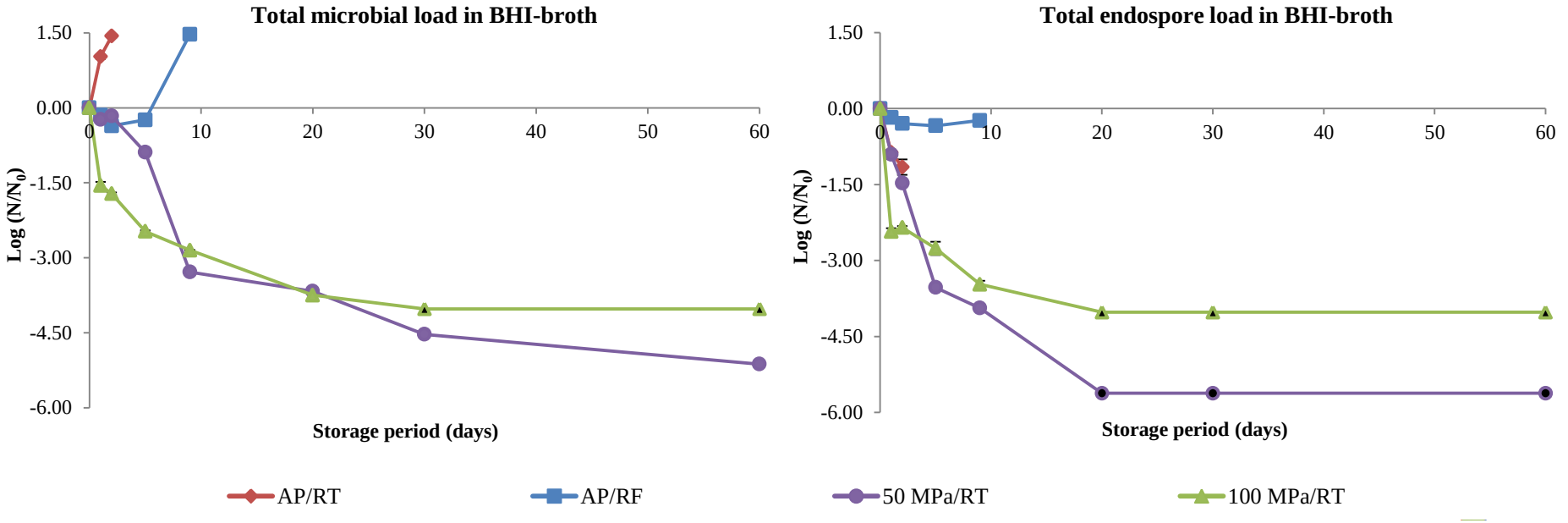
Samples kept at AP/RT faced a significant increase ($p<0.05$) on the TML, as expected. In fact, at the 2nd day, a total microbial load increase ($p<0.05$) of about 1.40 log units, which was accompanied by a TEL load reduction ($p<0.05$) of 1.16 log units (**Figure 14**), attributed to the germination and outgrowth of the endospores to vegetative forms, as previously observed in carrot juice. Additional microbiological analyses regarding AP/RT samples were not performed due to the advanced putrefaction state observed for this condition.

At AP/RF conditions, the TML of the unheated samples was, generally, statistically similar ($p>0.05$) to the initial load until the 5th day of storage experiments, inclusive, being thereafter observed a significant increase ($p<0.05$) of 1.47 log units at the 9th day, while the TEL loads on heated samples decreased ($p<0.05$) about 0.34 log units by the 5th day of storage reaching, at the 9th day, a similar ($p>0.05$) microbial load when compared to the initial one. Further experiments at AP/RF conditions did not take place due to the severe spoilage state of such samples.

Contrarily to AP storage (at both RT and RF conditions), HS/RT at 50 and 100 MPa enhanced both TML and TEL loads inactivation along storage, as seen in the **Figure 14**, that were more accentuated at higher pressures (100 MPa).

One day at 50 MPa yielded a TML inactivation ($p>0.05$) of about 0.23 log units that was accompanied by an TEL load decrease ($p<0.05$) of about 0.91 log units. On the subsequent days, these differences between TML and TEL (unheated and heated samples, respectively) were even more accentuated. At the 5th day of HS/RT at 50 MPa induced significant differences ($p<0.05$) between unheated and heated samples (**Figure 15**), wherein TML and TEL loads depletion of 0.89 and 3.54 log units, respectively, were found, comparatively to the initial load. This suggests that the TEL germinated (thus causing the loss of resistance mechanisms, given the TEL loads reduction on heated samples) but were not able to grow under pressure (observed by the TML reductions on unheated samples), but were not quickly inactivated (TML), as observed on carrot juice

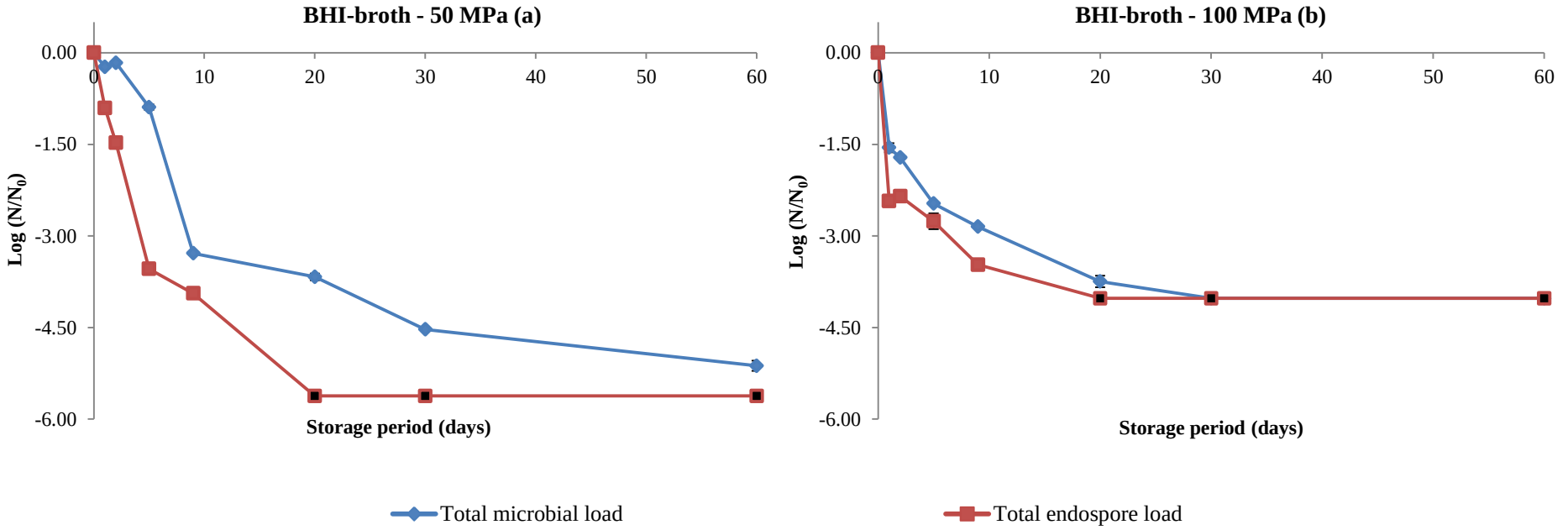
(**Figure 12** and **Figure 13**), especially for HS/RT at 100 MPa, which is supported by the statistical differences ($p<0.05$) between TML and TEL loads.



Condition/ Storage period (days)	1	2	5	9	20	30	60
AP/RT	aD	bC	-	-	-	-	-
AP/RF	bCε	aB	abBε	cC	-	-	-
50 MPa/RT	fB	fB	eB	dB	cA	bA	aA
100 MPa/RT	fA	eA	dA	cA	bA	aB	aB

Condition/ Storage period (days)	1	2	5	9	20	30	60
AP/RT	bB	aC	-	-	-	-	-
AP/RF	aBε	bD	bC	aCε	-	-	-
50 MPa/RT	eC	dB	cA	bA	aA	aA	aA
100 MPa/RT	dA	dA	cB	bB	aB	aB	aB

Figure 14: Total microbial load (unheated samples) and total endospore load (heated samples) evolution on BHI-broth kept at atmospheric pressure (AP) and naturally variable/uncontrolled room temperature (18-23 ºC, AP/RT), AP and refrigeration (4 ºC, AP/RF) and hyperbaric storage (50 and 100 MPa, HS) at naturally variable/uncontrolled RT. Black filled symbols mean that the quantification limit (2.00 log CFU/mL) was reached. In the table, different upper/lower case letters (A-D)/(a-f) indicate significant differences ($p<0.05$) between different storage conditions/storage periods. The Greek letter ε indicates values that are not statistically different ($p>0.05$) from the initial value.



Case/Storage period (days)	1	2	5	9	20	30	60
Total microbial load	fB	fB	eB	dB	cB	bB	aB
Endospore load	eA	dA	cA	bA	aA	aA	aA

Case/Storage period (days)	1	2	5	9	20	30	60
Total microbial load	fB	eB	dB	cB	bB	aA	aÂ
Endospore load	dA	dA	cA	bA	aA	aA	aA

Figure 15: Comparison between the total microbial load (unheated samples, TML) and total endospore load (heated samples, TEL) in BHI-broth (pH 6.00) kept at 50 and 100 MPa (a and b, respectively) for 60 days, at naturally variable, uncontrolled RT. Black filled symbols mean that the quantification limit (2.00 log CFU/mL) was reached. In the table, different upper/lower case letters (A-B)/(a-f) indicate significant differences ($p<0.05$) between different cases/storage periods. At 100 MPa, it is noticeable a protective effect to the TML, probably given by the matrix itself, and supported by the statistical differences ($p<0.05$) between TML and TEL loads between storage periods. This figure shows, partially, the same data shown on the previous figure, in order to facilitate the comparison between TML and TEL for each storage pressure.

This might be attributed to a protective effect conferred by slightly lower a_W of BHI-broth (**Appendix A**). In fact, **Raso et al. (1998)** reported that at lower a_W, *B. cereus* endospores were more pressure-resistant than those suspended on matrixes with lower a_W (higher ungerminated fraction of *B. cereus* endospores were found at a_W = 0.92, ≈ 7 log CFU/mL, contrasting with ≈ 2 log CFU/mL at a_W = 1, in McIlvaine buffer (pH 7) after a pressure treatment at 250 MPa/25 ºC/15 min. In addition, the endospore inactivation observed for heated samples followed two linear trends, whose equations are Log (N/N_0) = -0.6963*(storage period (days)) – 0.0922, R^2=0.9966, up to the 5th day, and Log (N/N_0) = 0.1420*(storage period (days)) – 2.7551, R^2=0.9943 from the 5th to the 20th day of storage at 50 MPa, as seen in **Figure G1** (**Appendix G**).

After 20 days at 50 MPa/RT, the heated samples reached the quantification limit (of 2.00 log CFU/mL), which was maintained until the 60th day, while the unheated samples still presented microbial counts, but by the 30th day of storage the same limit was reached.

While stored at 100 MPa/RT, it was verified a progressive reduction of the TML and TEL loads of about 2.85 and 3.47 log units, respectively, at the 9th day of storage, being statistically different ($p<0.05$) from each other. Then, at the 30th day of HS/RT, the TML reached the quantification limit (which was reached by the TEL loads by the 20th day), remaining stable ($p>0.05$) until the end of the storage experiments.

As far as the author of this thesis is aware, this is the first study regarding the effect of HS/RT on *B. subtilis* endospores inoculated in three nutritionally different matrices, despite other studies concerning the effect of low pressures (in the HS range) but at higher temperatures (above 30-40 ºC) in different matrices, whose main purpose was to trigger endospore germination by combining mild pressure and temperatures **(Aoyama et al., 2005; Aoyama et al., 2004, 2005; Shigeta et al., 2007)** for a few min/h (from 30 min to 48 h), while the present work aimed to test the feasibility of a new preservation methodology on endospore germination (and outgrowth) control, in a range of pressures between 25 and 100 MPa, at RT, for longer periods of time, in fact during the whole storage period of a food product.

In short, HS/RT has, generally, shown to be equal to better than AP/RF controlling the development of *B. subtilis* endospores.

III.2. Dipicolinic acid release from the endospore core

As described on the **Chapter II – Materials and methods** section (**Dipicolinic acid release from the endospore core**) was assayed, unfortunately, the employed method did not allow quantifying the DPA for both *B. subtilis* and *A. acidoterrestris* endospores, probably due to interferences between Tb^{3+} with other components of each matrix, or due to the equipment detection limit. Another methodology to quantify DPA could have been used, such as high performance liquid chromatography (HPLC), as proposed by Tabor et al. (1976), although, the lack of time did not allow it.

Although, it was possible to quantify the amount of dipicolinic acid on the endospore stock after autoclaving at 121.1 ºC for 15 min, which was 1.53 ± 0.03 mM.

Chapter IV – Results and discussion (*A. acidoterrestris*)

THIS SECTION REPORTS ALL THE OBTAINED RESULTS REGARDING *A. ACIDOTERRESTRIS* ENDOSPORES INOCULATED IN COMMERCIAL APPLE JUICE.

IV.1. Microbiological analyses

The commercial apple juice was inoculated with *A. acidoterrestris* endospores, being the initial loads presented in the **Table I1** (**Appendix I**). There were not found statistical differences ($p>0.05$) between unheated and heated initial samples.

At AP/RT, both TML and TEL loads (unheated and heated samples) grew of about 0.50 and 0.59 log units after 2 days of storage, and, furtherly, by the 9th day, both total microbial and endospore loads decreased, probably due to nutrient exhaustion and cell death (Abel-Santos, 2014).

AP/RF storage inhibited the development of the TML, whose loads remained statistically similar ($p>0.05$) to the initial one along storage (except on the 30th day), while the TEL load faced a small but statistically relevant ($p<0.05$) increase until the 5th day, and then decreased ($p<0.05$) until the 30th day.

Contrarily to AP/RF, HS/RT enhanced both TML and TEL loads inactivation on all storage pressures, resulting in microbiological shelf-lives extensions.

While 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 loads (2.40 and 2.20 log units, respectively), being statistically similar ($p>0.05$). Then, by the 20th day, the microbial load stabilized and remained similar ($p>0.05$) until the end of the storage experiments.

The major differences between TML and TEL loads evidenced in the **Figure 17 (a)** on the first two days of storage at 25 MPa suggest that the endospore germination was triggered (reinforced by the reduction of the TEL), although, it probably did not proceed to outgrowth of the vegetative cells (given the TML reductions). Two hypothetical scenarios might explain this phenomenon, in which the endospores have completed the germination process and originated a competent vegetative cell which, due to the pressure hurdle, was not able to undergo cell division, or the endospores surpassed the dormancy and proceeded to the stage II of germination, wherein the heat-resistance is quite lower than in the previous states, thus becoming more heat-sensitive and, as a result, the TEL decreased more than the TML. Taking into account that, on the subsequent days of storage both TML and TEL did not present (generally) statistical differences ($p<0.05$) between them, it might suggest the fraction of vegetative cells (or partially germinated endospores) that were heat sensitive were killed by pressure, as it might have occurred juice nutrient exhaustion,

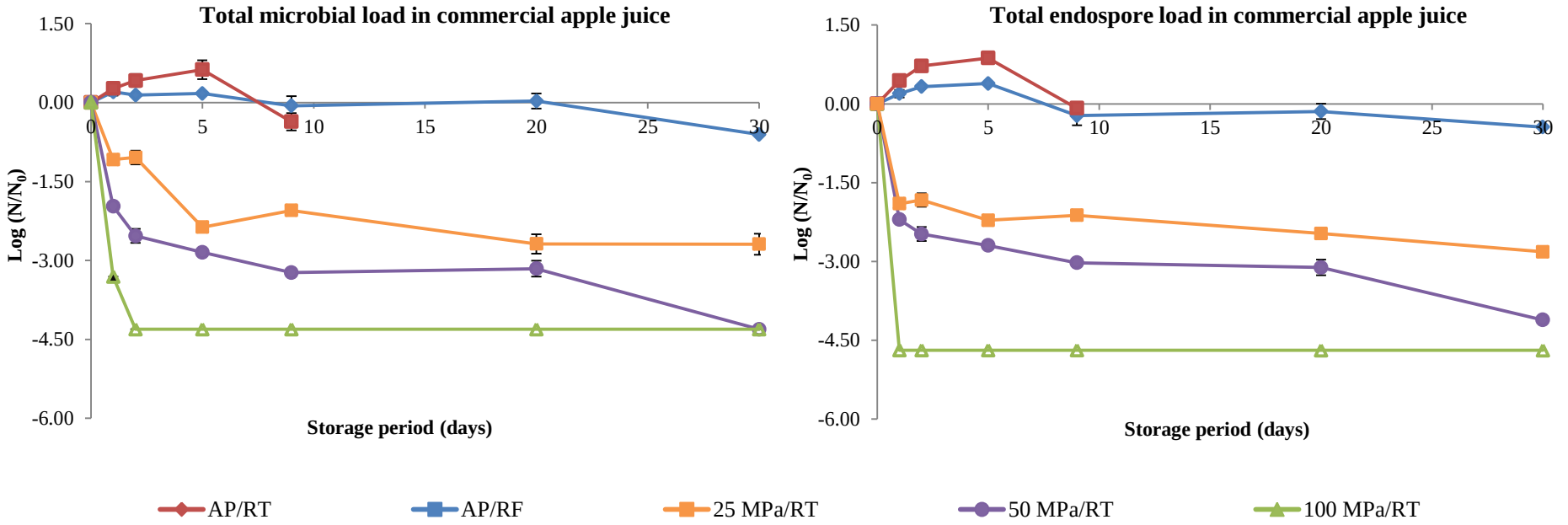
which could have been mobilized to resist to the pressure hurdle, and, once the nutrients are absent, the cells cannot survive, leaving the sporulated forms of *A. acidoterrestris*. Nevertheless, more fundamental research in this field is needed to fully understand the extension of the endospore germination under pressure.

An accelerated 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 load reduction of ≈ 2.00 and ≈ 2.20 log units were observed. Then, a linear decay of the TML and TEL loads was observed, (Log (N/N_0) = -0.4093*(storage period (days)) - 1.6221), R^2 = 0.9845) and (Log (N/N_0) = -0.2367*(storage period (days)) - 1.9932), R^2 = 0.9794), respectively, as seen in the **Figure I1 (A-B)** (**Appendix I**). By the end of the storage experiments, both TML and TEL loads were below the detection limit (1.00 log CFU/mL), proving that HS/RT at 50 MPa was suitable to preserve the commercial apple juice.

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 loads, right after 1 day of storage, as seen in the **Figure 16**, being maintained until the end of the storage experiments.

When compared to carrot juice, a low acidic food product, HS/RT at 25 MPa was not suitable to hurdle endospore germination and outgrowth, due to the development of the TML observed at such conditions, while the commercial apple juice suffered a TML and a TEL load decrease along storage, possibly due to the contribution of the acidity hurdle (pH ≈ 3.5) of the apple juice.

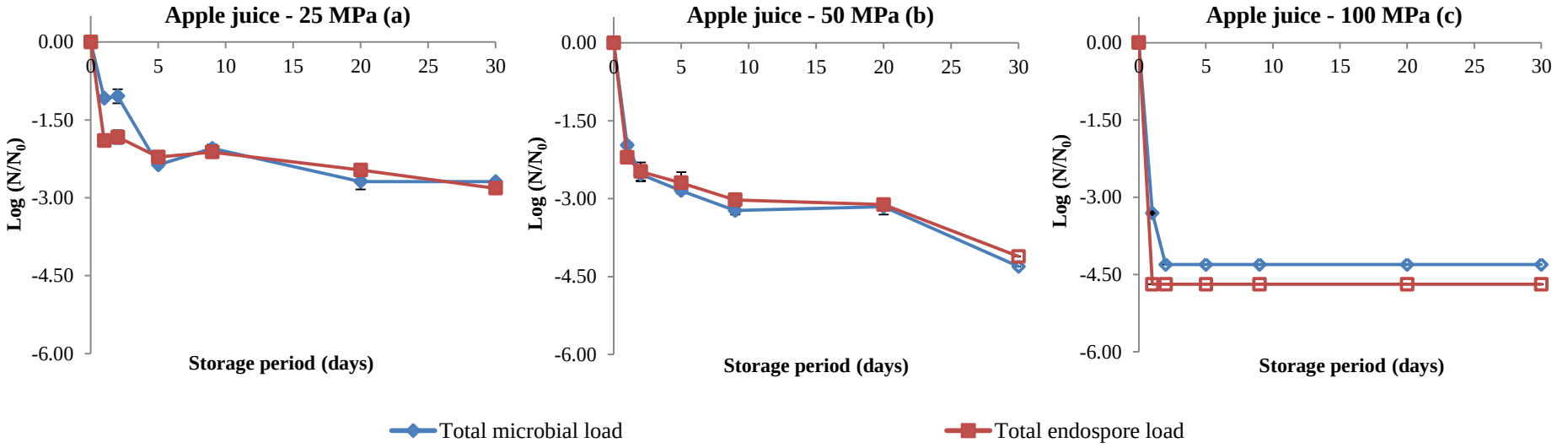
Contrarily to that observed for *B. subtilis* endospores in carrot juice, the possible presence of superdormant endospores was not so evident with *A. acidoterrestris* endospores in commercial apple juice, due to statistical similarities between unheated and heated samples, as seen in the **Figure 17 (a-c)**.



Condition/ Storage period (days)	1	2	5	9	20	30
AP/RT	bDε	bcD	cE	aD	-	-
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	bD	cE	cE	aDε	-	-
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 16: Total microbial load (unheated samples) and total endospore load (heated samples) evolution in commercial apple juice (pH 3.50) kept at atmospheric pressure (AP) and naturally variable/uncontrolled room temperature (18-23 ºC, AP/RT), AP and refrigeration (4 ºC, AP/RF) and hyperbaric storage at RT (25, 50 and 100 MPa, HS/RT). Dark filled/empty filled symbols mean that the quantification/detection limit (2.00 and 1.00 log CFU/mL, respectively) was reached. In the table, different upper/lower case letters (A-D)/(a-d) indicate significant differences ($p<0.05$) between different storage conditions/storage periods. The Greek letter ε indicates values that are not statistically different ($p>0.05$) from the initial value.



Storage period (days)	1	2	5	9	20	30
Total microbial load	cA	cB	bA	abA	aA	aA
Endospore load	dB	bcA	cB	abA	bB	aA

Storage period (days)	1	2	5	9	20	30
Total microbial load	eA	dA	cA	bA	bcA	aA
Endospore load	dA	cdA	cA	bA	bA	aA

Storage period (days)	1	2	5	9	20	30
Total microbial load	bB	aA	aA	aA	aA	aA
Endospore load	aA	aA	aA	aA	aA	aA

Figure 17: Total microbial load (unheated samples, TML) and total endospore load (heated samples, TEL) evolution in commercial apple juice (pH 3.50) kept at hyperbaric storage (HS) and naturally variable/uncontrolled room temperature (18-23 ºC, RT) at 25, 50 and 100 MPa (a, b and c, respectively), HS/RT. Empty filled/black filled symbols mean that the detection/quantification limit (1.00 and 2.00 log CFU/mL, respectively) was reached. In the table, different upper/lower case letters (A-B)/(a-e) indicate significant differences ($p<0.05$) between different storage conditions/storage periods. This figure shows, partially, the same data shown on the previous figure, in order to facilitate the comparison between TML and TEL for each storage pressure.

Although AP/RF was feasible to hinder the TEL germination outgrowth, HS/RT at all studied pressures (25, 50 and 100 MPa) equally hindered (during 30 days) plus inactivated both TML and TEL loads, thus allowing to extend the juice shelf-life. In fact, if the storage experiments were extended even more, it was probable that the TEL would germinate and outgrowth, thus increasing the TML.

Chapter V – Conclusions

THIS SECTION COMPRISES THE MAIN CONCLUSIONS ON THE SCOPE OF THIS THESIS.

This work aimed the evaluation of HS/RT effect on *B. subtilis* endospores on three nutritionally different matrices (McIlvaine buffer, carrot juice and BHI-broth), as well its effect on *A. acidoterrestris* endospores in an acidic matrix as commercial apple juice.

The composition of the inoculation matrix proved to be fundamental on food preservation by HS, by means that a nutrient-poor matrix such as McIlvaine buffer, with the same pH and a_W as the carrot juice, presented lower total microbial and endospore loads reductions at 100 MPa, while at 50 MPa HS showed to have an inhibitory effect similar to AP/RF.

In carrot juice, a storage pressure of 50 and 100 MPa performed better than RF (4 ºC) to preserve the carrot juice, resulting in shelf-life extensions, while a pressure level of 25 MPa did not hurdle the endospore germination and outgrowth, due to the total microbial load increase observed at such conditions.

The endospores of *B. subtilis* in BHI-broth and stored at 50 MPa faced a smaller total microbial load decrease than when stored at 100 MPa. A storage pressure of 25 MPa triggered the endospore germination and did not hinder the development of the total microbial load. In addition, a protective effect conferred by the BHI-broth to the total microbial load is suggested at higher pressures (100 MPa) by the significant differences ($p<0.05$) between unheated and heated samples, with the first ones presenting higher total microbial loads than the second ones, which are not verified on both McIlvaine buffer and carrot juice. This protective effect might be related with the slightly lower a_w of the BHI-broth, or probably due to its richer nutritional content, optimal for the endospore germination outgrowth in normal conditions.

These results are of great interest, since, in a practical point of view, HS/RT can preserve, for example, highly perishable (low acidity and high a_W, which do not preclude endospore germination), pasteurized food products, which need, currently, to be permanently kept at AP/RF conditions (which is energetically expensive and environmentally harmful), with HS/RT yielding not only considerable shelf-life extensions, but also potentiating reduced energetic costs, since the pressure pumps are only mobilized until the desired pressure is reached within the vessel.

Furthermore, an atypical case of an endospore able to germinate and outgrow in acidic, pasteurized food products, such as commercial apple juice, even at AP/RF conditions, (*A. acidoterrestris*), was studied, with HS/RT proving to be more feasible than

AP/RF to preserve the juice, allowing to extend its shelf-life by endospore inactivation to levels below the detection limit (1.00 log CFU/mL) in some cases.

In fact, all the evaluated storage pressures were suitable to preserve it, evidencing lower endospore loads along storage, whereas a storage pressure of 100 MPa evidenced a sharper total microbial load reduction to below the detection limit after 2 days of storage, at 50 MPa a similar load was observed on the last day of the storage experiments, while at 25 MPa a subtle total microbial load decrease was observed, contrarily to the conventional RF, that only inhibited the endospore germination and outgrowth.

The intrinsic acidity of the commercial apple juice might have complemented the pressure hurdle to the total microbial load development, since for the low acidic carrot juice, a storage pressure of 25 MPa not only triggered the endospore germination but also did not hindered the total microbial load development, suggesting that the pH of the food product might have, similarly to RF, an important role on the shelf-life of acidic products at HS/RT conditions, even though the carrot juice and apple juice cannot be fully compared due to the nutritional differences, as well by the differences between *A. acidoterrestris* and *B. subtilis* endospores.

In conclusion, HS/RT proved to be a feasible food storage methodology when it comes to bacterial endospores, which are a threat to the food industry, and, consequently, for consumers themselves. The fact that there is no temperature control, but instead pressure control, is, *per se*, an advantage over the conventional RF processed, inasmuch energy is only required on the compression phase of the storage vessel (pressure vessel), being kept along storage once the vessel is properly sealed, which means energetic savings, and, consequently, economic gains and a lower carbon footprint, without compromising food safety.

Chapter VI – Future work

THIS SECTION PROPOSES THE ESSENTIAL FUTURE WORK REGARDING THE CONCEPT OF HYPERBARIC STORAGE

Further experiments regarding the effect of HS on endospores are of upmost importance to validate HS as a safe preservation procedure. To do so, the future work should comprise the evaluation of HS on pathogenic spore-forming bacteria, such as *B. cereus* and *C. botulinum*, as well to verify if HS can inhibit the production of endemic toxins such as cereulide (produced by *B. cereus*).

The effect of this new preservation methodology should also be evaluated regarding fungi spores (ascospores), due to the inexistent literature regarding this subject, an also due to the importance that such biological structures play on food spoilage and poisoning.

Additionally, more fundamental studies concerning this subject are needed, such as microscopy analysis, to fully understand the endospore inactivation mechanisms that they undergo at HS conditions.

In a more practical perspective, different matrices (nutritionally, acidities and a_w's) should be also studied in order to evaluate the optimal storage pressure according with the aforementioned properties to ensure food safety, for, maybe in a near future, HS/RT to be a reliable replacement of the conventional RF processes, in what concerns endospore outgrowth control.

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Appendices – Complementary information

THIS SECTION COMPRISES ALL THE COMPLEMENTARY INFORMATION MENTIONED ALONG THE VARIOUS THESIS CHAPTERS.

Appendix A – Nutritional composition of the inoculation matrices

Table A1: Nutritional composition of raw carrot. Adapted from Instituto Nacional de Saúde (2006).

Amount	Per 100 g of edible portion	% Daily value
Calories (energy)	25 kcal (106 kJ)	1.25
Fats	0 g	0
Carbohydrates	4.4 g	1.8
Dietary fiber	2.6 g	8.7
Proteins	0.6 g	1.1
Salt	0.1 g	2
Vitamins	933 μg	0.8

Table A2: Composition of the BHI-broth, provided by the supplier (Oxoid).

Formula	g/L
Brain infusion solids	12.5
Beef heart infusion solids	5.0
Proteose peptone	10.0
Glucose	2.0
Sodium chloride	5.0
Dissodium phosphate	2.5
pH 7.4 ± 0.2 at 25 ºC	

Table A3: Nutritional content of the commercial apple juice used to inoculate *A. acidoterrestris* endospores. Information translated from the one provided by the supplier.

Amount	Per 100 mL	% Daily value
Calories (energy)	46 Kcal (197 kJ)	2.5
Fats	0 g	0
Saturated	0 g	0
Carbohydrates	11 g	4.5
Sugars	9.6 g	10.5
Fiber	0 g	0
Proteins	0 g	0
Salt	0.01 g	≈ 0
Vitamin C	6.00 mg	7.5

Figure A1: Commercial apple juice employed on the *A. acidoterrestris* endospores storage experiments. Courtesy of Auchan.

Appendix B – pH and a_W measurements (low acidic matrices)

Table B1: pH and water activity (aw) values of each matrix after autoclaving at 121.1 ºC for 15 min (expressed as mean ± standard deviation).

Matrix	pH	a_W
McIlvaine buffer	6.01 ± 0.01	0.967 ± 0.001
Carrot juice	6.00 ± 0.01	0.969 ± 0.001
BHI-broth	6.00 ± 0.01	0.947 ± 0.001

Appendix C – Culture media preparation

Table C1: Preparation scheme of the culture media used in the aim of this thesis.

Culture media	Amount of media powder* (g)
BAT-agar**	31.0
BHI-broth	37.0
BHI-agar	52.0
PCA	23.5
PDA	42.0
PDB	27.0

(*) Amount of powder for each 1.0 L of distilled water;
(**) Acidified with filter-sterilized sulphuric acid at 0.1 M after sterilization at 121.1 ºC for 15 min.

Appendix D – Standard curve for dipicolinic acid quantification

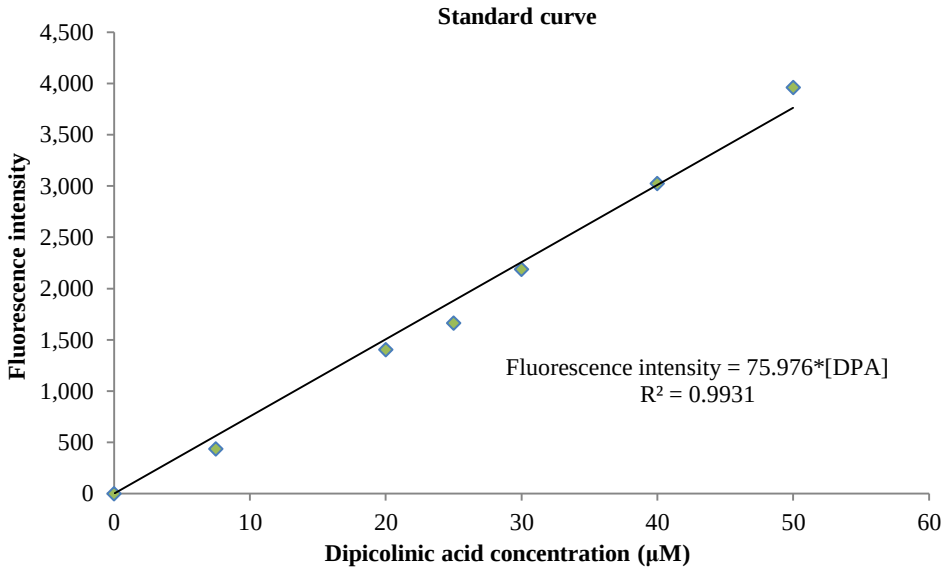


Figure D1: Standard curve of fluorescence intensity *versus* dipicolinic acid (DPA) concentration (µM).

Appendix E – Total microbial and endospore loads of *B. subtilis* in McIlvaine buffer and linear trends

Table E1: Total microbial (unheated samples) and total endospore (heated samples) loads (expressed as value ± standard deviation, in log CFU/mL) evolution in McIlvaine buffer (pH 6.00) throughout each storage condition.

Storage period (days)	Storage conditions	Unheated samples (Log CFU/mL)	Heated samples (Log CFU/mL)
0	Initial	6.01 ± 0.01	6.09 ± 0.03
	Initial (50 MPa)	7.68 ± 0.03	7.85 ± 0.03
1	AP/RT	6.05 ± 0.09	6.09 ± 0.01
	AP/RF	5.73 ± 0.03	6.25 ± 0.09
	50 MPa/RT	7.45 ± 0.01	7.62 ± 0.07
	100 MPa/RT	5.32 ± 0.04	5.25 ± 0.01
2	AP/RT	5.96 ± 0.02	5.98 ± 0.02
	AP/RF	5.99 ± 0.01	5.98 ± 0.04
	50 MPa/RT	7.44 ± 0.01	7.51 ± 0.01
	100 MPa/RT	5.05 ± 0.01	4.84 ± 0.02
5	AP/RT	5.99 ± 0.01	6.10 ± 0.01
	AP/RF	5.70 ± 0.03	6.08 ± 0.08
	50 MPa/RT	7.90 ± 0.09	7.76 ± 0.06
	100 MPa/RT	4.32 ± 0.07	4.28 ± 0.06
9	AP/RT	6.01 ± 0.01	6.09 ± 0.02
	AP/RF	5.99 ± 0.20	5.99 ± 0.15
	50 MPa/RT	7.82 ± 0.08	7.91 ± 0.04
	100 MPa/RT	4.26 ± 0.03	4.41 ± 0.01
20	AP/RT	-	-
	AP/RF	5.78 ± 0.07	6.12 ± 0.01
	50 MPa/RT	7.38 ± 0.01	7.42 ± 0.01
	100 MPa/RT	3.76 ± 0.12	3.87 ± 0.12
30	AP/RT	-	-
	AP/RF	5.89 ± 0.02	6.01 ± 0.04
	50 MPa/RT	7.22 ± 0.02	7.14 ± 0.04
	100 MPa/RT	3.88 ± 0.06	3.86 ± 0.06
60	AP/RT	-	-
	AP/RF	-	-
	50 MPa/RT	5.92 ± 0.20	6.21 ± 0.07
	100 MPa/RT	3.48 ± 0.05	3.68 ± 0.16

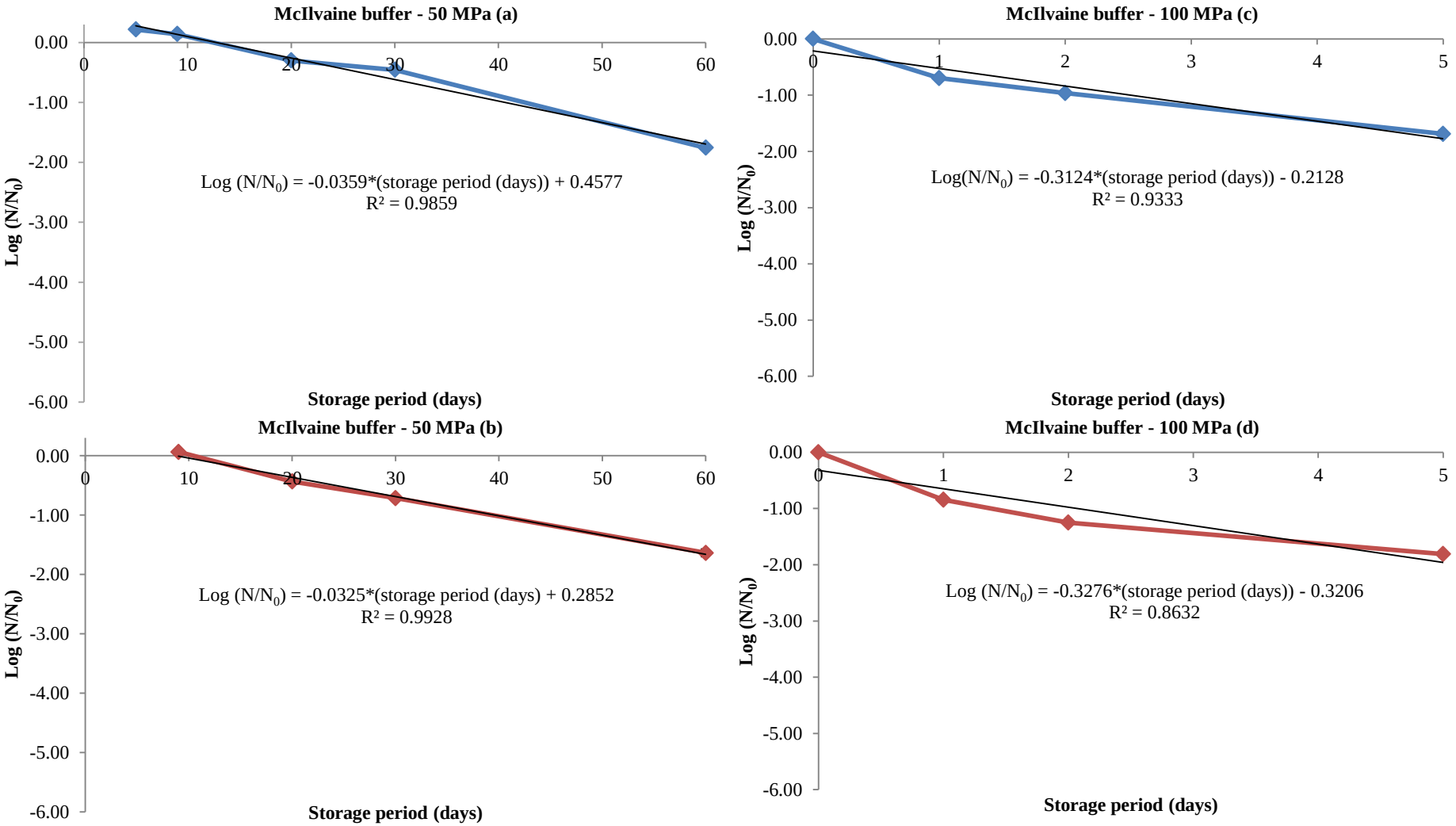


Figure E1: Linear trends observed concerning the reduction of the total microbial (blue line, a-b) and total endospore loads (heated samples, red line, c-d) of *B. subtilis* in McIlvaine buffer stored at 50 and 100 MPa, at variable/uncontrolled RT.

Appendix F – Total microbial and endospore loads of *B. subtilis* in carrot juice and linear trends

Table F1: Total microbial (unheated samples) and total endospore (heated samples) loads (expressed as value ± standard deviation, in log CFU/mL) evolution in carrot juice (pH 6.00) throughout each storage condition.

Storage period (days)	Storage conditions	Unheated samples (Log CFU/mL)	Heated samples (Log CFU/mL)
0	Initial	6.21 ± 0.30	5.99 ± 0.11
	Initial (25 MPa)	6.44 ± 0.01	6.37 ± 0.01
	Initial (50 MPa)	7.63 ± 0.02	7.62 ± 0.01
1	AP/RT	5.82 ± 0.02	5.48 ± 0.02
	AP/RF	6.20 ± 0.16	5.79 ± 0.01
	25 MPa/RT	6.69 ± 0.01	4.13 ± 0.05
	50 MPa/RT	7.04 ± 0.06	6.45 ± 0.03
	100 MPa/RT	4.08 ± 0.09	3.51 ± 0.08
2	AP/RT	5.91 ± 0.13	5.54 ± 0.13
	AP/RF	5.85 ± 0.05	5.83 ± 0.03
	25 MPa/RT	7.33 ± 0.02	2.27 ± 0.04
	50 MPa/RT	6.82 ± 0.01	5.78 ± 0.10
	100 MPa/RT	3.12 ± 0.08	3.11 ± 0.29
5	AP/RT	6.26 ± 0.17	5.53 ± 0.07
	AP/RF	6.09 ± 0.01	5.91 ± 0.05
	25 MPa/RT	6.12 ±0.19	2.05 ± 0.05
	50 MPa/RT	6.73 ± 0.06	4.51 ± 0.12
	100 MPa/RT	2.64 ± 0.02	2.71 ± 0.16
9	AP/RT	7.24 ± 0.04	7.16 ± 0.27
	AP/RF	6.02 ± 0.10	5.80 ± 0.09
	25 MPa/RT	6.31 ± 0.30	2.32 ± 0.17
	50 MPa/RT	5.65 ± 0.02	3.58 ± 0.01
	100 MPa/RT	2.16 ± 0.03	2.65 ± 0.02
20	AP/RT	-	-
	AP/RF	5.99 ± 0.04	6.06 ± 0.04
	25 MPa/RT	5.85 ± 0.25	2.00
	50 MPa/RT	3.24 ± 0.02	2.36 ±0.15
	100 MPa/RT	2.07 ± 0.08	2.27 ± 0.27
30	AP/RT	-	-
	AP/RF	6.20 ± 0.06	6.06 ± 0.04
	25 MPa/RT	5.89 ± 0.08	1.00
	50 MPa/RT	2.85 ± 0.03	2.33 ± 0.16
	100 MPa/RT	2.00	2.14 ± 0.14
60	AP/RT	-	-
	AP/RF	6.91 ± 0.06	5.12 ± 0.07
	25 MPa/RT	-	-
	50 MPa/RT	2.25 ± 0.01	2.54 ± 0.13
	100 MPa/RT	2.00	2.00

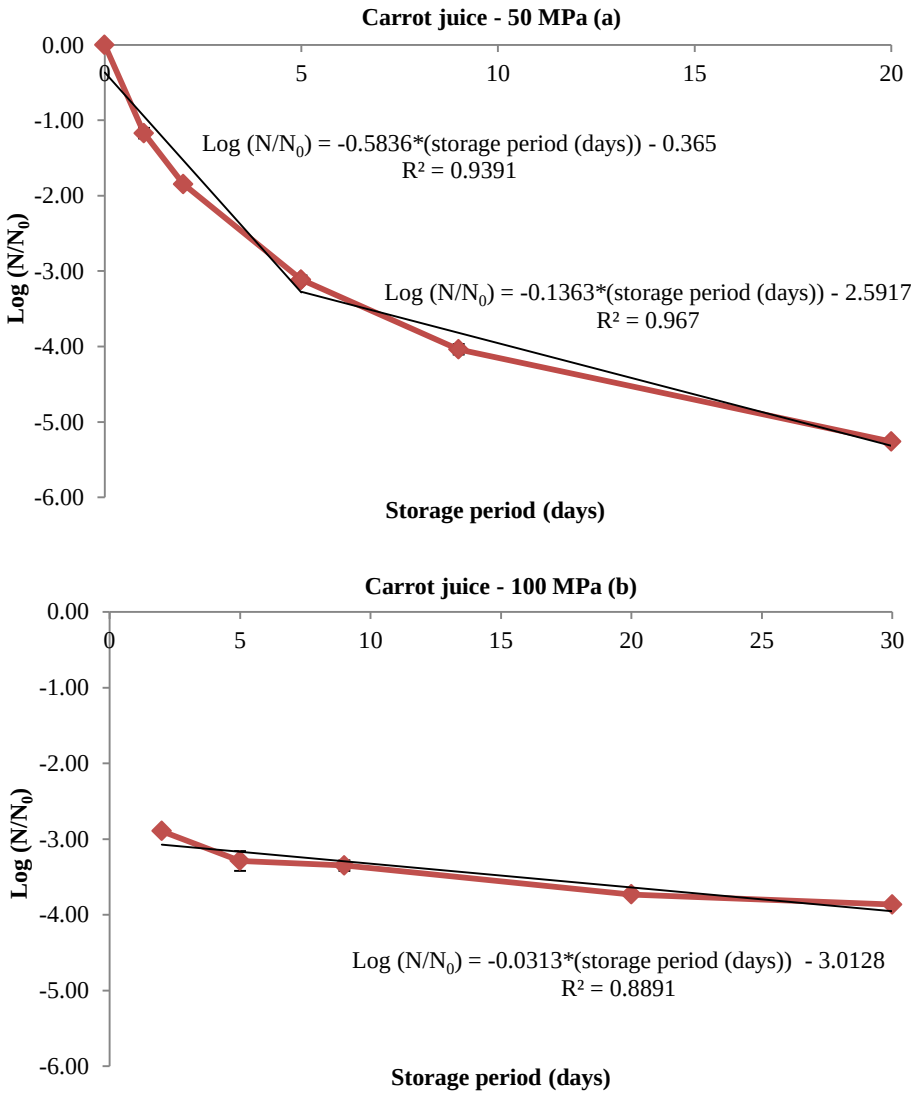


Figure F1: Linear trends observed concerning the reduction of total endospore loads (heated samples, red lines) of *B. subtilis* in carrot juice stored at 50 (a) and 100 (b) MPa, at variable/uncontrolled RT.

Appendix G – Total microbial and endospore loads of *B. subtilis* in BHI-broth and linear trends

Table G1: Total microbial (unheated samples) and total endospore (heated samples) loads (expressed as value ± standard deviation, in log CFU/mL) evolution in BHI-broth (pH 6.00) throughout each storage condition.

Storage period (days)	Storage conditions	Unheated samples (Log CFU/mL)	Heated samples (Log CFU/mL)
0	Initial	6.02 ± 0.03	6.05 ± 0.04
	Initial (AP/RT - RF)	6.70 ± 0.08	6.67 ± 0.02
	Initial (50 MPa)	7.58 ± 0.01	7.62 ± 0.01
1	AP/RT	7.73 ± 0.05	5.80 ± 0.02
	AP/RF	6.56 ± 0.04	6.49 ± 0.04
	50 MPa/RT	7.35 ± 0.01	6.72 ± 0.09
	100 MPa/RT	4.47 ± 0.08	3.59 ± 0.07
2	AP/RT	8.14 ± 0.04	5.51 ± 0.15
	AP/RF	6.35 ± 0.01	6.37 ± 0.09
	50 MPa/RT	7.42 ± 0.01	6.15 ± 0.06
	100 MPa/RT	4.30 ± 0.03	3.67 ± 0.03
5	AP/RT	-	-
	AP/RF	6.47 ± 0.03	6.33 ± 0.05
	50 MPa/RT	6.69 ± 0.05	4.09 ± 0.01
	100 MPa/RT	3.55 ± 0.02	3.26 ± 0.13
9	AP/RT	-	-
	AP/RF	8.18 ± 0.11	6.43 ± 0.03
	50 MPa/RT	4.30 ± 0.03	3.68 ± 0.07
	100 MPa/RT	3.17 ± 0.01	2.55 ± 0.07
20	AP/RT	-	-
	AP/RF	6.06 ± 0.03	6.12 ± 0.02
	50 MPa/RT	3.91 ± 0.06	2.00
	100 MPa/RT	2.27 ± 0.10	2.00
30	AP/RT	-	-
	AP/RF	7.32 ± 0.03	6.09 ± 0.06
	50 MPa/RT	3.05 ± 0.03	2.00
	100 MPa/RT	2.00	2.00
60	AP/RT	-	-
	AP/RF	-	-
	50 MPa/RT	2.46 ± 0.09	2.00
	100 MPa/RT	2.00	2.00

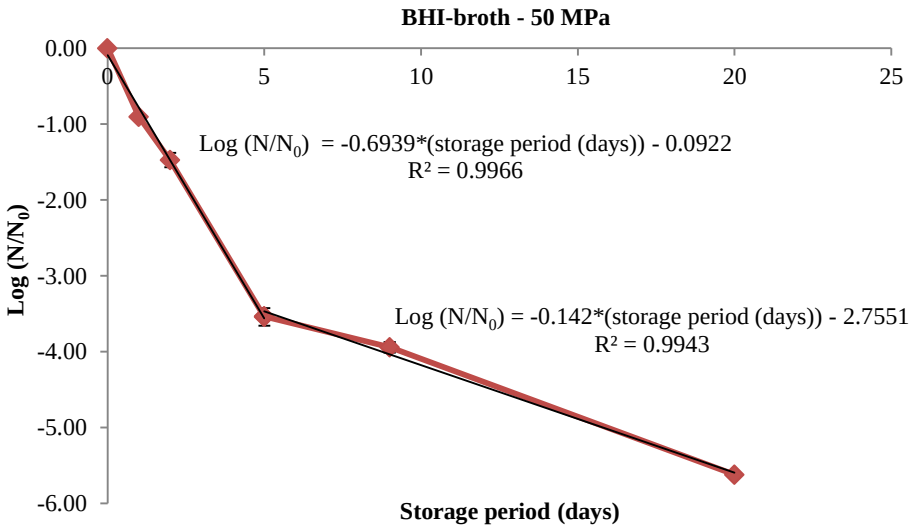
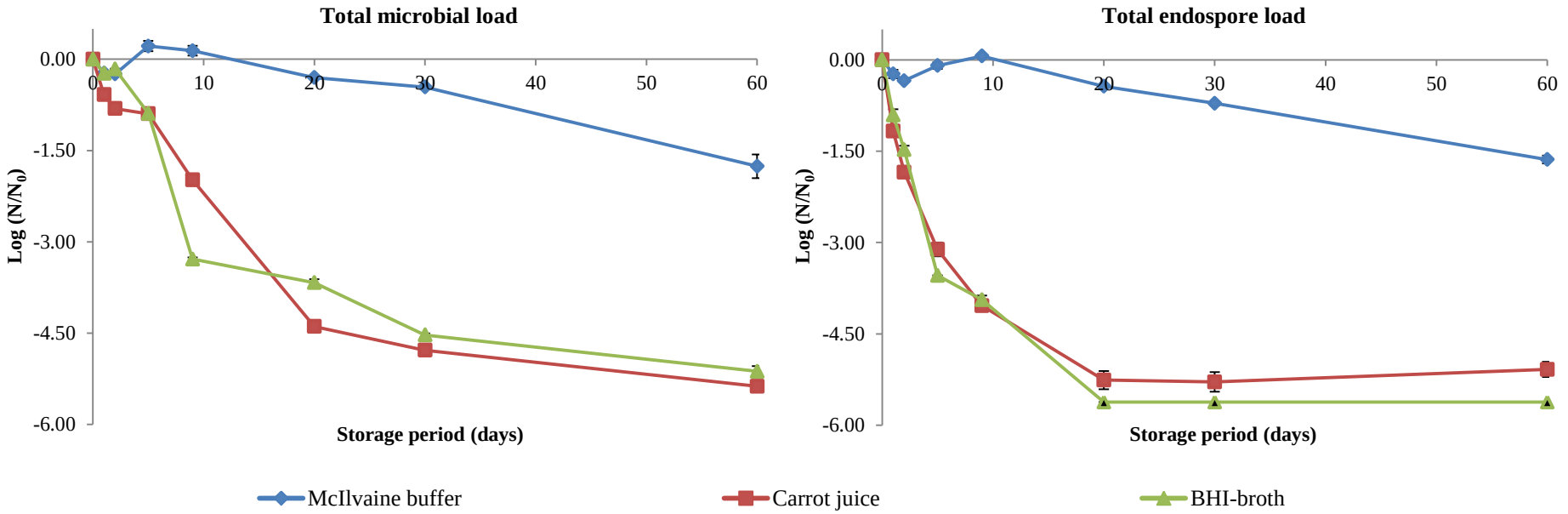


Figure G1: Linear trend observed concerning the reduction of the total endospore load (heated samples) of *B. subtilis* in BHI-broth stored at 100 MPa, at variable/uncontrolled RT.

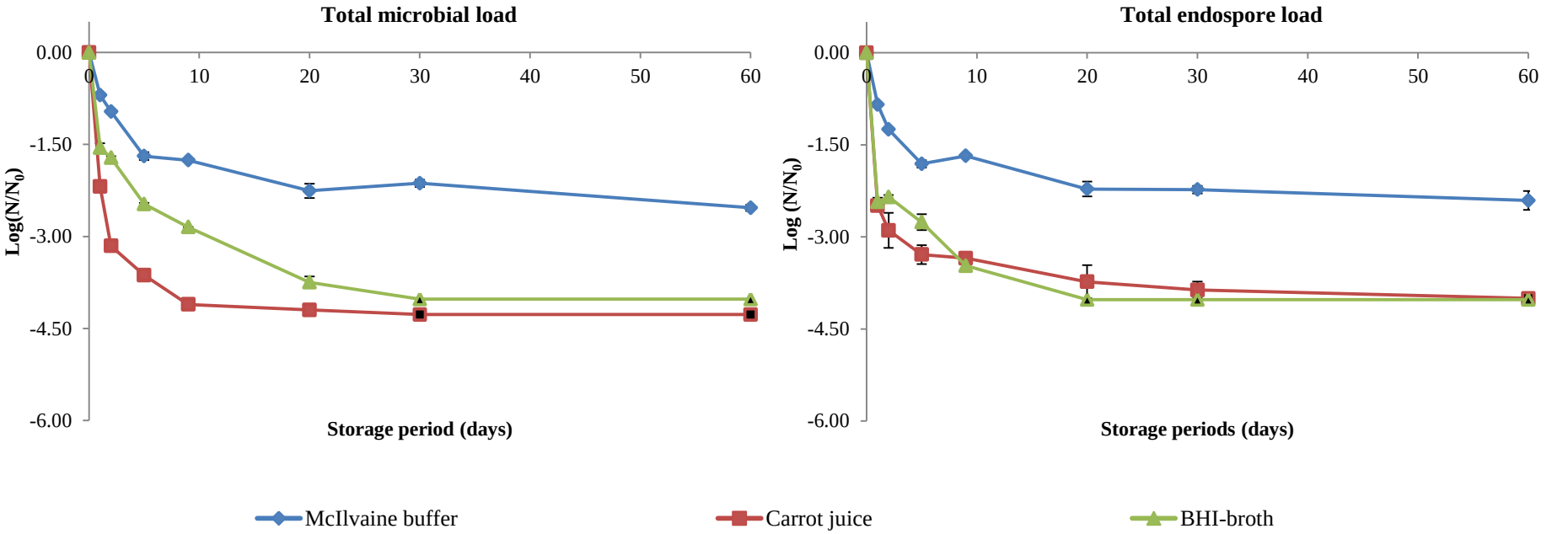
Appendix H – *B. subtilis* endospore behaviour evolution on each inoculation matrix at HS/RT (50 and 100 MPa)



Storage period (days)	1	2	5	9	20	30	60
McIlvaine buffer	cB	cBε	dB	dC	bcC	bC	aC
Carrot juice	gA	fA	eA	dB	cA	bA	aA
BHI-broth	fB	fBε	eA	dA	cB	bB	aB

Storage period (days)	1	2	5	9	20	30	60
McIlvaine buffer	cC	cC	cdCε	dBε	cC	bC	aC
Carrot juice	eA	dA	cB	bA	aB	aB	aB
BHI-broth	eB	dB	cA	bA	aA	aA	aA

Figure H1: Matrix comparison denoting the differences on the total microbial load (unheated samples) and total endospore load (heated samples) evolution in McIlvaine buffer, carrot juice and BHI-broth (pH 6.00) kept at 50 MPa throughout 60 days, at naturally variable, uncontrolled RT. Black filled symbols mean that the quantification limit (2.00 log CFU/mL) was reached. In the table, different upper/lower case letters (A-C)/(a-g) indicate significant differences ($p<0.05$) between different storage conditions/storage periods. The Greek letter ε indicates values that are not statistically different ($p>0.05$) from the initial value.



Storage period (days)	1	2	5	9	20	30	60
McIlvaine buffer	eC	dC	cC	cC	bC	bC	aC
Carrot juice	dA	cA	bA	aA	aA	aA	aA
BHI-broth	eB	eB	dB	cB	bB	aB	aB

Storage period (days)	1	2	5	9	20	30	60
McIlvaine buffer	dB	cC	bC	bB	aB	aB	aB
Carrot juice	dA	cA	bA	bA	aA	aA	aA
BHI-broth	cdA	dB	cB	bA	aA	aA	aA

Figure H2: Matrix comparison denoting the differences on the total microbial load (unheated samples) and total endospore load (heated samples) evolution in McIlvaine buffer, carrot juice and BHI-broth (pH 6.00) kept at 100 MPa throughout 60 days, at naturally variable, uncontrolled RT. Black filled symbols mean that the quantification limit (2.00 log CFU/mL) was reached. In the table, different upper/lower case letters (A-C)/(a-e) indicate significant differences ($p<0.05$) between different storage conditions/storage periods. The Greek letter ε indicates values that are not statistically different ($p>0.05$) from the initial value.

Appendix I – Total microbial and endospore loads of *A. acidoterrestris* in commercial apple juice and linear trends

Table I1: Total microbial (unheated samples) and total endospore (heated samples) loads (expressed as value ± standard deviation, in log CFU/mL) evolution in commercial apple juice (pH 3.50) throughout each storage condition.

Storage period (days)	Storage conditions	Unheated samples (Log CFU/mL)	Heated samples (Log CFU/mL)
0	Initial	5.29 ± 0.15	5.11 ± 0.03
	Initial (AP/RT)	4.96 ± 0.01	4.86 ± 0.06
	Initial (25 MPa)	5.66 ± 0.01	6.03 ± 0.02
	Initial (100 MPa)	5.44 ± 0.11	5.68 ± 0.08
1	AP/RT	5.23 ± 0.12	5.30 ± 0.11
	AP/RF	5.51 ± 0.07	5.31 ± 0.07
	25 MPa/RT	4.58 ± 0.09	4.13 ± 0.09
	50 MPa/RT	3.34 ± 0.05	2.91 ± 0.03
	100 MPa/RT	2.00	1.00
2	AP/RT	5.38 ± 0.13	5.58 ± 0.11
	AP/RF	5.45 ± 0.02	5.44 ± 0.19
	25 MPa/RT	4.62 ± 0.13	4.20 ± 0.13
	50 MPa/RT	2.77 ± 0.14	2.64 ± 0.18
	100 MPa/RT	1.00	1.00
5	AP/RT	5.59 ± 0.18	5.73 ± 0.02
	AP/RF	5.48 ± 0.01	5.50 ± 0.06
	25 MPa/RT	3.29 ± 0.04	3.81 ± 0.11
	50 MPa/RT	2.46 ± 0.02	2.42 ± 0.21
	100 MPa/RT	1.00	1.00
9	AP/RT	4.60 ± 0.16	4.78 ± 0.08
	AP/RF	5.25 ± 0.19	4.89 ± 0.04
	25 MPa/RT	3.61 ± 0.04	3.91 ± 0.01
	50 MPa/RT	2.08 ± 0.08	2.09 ± 0.09
	100 MPa/RT	1.00	1.00
20	AP/RT	-	-
	AP/RF	5.34 ± 0.15	4.97 ± 0.05
	25 MPa/RT	2.97 ± 0.19	3.56 ± 0.06
	50 MPa/RT	2.15 ± 0.15	2.00
	100 MPa/RT	1.00	1.00
30	AP/RT	-	-
	AP/RF	4.70 ± 0.01	4.67 ± 0.01
	25 MPa/RT	2.97 ± 0.20	3.21 ± 0.01
	50 MPa/RT	1.00	1.00
	100 MPa/RT	1.00	1.00

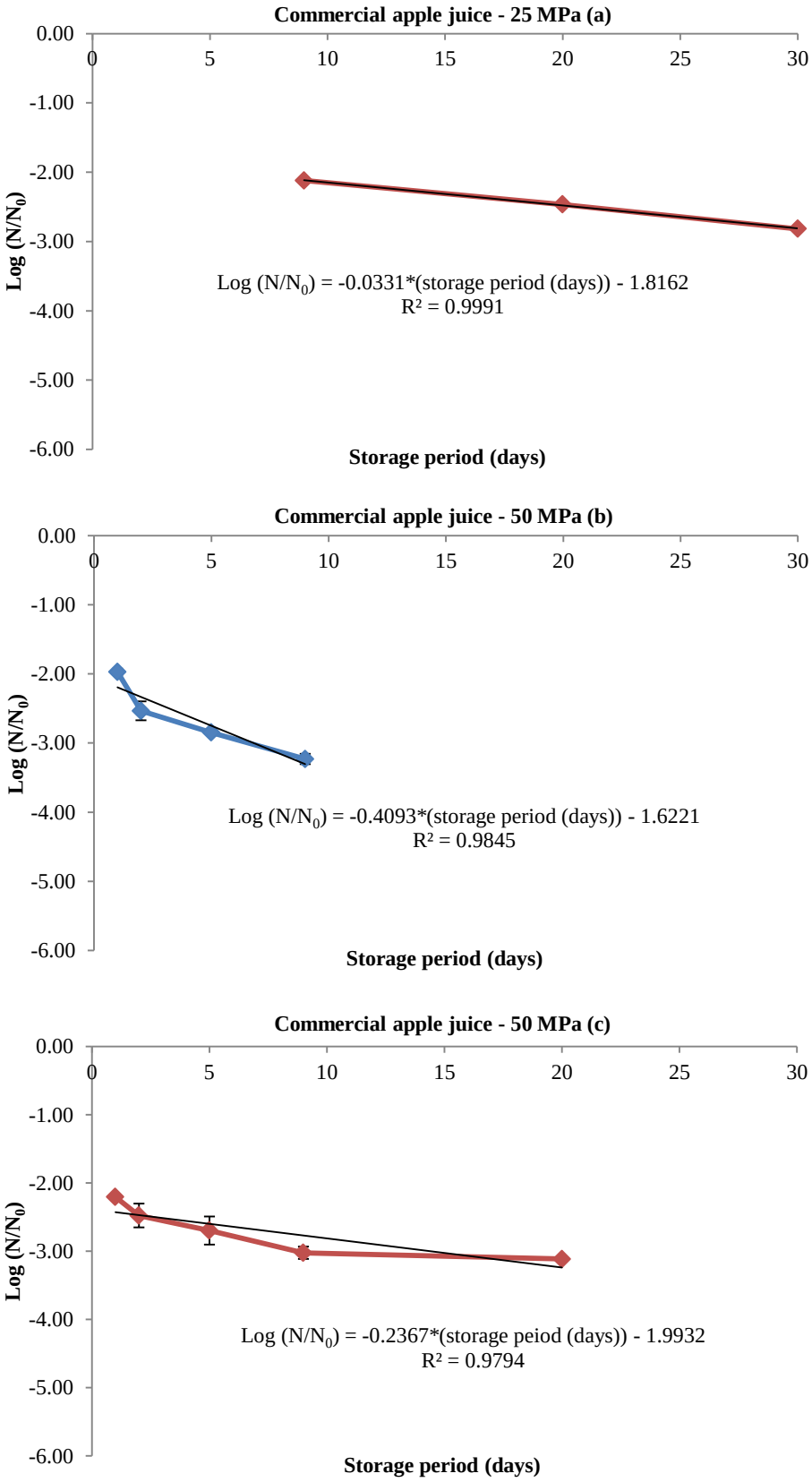


Figure I1: Linear trends observed concerning the reduction of the total microbial (unheated samples, b) and endospore loads (heated samples, a, c) of *A. acidoterrestris* in commercial apple juice stored at 25 and 50 MPa, at variable/uncontrolled RT.