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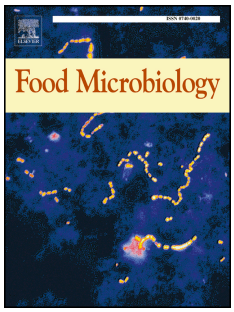

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Research paper

Enhanced control of *Bacillus subtilis* endospores development by hyperbaric storage at variable/uncontrolled room temperature compared to refrigeration

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Abstract

The effect of hyperbaric storage on *Bacillus subtilis* endospores, as a new food preservation methodology with potential to replace the conventional refrigeration processes, was assessed and compared to refrigeration. To do so, three different matrices (McIlvaine buffer, carrot juice and brain-heart infusion broth, BHI-broth) were inoculated with *B. subtilis* endospores and stored at 25, 50 and 100 MPa at variable/uncontrolled room temperature (18-23 ºC), under refrigeration (4 ºC), and room temperature at atmospheric pressure (0.1 MPa), up to 60 days. Two different quantification procedures were performed to assay both vegetative and endospores (unheated samples) and endospores (heated samples), to assess germination under pressure.

The results showed that hyperbaric storage yielded pronounced endospore loads reductions in carrot juice and BHI-broth at 50 and 100 MPa, while in McIlvaine buffer, lower endospore loads reductions were observed. At 25 MPa, the endospores germinated and outgrew in carrot juice. Under refrigeration conditions, both carrot juice and BHI-broth underwent endospore germination and outgrowth after 60 and 9 days of storage, respectively, while in McIlvaine buffer there were no endospore outgrowth.

These results suggest that hyperbaric storage at room temperature might not only be a feasible preservation procedure regarding endospores, but also that the food product (matrix characteristics) seems to influence the microbial inactivation that occurs during HS.

Keywords: Hyperbaric storage, refrigeration, endospores, *Bacillus subtilis*.

1. Introduction

Environmental concerns towards global warming are raising issues concerning the need of environmentally friendlier domestic/industrial practises. Regarding food industry, it is responsible for considerable emissions of carbon dioxide (CO_2), along with other greenhouse-effect gases. For instance, **James and James, (2010)** reported that 35 to 50% of the energetic consumptions in super and hypermarkets are due to the refrigeration (RF) and freezing facilities, being responsible for approximately 1% of the CO_2 emissions worldwide. RF is also 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)**. Thus, the adoption of alternative and more efficient food preservation procedures than RF are required, without compromising food quality and safety.

When it comes to food safety, pasteurized low acidic and high water activity (a_W) food products are to be permanently kept at RF temperatures in order to slowdown/inhibit the germination and outgrowth of bacterial spores. Pasteurization only destroys vegetative microorganisms, being many endospores resistant structures **(Soni et al., 2016)**, which limit the product shelf-life. So, a preservation methodology that is not only environmentally-friendlier but that could perform equally or even better than RF to slowdown/inhibit endospore germination and outgrowth is of upmost interest.

Lately, a new preservation methodology is being increasingly studied with potential to be a feasible alternative to RF. Under the name of hyperbaric storage (HS), it states that instead of controlling the storage temperature, it is more advantageous to control the storage pressure. Since energy is only required for the short compression and decompression phases of the pressure vessel, and not to keep it along storage, together with the needless temperature control (performed at naturally variable/uncontrolled room temperature, RT) **(Fernandes et al., 2014)**., This allows substantial energetic savings and, consequently, economic gains and reduced CO_2 emissions. In fact, **Bermejo-Prada et al. (2017)** demonstrated that keeping 800 Kg of strawberry juice under HS/RT conditions for 15 days had an energetic cost of 0.002$, against 0.034$ of RF. Still, equipment costs for HS were estimated by the same authors as being currently higher compared to RF. In an industrial point of view, the aforecited author also stated that, if a liquid food product is to be stored under HS/RT conditions, it could be used as the pressurization fluid itself.

HS performance at room-like temperatures is being increasingly investigated regarding the preservation of highly perishable food products (low acidity and high a_W), namely watermelon juice **(Fidalgo et al., 2014; Lemos et al., 2017; Pinto et al., 2017, 2016; Santos et al., 2015)**, carrot soup **(Moreira et al., 2015)**, *requeijão* (Portuguese whey cheese) **(Duarte et al., 2015)**, cooked ham **(Fernandes et al., 2015)**, raw bovine meat **(Freitas et al., 2016)** and tilapia fillets **(Ko and Hsu, 2002)**. Moreover, the effect of HS/RT has been also extensively evaluated for strawberry juice (acidic food product) when it comes to its microbiological, physicochemical and enzymatic parameters **(Bermejo-Prada et al., 2016, 2015; Bermejo-Prada and Otero, 2016; Segovia-Bravo et al., 2012)**. Moreover, it was recently proved that HS/RT performed similarly (50 MPa) to better (75 and 100 MPa) than the conventional RF regarding the development of pathogenic surrogated microorganisms (*Escherichia coli* and *Listeria innocua*) **(Pinto et al., 2017)**.

All these studies concluded that HS/RT performed similarly or even better than RF concerning the preservation of the quality attributes (colour, volatile profiles (strawberry juice), phenolic compounds, among others) and microbial development control, resulting in potential shelf-life extensions when compared to RF **(Freitas et al., 2016; Lemos et al., 2017; Pinto et al., 2017, 2016)**.

When it comes to enzymatic activity, **Bermejo-Prada et al. (2015)** reported a significant increase of polyphenol oxidase (PPO) activity on strawberry juice stored under different HS/RT conditions (50 and 200 MPa/15 days) compared to RF storage. Contrarily, significant peroxidase (POD) inactivation on longer HS/RT periods (200 MPa/15 days) were found, while pectin methylesterase (PME) catalytic activity was not affected by HS/RT compared to samples stored under RF **(Bermejo-Prada et al., 2016)**. These results are generally in agreement with those reported by **Pinto et al. (2017)**, who evaluated the impact of HS/RT (50, 75 and 100 MPa/10 days) the enzymatic parameters of PPO, POD and PME of watermelon juice.

The aforementioned studies only reported the effect of HS on vegetative microorganisms, and even though information regarding the effect of low pressures on some *Bacillus* spp. and *Clostridium* spp. endospores is available, the cases reported studied only short periods of time (few minutes/hours). For example, **Aoyama et al. (2005)** reported a germination rate of about 4 and 1.5 log-cycles at 100 MPa for 1 h, at 40 and 60 ºC, respectively for *Bacillus subtilis* endospores suspended in glucose broth, as well the reduction of about 1 log cycle on endospore counts at 80 MPa for 1 h at 60

ºC in phosphate buffer. However, literature concerning the HS effect (25-220 MPa over days of storage) on endospores is unavailable.

In fact, only three related papers are available, as the authors are aware, concerning this subject, suggesting that a combination of mild pressures (40 to 100 MPa) and moderate temperatures (30 to 80 ºC) for periods up to 4 days enhances the germination and inactivation of *Bacillus* spp. and *Clostridium* spp. **(Aoyama et al., 2005, 2004; Shigeta et al., 2007).** The authors of the aforecited studies meant to trigger endospore germination by combining low hydrostatic pressures with moderate/higher temperatures (than those of the HS range), and with a different final objective, consisting only in endospore germination induction for subsequent inactivation by further processing.

The spore-former *B. subtilis* is a gram-positive, facultative aerobic, non-pathogenic and rod-shaped bacteria whose endospores are widely used for food processing design. In fact, they are used as surrogated endospores of the pathogenic *B. cereus* (that are quite heat-resistant and its vegetative form produces cereulide, a heat-resistant emetic toxin) resulting in food poisoning illness (such as vomits and nausea) **(Agata et al., 2002; Checinska et al., 2015)**. *B. cereus,* along with *B. subtilis* endospores, are prevalent in low acidic food products such as meat **(Soni et al., 2016)**, raw and pasteurized milk **(Christiansson et al., 1999; Eneroth et al., 2001)** and carrot juice **(Aneja et al., 2014)**, among others. These products need to be preserved at RF conditions to inhibit endospore germination and outgrowth, since both pH and a_W do not hurdle the microbial development on the aforementioned products.

Given the importance of these biological structures on food safety, HS/RT of three different matrices was performed, consisting of McIlvaine buffer (pH 6.00), carrot juice and brain-heart infusion broth (BHI-broth, a general, non-selective culture media) (both at pH 6.00). Each matrix was inoculated with *B. subtilis* endospores and stored at 25, 50 and 100 MPa for up to 60 days at naturally variable/uncontrolled RT (18-23 ºC) and compared with atmospheric pressure (AP) storage at both RT and RF (4 ºC). These three different matrices were used since the easiness of *B. subtilis* endospores germination increases in the order McIlvaine buffer (a nutrient-free matrix), carrot juice (an intermediate nutrient matrix) and BHI-broth (optimal growth matrix), allowing to evaluate the endospore behaviour at HS/RT under very different conditions, as well the matrix composition influence on the endospore behaviour under pressure.

2. Materials and methods

2.1. Reagents and solutions

Physiological solution (0.9% NaCl) and citric acid were purchased from Applichem Panreac (Darmstadt, Germany), BHI-broth and BHI-agar were obtained from Oxoid (Cheshire, United Kingdom), and sodium phosphate dibasic was purchased from Riedel-de Haën (Seelze, Germany).

2.2. Matrices preparation

The McIlvaine citrate-phosphate buffer (0.2 M of Na_2HPO_4 and 0.1 M of citric acid) at pH 6.00 and BHI-broth were prepared according to **McIlvaine, (1921)** and the instructions provided by the supplier, respectively.

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.

The inoculation matrices were sterilized at 121.1 ºC for 15 min and were used on the same day of its preparation. Moreover, 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 hydrostatic pressure**,** the pH of both carrot juice and BHI-broth were adjusted to 6.00 with sterile citric acid (0.1 M), while the a_W was just measured using a hygrometer (Lab Swift – a_W, Novasina AG, Switzerland), being verified a similar value for the three matrices.

2.3. Endospore preparation

The endospore preparation was carried out as performed 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. Hereafter, the liquid culture was aseptically spread-plated onto BHI-agar plates and incubated at 30 ºC for 24 h. The sporulation was

verified by phase-contrast microscopy, and it took 15 days to achieve more than 95% of bright-phase endospores. Then, 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. The endospores were afterwards 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.

2.4. Endospores inoculation

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), 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 each matrix, at a concentration of about 10^6- 10^7 cells/mL.

The endospores used in this study were not heat-activated to avoid changes on their pressure resistance, in order to simulate the worst-case scenario on food preservation (low acidic and elevated a_W matrices containing non-heat-activated endospores that are known to be more pressure-resistant than those heat-activated, thus the germination process could only be triggered by nutrients and/or hydrostatic pressure) **(Vercammen et al., 2012)**.

2.5. Storage conditions

The storage experiments were carried out at 25, 50 and 100 MPa for 60 days at naturally variable/uncontrolled RT (18-23 ºC), using a high pressure equipment (SFP FPG13900, Stanstead Fluid Power, Stanstead, United Kingdom). This equipment has a pressure vessel of 30 mm inner diameter and 500 mm height, and a mixture of propylene glycol and water (40:60 v/v) was used as pressurization fluid. Simultaneously, two control samples were kept at atmospheric pressure (AP) and RT (AP/RT) and at RF (4 ºC), submersed in the same pressurization fluid and kept in the dark. Storage experiments at 25 MPa only took place for carrot juice, since the main goal of the present work was to infer the HS/RT feasibility in a highly perishable food product.

2.6. Determination of endospore germination and inactivation

To assess both germinated (vegetative cells) and ungerminated spores (dormant cells) after each storage condition, an aliquot of each matrix was heated at 80 ºC for 20 min to inactivate vegetative bacteria **(Reineke et al., 2013b; Wuytack et al., 1998)**, allowing to quantify not only both germinated and non-germinated spores (unheated samples, that will be termed total microbial load TML) as well non-germinated spores (heated samples, that will be termed as total endospore loads TEL). Then, decimal dilutions were performed (1.0 mL of each sample for 9.0 mL of physiological solution) that were plated in BHI-agar and incubated at 30 ºC for 24 h. 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.

2.7. Statistical analysis

All 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 HSD test at 5% of significance and were expressed as mean ± standard deviation.

3. Results and discussion

Since statistical similarities were observed between the initial TML and TEL loads in each matrix (supplementary material), it can be concluded that almost all cells were inoculated as endospores, being, thus, the initial load inoculated in each matrix referred to as TEL. Moreover, as the results are expressed as log (N/N_0), as aforementioned, the initial endospore loads are displayed in the supplementary material.

3.1. McIlvaine buffer

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, 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 TEL

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. Also for AP/RF samples, the TML loads on unheated samples evidenced, globally, no significant differences ($p>0.05$) between unheated and heated samples, **Figure 1** (**a-b**), due to the lack of nutrients.

HS/RT at 50 MPa performed similarly to AP/RF maintaining the TML load on unheated samples, at least until the 2nd day of storage experiments, wherein statistical similarities ($p>0.05$) were observed between conditions and storage periods. Then, both TML and TEL decreased ($p<0.05$) more pronouncedly (about 1.76 and 1.64 log units, respectively) from the 5th to the 60th day of storage. HS/RT at 100 MPa yielded a more remarkable TML and TEL loads reduction along storage. Five days of HS/RT resulted in a similar ($p>0.05$) TML and TEL load reduction of 1.7 and 1.8 log units on both unheated and heated samples, respectively, when compared to the initial values ($p<0.05$). Both TML and TEL loads inactivation rates observed at 100 MPa slowed down along storage from that day onwards (**Figure 1 a-b**), being practically the same ($p>0.05$) on the remaining days of storage experiments.

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 AP/RF storage, while at 100 MPa was more evident endospore inactivation throughout storage, which means that, for a nutrient-free matrix as McIlvaine buffer, a storage pressure of at least 50 MPa should be set to perform HS/RT instead of AP/RF.

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 6.0) after being kept under pressure (80 MPa) for 1 h at 25 and 30 ºC. More pronounced reductions were 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 endospore germination and inactivation **(Aoyama et al., 2005; Shigeta et al., 2007)**.

3.2. Carrot juice

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 these samples did not take place further.

The AP/RF storage allowed to maintain both TML and TEL loads at similar levels ($p>0.05$) when compared to the initial values until the 30th day of storage **(Figure 2 a-b)**, inclusive. Then, at the 60th day, the TML increased ($p<0.05$) about 0.64 log units, which was accompanied by a significant TEL reduction ($p<0.05$) of 0.90 log units on heated samples, attributed to the germination and outgrowth of TEL (thus reducing the endospore load) **(Abel-Santos, 2014)**.

At 25 MPa, HS/RT yielded a significant increase ($p<0.05$) of the TML (≈0.9 log units) right after 2 days of storage, which was accompanied by an accentuated TEL reduction ($p<0.05$) of about 4.0 log units. This remarkable reduction on the TEL loads might be related to a combined effect of nutrient and hydrostatic pressure-induced germination (also known as nutrient-like physiological germination) and loss of defence mechanisms, such as was reported for heat resistance **(Reineke et al., 2013a)**, with this pressure level (25 MPa) not hurdling the microbial development. Further experiments at 25 MPa/RT did not take place due to the severe spoilage state of samples.

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). By the 60th day, the TML was reduced ($p<0.05$) of about 5.4 log units comparatively to the initial load, compared with a reduction ($p<0.05$) of 5.1 log units for the TEL. A storage pressure of 50 MPa seems to unleash endospores germination, given the more pronounced reduction of the TEL loads when compared to the TML, although, outgrowth might not be fulfilled, possibly due to the pressure hurdle. For example, the same storage pressure (at RT) allows microbial proliferation (≥ 2 log units) in food products such as watermelon juice **(Lemos et al. 2017; Pinto et al. 2017)**, raw bovine meat **(Freitas et al. 2016)** and salmon **(Fidalgo et al. 2018)**, leading to food spoilage, similarly to AP/RF

storage, while in carrot juice in the present work there was no microbial development at 50 MPa.

Figure 2 (a-b) 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, TML reached the quantification limit (of 2.00 log CFU/mL), which was maintained until the 60th day for unheated samples, pointing to a potential microbiological shelf-life extension. A similar behaviour was observed for TEL

The higher TEL on heated samples (when compared to unheated samples, namely at 100 MPa), in some cases (supplementary material), 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 hydrostatic pressure-induced germination processes **(Reineke et al., 2013; Setlow et al., 2012; Wei et al., 2010)**, as aforementioned.

The composition of the food matrix (or food-like matrix) is known to play a key-role on the 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, but also at high pressure. For instance, **Aoyama et al. (2005a)** reported *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 the composition of the inoculation media. In another study, **Shigeta et al. (2007)** induced *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 maximum 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 **(Reineke et al., 2013a)**, which means that nutrient-rich matrices are more likely to evidence higher endospore germination rates under hydrostatic pressure than nutrient-poor matrices.

3.3. BHI-broth

Samples kept at AP/RT faced a significant increase ($p<0.05$) of TML, as expected. In fact, at the 2nd day, a total microbial load increase of about 1.40 log units was verified, which was accompanied by a TEL load reduction ($p<0.05$) of 1.16 log units (**Figure 3 a-b)**, 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 of the samples.

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, being thereafter observed a significant increase ($p<0.05$) of 1.47 log units at the 9th day, while the TEL remained, generally, similar ($p>0.05$) to the initial one. Further experiments at AP/RF conditions did not take place due to the severe spoilage state of the samples.

Contrarily to AP storage (at both RT and RF conditions), HS/RT at 50 and 100 MPa caused both TML and TEL inactivation along storage, as seen in **Figure 3 (a-b)**, that were more accentuated at 100 MPa. One day at 50 MPa yielded a TML inactivation ($p>0.05$) of about 0.23 log units that was accompanied by a TEL load decrease ($p<0.05$) of about 0.91 log units. At the 5th day of HS/RT at 50 MPa significant differences ($p<0.05$) between unheated and heated samples, wherein TML and TEL reductions of 0.89 and 3.54 log units, respectively, were found. This suggests that the endospores 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 also not quickly inactivated (TML), as observed on carrot juice, especially for HS/RT at 100 MPa, which is supported by the statistical differences ($p<0.05$) between TML and TEL loads. This might be possibly attributed to a protective effect conferred by the BHI-broth nutritional richness, although, more studies in this field are needed to understand endospore inactivation at HS/RT conditions in nutritionally distinct matrices. After 30 days at 50 MPa/RT, both TML and TEL loads reached the quantification limit (of 2.00 log CFU/mL).

For storage at 100 MPa/RT, it was verified a progressive reduction of the TML and TEL loads. At the 30th day of HS/RT, the TML reached the quantification limit (the

same level was reached by the TEL loads by the 20th day), and these values remained thereafter until the end of the storage experiments.

As far as the authors are 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 40 ºC) in different matrices. The main purpose of these other studies was to trigger endospore germination by combining mild pressure and temperatures **(Aoyama et al., 2005; Aoyama et al., 2004, 2005; Shigeta et al., 2007)**, while the present work aimed to test the feasibility of a new preservation methodology on endospores, at RT and for longer periods of time.

In short, HS/RT at 50 and 100 MPa showed to be better than AP/RF controlling the development of *B. subtilis* endospores, since these pressures caused endospore inactivation, while AP/RF allowed endospore germination and outgrowth, thus pointing for HS to be potentially able to extend the shelf-life of pasteurized foods compared to RF.

4. Conclusion

Preservation by HS performed at 50 and 100 MPa at naturally variable/uncontrolled RT for 60 days caused *B. subtilis* endospores reductions on all the studied matrices, with this decrement increasing in the order McIlvaine buffer>carrot juice>BHI-broth, with the cause for this being hypothesized to be due to the increment of the nutritional conditions for *B. subtilis* ATCC 6633 germination in the same order.

Contrarily, AP/RF storage kept the endospore counts throughout storage in McIlvaine buffer and carrot juice, but allowed germination on BHI-broth, while AP/RT storage promoted endospore germination and outgrowth faster, as expected.

These results are of great importance and potential for preservation of low acidity/high a_W pasteurized foods, whose shelf-life is limited by endospore development under RF, since HS at 50 and 100 MPa at naturally variable/uncontrolled RT resulted in endospores load reductions in the three matrices studied. This opens the possibility for considerable shelf-life extensions of these products by HS/RT, with the additional advantage of HS/RT being *quasi* energetically costless, since energy is only required for compression and decompression of the pressure vessel, and not during storage. Further experiments are of interest to fully explore the potential of HS/RT for pasteurized foods

preservation, namely the study of other spores and the estimation of achievable shelf-life. However, equipment development for practical applications of HS/RT remains a challenge.

Conflict of interest

The authors of this research paper declare no conflict of interest.

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Captions:

Figure 1: Total microbial load (unheated samples, a) and total endospore load (heated samples, b) 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 times. The Greek letter ε indicates values that are not statistically different ($p>0.05$) from the initial value.

Figure 2: Total microbial load (unheated samples, a) and endospore load (heated samples, b) 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. Black filled symbols mean that the quantification limit (2.00 log CFU/mL) was reached. Different upper/lower case letters (A-D)/(a-d) indicate significant differences ($p<0.05$) between different storage conditions/storage times. The Greek letter ε indicates values that are not statistically different ($p>0.05$) from the initial value.

Figure 3: Total microbial load (unheated samples, a) and endospore load (heated samples, b) 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.

Figure 1:

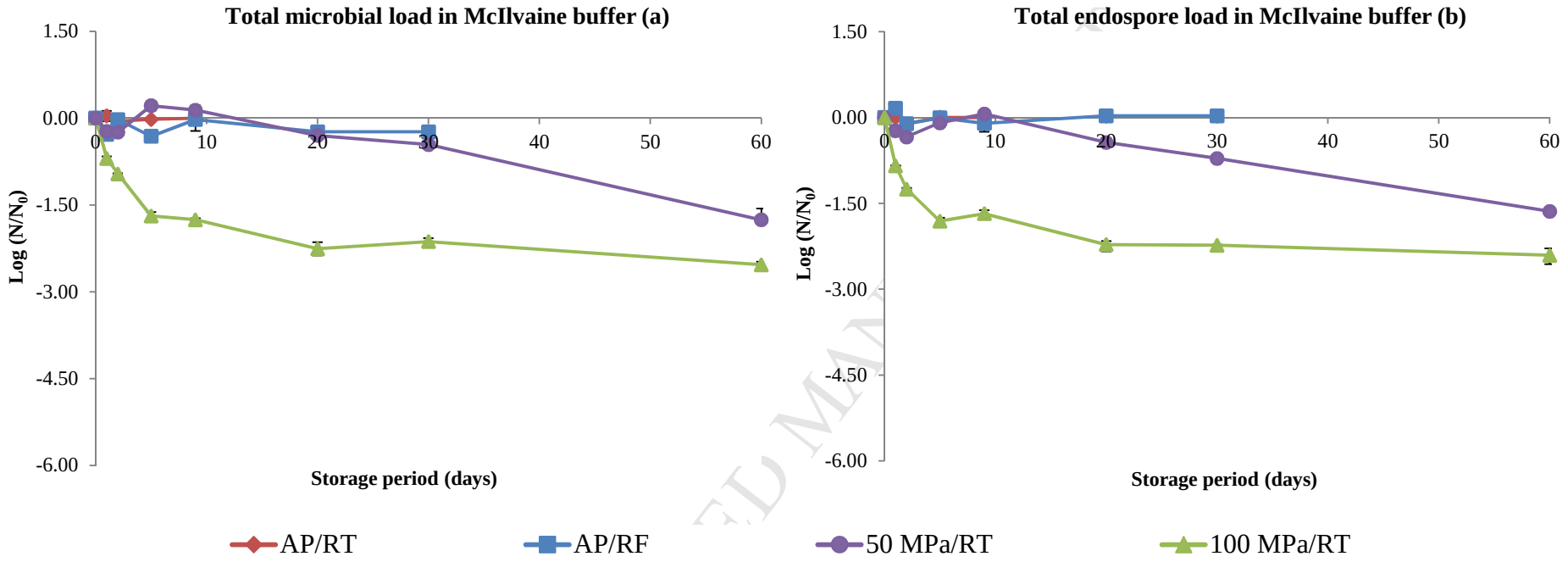


Figure 2:

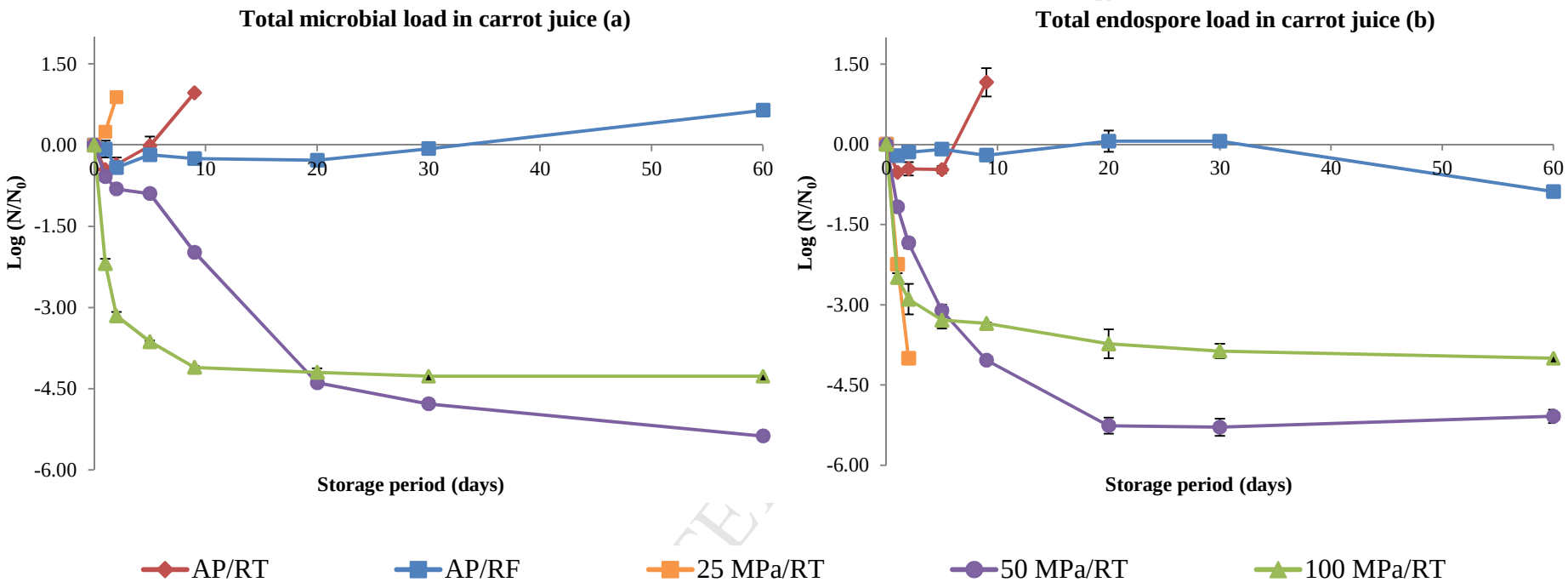
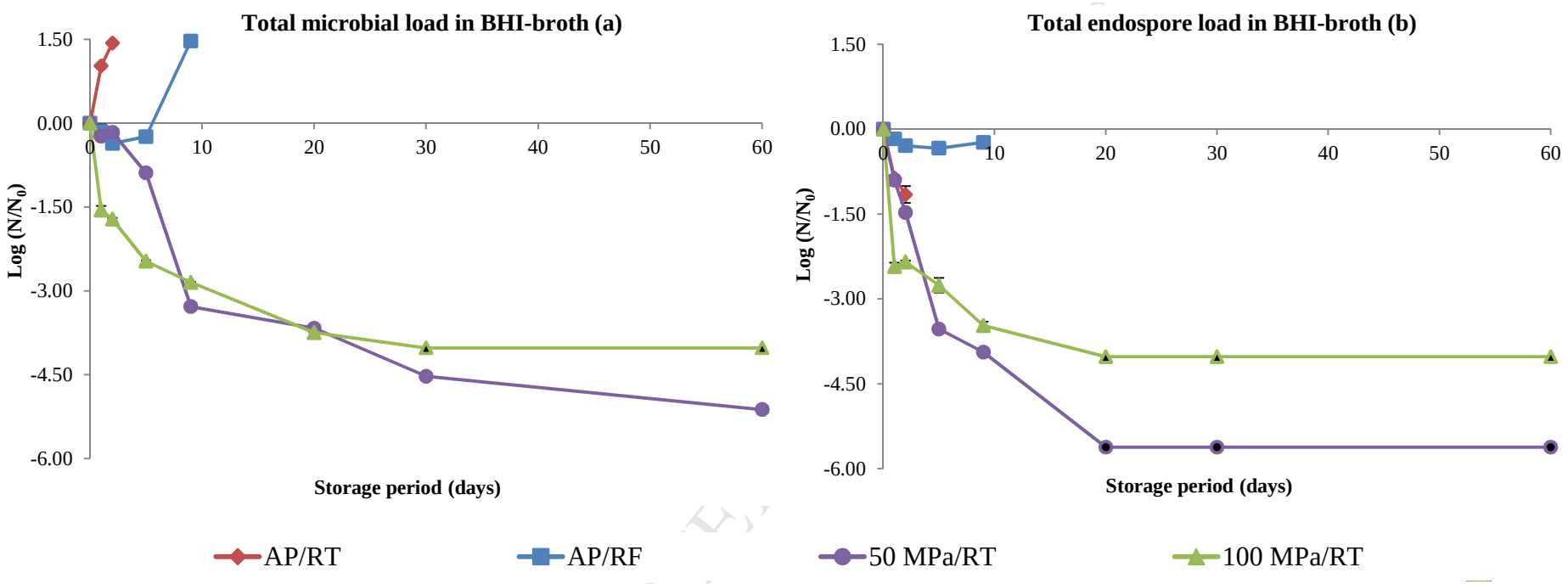


Figure 3:


Total microbial load in BHI-broth (a)
Total endospore load in BHI-broth (b)
Log (N/N0)
Storage period (days)
1.50
0.00
-1.50
-3.00
-4.50
-6.00
0
10
20
30
40
50
60
AP/RT
AP/RF
50 MPa/RT
100 MPa/RT

Supplementary material

Tables:

Table 1: Initial endospore loads on unheated and heated samples in each matrix before the storage experiments (expressed as mean ± standard deviation, in Log CFU/mL). The information within parenthesis in the storage conditions column refers to the initial load at the respective storage condition.

Matrix	Storage conditions	Unheated samples (Log CFU/mL)	Heated samples (Log CFU/mL)
McIlvaine buffer	Initial	6.01 ± 0.01	6.09 ± 0.03
	Initial (50 MPa)	7.68 ± 0.03	7.85 ± 0.03
Carrot juice	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
	Initial (100 MPa)	6.02 ± 0.03	6.05 ± 0.04
BHI-broth	Initial (AP/RT and RF)	6.70 ± 0.08	6.67 ± 0.02
	Initial (50 MPa)	7.58 ± 0.01	7.62 ± 0.01

Table 2: 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	0.984 ± 0.001
Carrot juice	6.00 ± 0.01	0.979 ± 0.001
BHI-broth	6.00 ± 0.01	0.977 ± 0.001

Table 3: Statistical analyses of the results obtained after each storage condition/period for each matrix (on the left, total microbial load; on the right, total endospore load). Different upper/lower case letters (A-D)/(a-f) indicate significant differences ($p<0.05$) between different storage conditions/storage times, while similar upper/lower case letters indicate no significant differences ($p>0.05$). The Greek letter ε indicates values that are not statistically different ($p>0.05$) from the initial value.

McIlvaine buffer

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

Carrot juice

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

BHI-broth

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

Highlights:

- Hyperbaric storage/room temperature (HS/RT) avoided *Bacillus subtilis* spores growth
- At 50/100 MPa, HS/RT reduced spore loads in McIlvaine buffer, carrot juice and BHI
- Spores in carrot juice and BHI reached the quantification limit (2.00 log CFU/mL)
- Globally, HS/RT enhanced *B. subtilis* spores germination control *versus* RF
- HS can extend pasteurized foods shelf-life by spore inactivation