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Germination and inactivation of *Bacillus coagulans* and *Alicyclobacillus acidoterrestris* spores by high hydrostatic pressure treatment in buffer and tomato sauce

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ABSTRACT

Acidothermophilic bacteria like *Alicyclobacillus acidoterrestris* and *Bacillus coagulans* can cause spoilage of heat-processed acidic foods because they form spores with very high heat resistance and can grow at low pH. The objective of this work was to study the germination and inactivation of *A. acidoterrestris* and *B. coagulans* spores by high hydrostatic pressure (HP) treatment at temperatures up to 60 °C and both at low and neutral pH. In a first experiment, spores suspended in buffers at pH 4.0, 5.0 and 7.0 were processed for 10 min at different pressures (100–800 MPa) at 40 °C. None of these treatments caused any significant inactivation, except perhaps at 800 MPa in pH 4.0 buffer where close to 1 log inactivation of *B. coagulans* was observed. Spore germination up to about 2 log was observed for both bacteria but occurred mainly in a low pressure window (100–300 MPa) for *A. acidoterrestris* and only in a high pressure window (600–800 MPa) for *B. coagulans*. In addition, low pH suppressed germination in *A. acidoterrestris,* but stimulated it in *B. coagulans*. In a second series of experiments, spores were treated in tomato sauce of pH 4.2 and 5.0 at 100 – 800 MPa at 25, 40 and 60 °C for 10 min. At 40 °C, results for *B. coagulans* were similar as in buffer. For *A. acidoterrestris*, germination levels in tomato sauce were generally higher than in buffer, and showed little difference at low and high pressure. Remarkably, the pH dependence of *A. acidoterrestris* spore germination was reversed in tomato sauce, with more germination at the lowest pH. Furthermore, HP treatments in the pH 4.2 sauce caused between 1 and 1.5 log inactivation of *A. acidoterrestris*. Germination of spores in the high pressure window was strongly temperature dependent, whereas germination of *A. acidoterrestris* in the low pressure window showed little temperature dependence. When HP treatment was conducted at 60 °C, most of the germinated spores were also inactivated. For the pH 4.2 tomato sauce, this resulted in up to 3.5 and 2.0 log inactivation for *A. acidoterrestris* and *B. coagulans* respectively. We conclude that HP treatment can induce germination and inactivation of spores from thermoacidophilic bacteria in acidic foods, and may thus be useful to reduce spoilage of such foods caused by these bacteria.

1. Introduction

Food pasteurization by HP has become a mature technology in recent years, and the number of HP treated foods on the market is rapidly increasing worldwide. Because it is a cold process, HP does not cause heat-related deterioration phenomena like loss of vitamins and nutrients, discoloration and textural and organoleptic changes, and HP treated foods are therefore often of superior quality. In general, vegetative bacteria (as well as yeasts and molds) can be efficiently inactivated at pressures in the range of 400–600 MPa without heating, although there is still an issue with pressure-resistant strains that may survive this treatment (Benito et al., 1999; Hauben et al., 1997). Bacterial spores, on the other hand, are more resistant to HP as is the case for other inimical treatments, and have been reported to withstand pressures of at least 1000 MPa, which is 400 MPa higher than the maximum pressure currently achievable in commercial food processing (Smelt, 2002). However, HP treatment can induce germination of spores (Clouston and Wills, 1969; Gould and Sale, 1970; Nakayama et al., 1996; Sale et al., 1970; Smelt, 2002). Once germinated, the spores lose their characteristic resistance and can be inactivated by a second treatment such as pressure or heat, and HP pasteurization approaches making use of this behavior have been proposed. For example, a 6-D reduction of *B. cereus* spores could be achieved in milk, either by a single treatment at 500 MPa/60 °C for 30 min, or by a sequential treatment for 30 min at 200 MPa/45 °C to induce spore germination, followed by mild heat treatment at 60 °C for 10 min to kill the germinated spores (Van Opstal et al., 2004). A fundamental limitation to this approach is that HP-induced spore germination is only moderate or even completely absent at or below room temperature (Nakayama et al., 1996; Sale et al., 1970).

Although growth of most sporeforming bacteria is inhibited by low pH, there are some notable exceptions such as *Clostridium butyricum*,

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B. coagulans and *A. acidoterrestris*, which can cause spoilage of thermally processed acidic foods (Silva and Gibbs, 2004). In the current study, we will focus on the latter two, which are considered as thermoacidophiles. *B. coagulans* has an optimum and maximum growth temperature of respectively 33 – 45 °C and 55 – 60 °C. Its spores are able to germinate and grow at pH-values as low as 4.0, and as such it is frequently isolated from spoiled canned acidic or acidified vegetables, in particular tomato products (Evancho and Walls, 2001; Mallidis et al., 1990). *A. acidoterrestris* is an obligate acidophile, which grows optimally at pH 3.5 to 4.0 and has a minimum and maximum pH for growth of 2.5 and approximately 5.5. *A. acidoterrestris* spores are generally more heat resistant than those of other acidophilic sporeformers, and cause spoilage of processed fruit and vegetables juices (Evancho and Walls, 2001; Silva and Gibbs, 2004; Splittstoesser et al., 1994). In addition, *A. acidoterrestris* is able to grow over a temperature range of 25 to 60 °C (Yamazaki et al., 1996).

Only few studies have addressed spore germination and inactivation of *B. coagulans* and *A. acidoterrestris* by HP. Sale et al. (1970) studied the effect of HP up to 800 MPa on the inactivation of spores from different *Bacillus* and *Clostridium* spp. including *B. coagulans* in buffer. In general inactivation proceeded more rapidly at high than at low temperatures (range 25 – 75 °C) and at moderate as opposed to extreme pH-values (range pH 3.0 – 11.5). In a separate study, the same research group reported that HP mediated germination of some bacterial spores including *B. coagulans*, shows a temperature optimum at 50 – 70 °C depending on the organism and the conditions, and a pH optimum around neutrality (Gould and Sale, 1970). However, only pressures up to 100 MPa were used in this study. HP up to 600 MPa which are more relevant to food processing also induce spore germination, but at least in *B. subtilis* the process of germination seems to be aborted at an early stage, resulting in partially germinated spores which retain resistance to some stresses (Wuytack et al., 1998). Remarkably, more recent HP studies with *B. coagulans* only report spore inactivation, and have not specifically addressed germination. Roberts and Hoover (1996) studied the effect of HP (400 MPa; 25, 45 and 75 °C; 15 and 30 min) on spores of *B. coagulans* in McIlvaine citrate buffer (pH 4.0-7.0) and observed the highest sensitivity at low pH and high process temperatures. In another study, the effect of HP (100 MPa, 65–85 °C, 3–12 h) on the inactivation of *B. coagulans* spores in ketchup (pH 4.0) and neutralized ketchup (pH 7.0) and in potage (pH 7.0) and acidified potage (pH 4.0) was studied (Islam et al., 2006). *B. coagulans* spores tended to be more resistant in neutralized foods than in acid foods during heat and HP treatment and to be more resistant in ketchup than in potage during HP treatment. Finally, Wang et al. (2009) found that *B. coagulans* spores were much more resistant to HP (400–600 MPa) at moderately elevated temperature (70 and 80 °C) in ultra-high temperature treated (UHT) milk than in phosphate buffer (100 mM, pH 6.7).

The few published HP studies with *A. acidoterrestris* also only document spore inactivation, not germination. Lee et al. (2002) reported that spore reduction by HP (207, 414 or 621 MPa, 10 min) was strongly temperature dependent. While viability was not appreciably reduced at room temperature, increasing levels and rates of inactivation were observed at 45, 71 and 90 °C respectively. In a follow-up study, inactivation of *A. acidoterrestris* spores by HP was shown to be suppressed by high solute concentration in apple juice concentrates (Lee et al., 2006).

It can be concluded that data about the effect of HP on the spores of thermoacidophilic bacteria are fragmentary. In particular, little is known about the HP-induced germination of these spores at low pH under different process conditions and in real foods. This is a relevant issue because HP inactivation of spores at moderate temperature is dependent on prior germination, and spore germination of neutrophilic *B. subtilis* is completely inhibited at low pH (Wuytack and Michiels, 2001). Therefore, the objective of this work was to investigate the effect of HP (100–800 MPa) at different temperatures (25–60 °C) on the inactivation and the germination of *B. coagulans* and *A. acidoterrestris* spores at low pH in buffer and tomato sauce.

2. Materials and methods

2.1. Preparation of tomato sauce

Tomato sauce was manually prepared by mixing the different ingredients (Table 1) in a sterile beaker, heating in a water bath at 70 °C without agitation to a center temperature of 65 °C, and then stirring for 10 minutes. When necessary, the pH was adjusted to 5.0 with 3.0 M NaOH after cooling.

2.2. Bacterial strains and spore production

B. coagulans LMG6326 and *A. acidoterrestris* LMG16906 were obtained from the LMG culture collection (Ghent, Belgium) and grown respectively on Nutrient Agar (NA) composed of Nutrient Broth No.2 (25 g/l NB; Oxoid, Basingstoke, U.K.) and Agar No.1 (12 g/l; Lab M, Lancashire, U.K.) at 37 °C or *Bacillus acidoterrestris* Thermophilic Agar (BAT; Merck, Darmstadt, Germany) at 43 °C. To induce sporulation, a single colony of *B. coagulans* or *A. acidoterrestris* was grown in 4 ml NB or BAT broth at respectively 37 or 43 °C with shaking (200 rpm). After 48 h, 100 µl of this suspension was transferred into 900 µl sterile distilled water and surface-plated on respectively NA with 0.1 mM $MnSO_4$ or Potato Dextrose Agar (PDA; Oxoid, Basingstoke, U.K.). After 10 days of incubation at respectively 37 or 43 °C, spores were harvested by rubbing the surface of the plates with a sterile swab and 5 ml sterile distilled water. The spores were washed three times and stored in sterile distilled water at a concentration of respectively 10^6 and 10^7 spores/ml at −20 °C until use. Plating of these spore suspensions before and after a heat treatment (70 °C/30 min for *B. coagulans* (Sale et al., 1970) and 80 °C/10 min for *A. acidoterrestris* (Sinigaglia et al., 2003)) indicated that they contained less than 20% vegetative cells. For the HP experiments, non-heat treated spore suspensions were used to avoid any changes in pressure resistance. Just before the experiments, spores were suspended either in citric acid buffer (20 mM, pH 4.0 or 5.0), potassium phosphate buffer (10 mM, pH 7.0) or tomato sauce (pH 4.2 or 5.0) at approximately 10^5 - 10^6 spores/ml.

2.3. High hydrostatic pressure treatment

HP treatments were carried out in an eight 8 ml vessel HP equipment (HPIU-10000, 95/1994, Resato, Roden, the Netherlands). The temperature of the vessels was controlled by a water circuit connected to a cryostat. A mixture of glycols (TR15, Van Meeuwen, Weesp, the Netherlands) was used as pressure transferring liquid. Six to eight hundred µL of the spore suspensions were sealed in sterile polyethylene bags and treated at 100 to 800 MPa and 25 to 60 °C (initial temperature) during 10 minutes. Pressure was built up slowly (approximately 100 MPa/min) to minimize adiabatic heating and decompression was immediate. Under these conditions, a temperature increase of approximately 5–8 °C occurs in the pressure-

Table 1
Ingredients of tomato sauce.

Ingredient	% (w/w)
Double concentrated tomato puree (Elvea, Antwerp, Belgium)	25
Tomato passata (Elvea, Antwerp, Belgium)	14
Sodium chloride (Fisher Scientific, Leichestershire, U.K.)	1
Modified corn starch (Resistamyl 342, Tate and Lyle, Koog aan de Zaan, The Netherlands)	3.2
Distilled water	56.8

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transmitting fluid during pressurization, depending on the pressure applied (Van Opstal et al., 2005).

2.4. Determination of inactivation and germination of spores

Each HP treated sample was split in two 300 µl portions, of which one was immersed in a water bath at 70 °C for 30 min (*B. coagulans*) or 80 °C for 10 min (*A. acidoterrestris*) to inactivate germinated spores, and the other one was not heated. Both portions were then plated on NA (*B. coagulans*) and BAT (*A. acidoterrestris*) agar to count survivors. Pressure-induced inactivation was the difference between the plate count before and after the HP treatment, pressure-induced germination was the difference between the plate count before HP treatment and after HP and heat treatment, expressed in log CFU/ml. The lower detection limit of plating was 10 CFU/ml.

2.5. Reproducibility of results

All experiments were conducted in triplicate using a single spore suspension but independent HP treatments, and data are presented as mean values ± standard deviation. Significant differences were analyzed by Student's *t*-test using a 5% level of significance ($P<0.05$).

3. Results

3.1. Germination and inactivation of B. coagulans and A. acidoterrestris spores by HP treatment at 40 °C in buffer at neutral and low pH

To study the effect of low pH on the HP-induced germination and inactivation of *A. acidoterrestris* and *B. coagulans* spores, spore suspensions of *B. coagulans* and *A. acidoterrestris* in citric acid buffer (20 mM, pH 4.0 and 5.0) or potassium phosphate buffer (10 mM, pH 7.0) were subjected to HP treatments at 100 to 600 MPa and at 40 °C for 10 minutes. Since for *B. coagulans* germination was mostly observed at higher pressures, the pressure range was further extended to 800 MPa for this organism. The temperature of 40 °C was chosen for this experiment to stimulate germination without causing a thermal pasteurization effect. In those bacterial spores where it has been studied, HP germination is limited or absent at room temperature but rapidly increases at process temperatures of 40 °C or more (Van Opstal et al., 2004; Wuytack et al., 1998).

The results show that the HP treatments, irrespective of pH value, caused no significant inactivation of *A. acidoterrestris* spores (Fig. 1B). However, a considerable degree of spore germination was observed, especially in the low pressure range (100 – 300 MPa) (Fig. 1A). A maximum of about 2.5 log germination occurred at 200 MPa in the pH 7.0 buffer. Germination was lower in the high pressure range and was positively related to pH at all pressures, except perhaps at 200 MPa where more germination –although not significantly- was observed at pH 4.0 than at pH 5.0. At 600 MPa there was still between 0.6 and 1 log germination.

B. coagulans showed a markedly different behavior in most aspects. The only point of similarity was the lack of HP inactivation over the entire pressure range and at all pH values, except perhaps at 800 MPa in pH 4.0 buffer where close to 1 log inactivation was observed (Fig. 1D). However, two additional effects were observed. First, exposure to the low pH buffer reduced the spore counts of *B. coagulans*, even without HP or heat treatment, by up to about 0.8 log at pH 4.0. This was not due to residual vegetative cells in the spore suspension since heat treatment of the pH 7.0 spore suspension did not significantly reduce the counts. Second, at pH 7.0, spore counts increased by about 0.7 – 0.8 log after HP treatment at all pressures, indicating HP-induced activation of 'superdormant' spores. Remarkably, these superdormant spores were not activated by the heat treatment (70 °C/30 min) applied in this work (compare white bar at 0.1 MPa in Fig. 1C and D). It cannot be excluded, of course, that they would have been activated by a higher intensity heat treatment. HP treatment also induced germination of *B. coagulans* spores, but unlike in *A. acidoterrestris*, germination occurred only in the high pressure range (500 – 800 MPa) and was inversely related to buffer pH (Fig. 1C). The maximal degree of germination was about 1.8 log and occurred at 700 MPa in the pH 4.0 buffer. As was the case for *A. acidoterrestris*, germinated spores were not inactivated during the HP treatment.

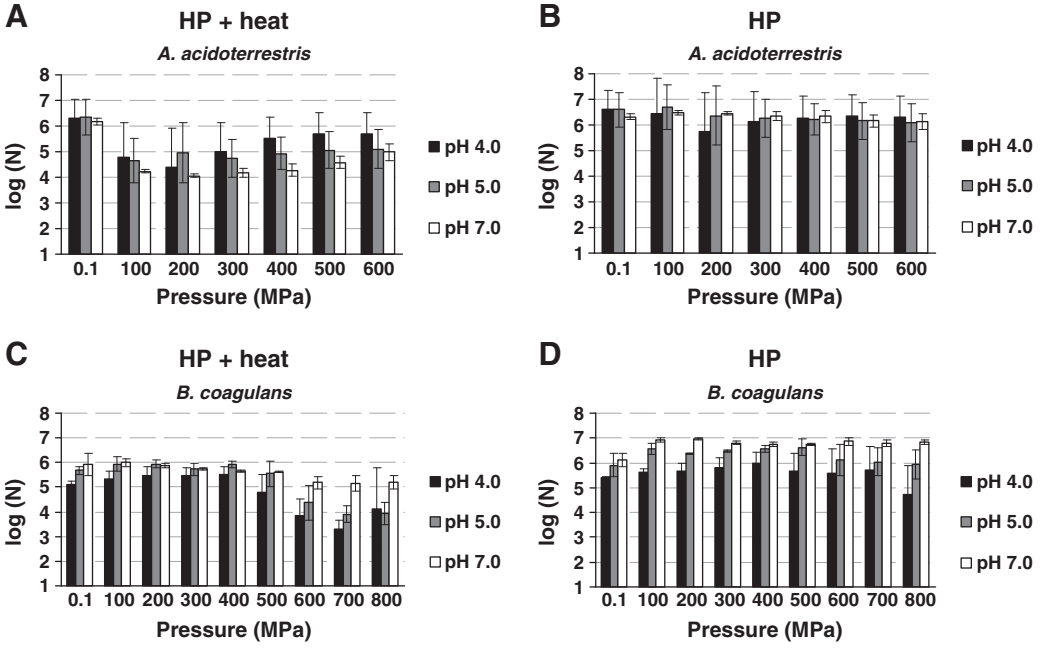

Fig. 1. Plate count (log N) after HP and heat treatment (A and C) and after HP treatment only (B and D) of *A. acidoterrestris* (A and B) and *B. coagulans* (C and D) spore suspensions. HP treatment was at 40 °C for 10 min in citric acid buffer (20 mM, pH 4.0 or 5.0) and potassium phosphate buffer (10 mM, pH 7.0). Heat treatment was 70 °C for 30 min (*B. coagulans*) or 80 °C for 10 min (*A. acidoterrestris*).

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3.2. Germination and inactivation of B. coagulans and A. acidoterrestris spores by HP treatments at 40 °C in tomato sauce

The above findings with spore suspensions in buffer were validated in a real acidic food by conducting a similar experiment in tomato sauce at pH 4.2 and 5.0. Additionally, HP treatments were not only conducted at 40 °C but also at 25 and 60 °C to assess the effect of process temperature on spore germination and inactivation. The results for *A. acidoterrestris* and *B. coagulans* are shown respectively in Figs. 2 and 3.

For the HP treatment at 40 °C, several effects observed in buffer were confirmed in tomato sauce, but there were also differences, particularly for *A. acidoterrestris*. Like in buffer, HP treatment alone did not inactivate *A. acidoterrestris* spores in tomato sauce at pH 5.0. However, unlike in buffer at pH 4.0, there was from 1 to 1.5 log inactivation in tomato sauce at pH 4.2 (Fig. 2B and D). Overall, there was more germination of *A. acidoterrestris* spores in tomato sauce than in buffer, with little difference at low and high pressure. Remarkably, there is a trend for germination to be more pronounced at pH 4.2 than at pH 5.0 (however only significant at 5% level for 200 MPa treatment), which is opposite from what was seen in buffer (Fig. 2A and C). For *B. coagulans*, observations for the HP treatment at 40 °C in tomato juice concurred with those in buffer: no inactivation -even some activation- upon pressure treatment alone (Fig. 3B and D), and germination only at 600 – 800 MPa and more pronounced at the lower pH value (Fig. 3A and C).

3.3. Effect of HP process temperature on germination and inactivation of B. coagulans and A. acidoterrestris in tomato sauce

Comparison of the results of HP treatments in tomato sauce at 40 °C with those at 25 and 60 °C indicate a strong temperature effect on spore germination and inactivation for both organisms. Since 60 °C is well below the temperature range where thermal inactivation of spores occurs, we assume that any spore inactivation caused by HP treatment at this temperature is due to spore germination followed by heat inactivation of the germinated spores. In *A. acidoterrestris,* higher process temperatures did not (pH 4.2) or only moderately (pH 5.0) stimulate germination in the low pressure region, but strongly stimulated germination in the high pressure region. At all temperatures and pressures, germination of *A. acidoterrestris* was higher at pH 4.2 than at pH 5.0 (Fig. 2A and C). Inactivation of *A. acidoterrestris* by HP treatment depended on the process temperature but not on the pressure level applied. There was little or no inactivation at 25 °C, 1 – 1.5 log at 40 °C, and 2.5 – 3.5 log at 60 °C (Fig. 2B and D). However, even at 60 °C not all germinated spores were inactivated in the HP process. Finally, spore inactivation of *A. acidoterrestris* was always higher at pH 4.2 than at pH 5.0 with few exceptions, probably as a direct consequence of the higher germination at this low pH.

Similar effects of process temperature were seen for *B. coagulans* spores. An increase in process temperature strongly stimulated spore germination at high pressures (600–800 MPa), but germination in the low pressure range -which was already absent at 40 °C- was also not induced even at 60 °C (Fig. 3A and C). A process temperature of 60 °C also resulted in spore inactivation under HP, although, as was the case for *A. acidoterrestris*, not all germinated spores were inactivated (Fig. 3B and D). Finally, a remarkable observation for *B. coagulans* is that the spore activation induced at 40 °C (even at 0.1 MPa) is suppressed at 60 °C.

4. Discussion

In this work we have studied germination and inactivation of spores from the thermophilic and aciduric *B. coagulans* and *A. acidoterrestris* by HP at moderate temperature in low pH buffer and in tomato sauce. A number of HP studies on these spores have been done previously, but this is the first comprehensive study involving a large number of pressure and temperature combinations in the HP pasteurization domain ($T \leq 60$ °C) and addressing not only spore inactivation but also spore germination.

In a first experiment, spore inactivation and germination at different pressures from 100 to 600 or 800 MPa was analyzed in buffers at pH 4.0, 5.0 and 7.0 for comparison (Fig. 1). A process temperature of 40 °C was chosen for this experiment because bacterial spores often show little or no germination at or below room temperature (Oh and Moon, 2003; Van Opstal et al., 2004; Wuytack et al., 1998). A general conclusion from this experiment for both bacteria is that HP treatment at 40 °C fails to cause any significant inactivation of the spores, except perhaps for *B. coagulans* at the most extreme condition (pH 4.0, 800 MPa). However, the application of an additional heat treatment after the HP treatment but prior to plating, revealed a considerable degree of spore germination that was dependent on the conditions and that showed a strikingly different pattern for both organisms. *A. acidoterrestris* spores germinated at all pressures but most extensively at low pressures (100–300 MPa), whereas *B. coagulans* germinated only at high pressures (500–800 MPa). Furthermore, both bacteria also showed an opposite pH dependence of spore germination in buffer, with *A. acidoterrestris* germination being somewhat reduced, and *B. coagulans* germination

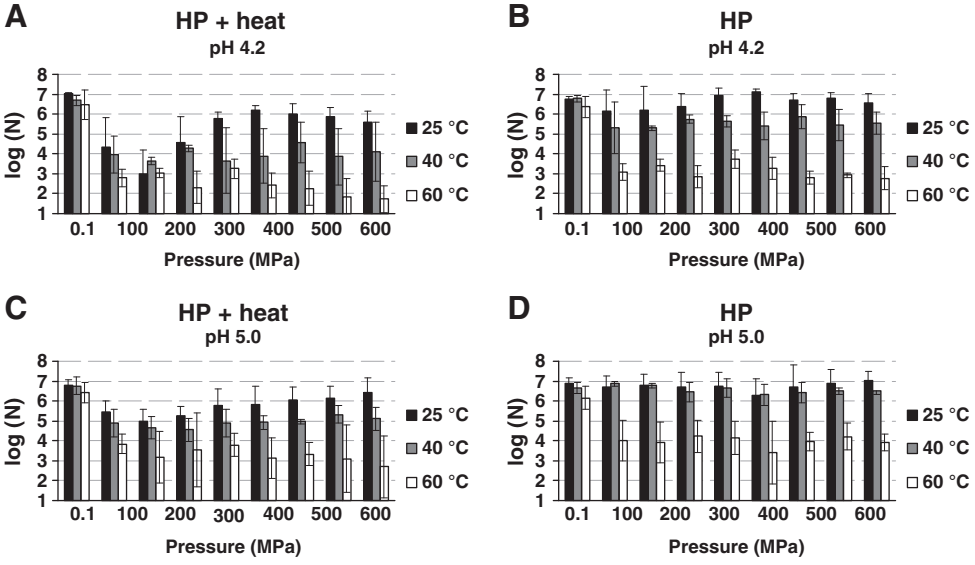

Fig. 2. Plate count (log N) of *A. acidoterrestris* spores after HP and heat treatment (A and C) and after HP treatment only (B and D) in tomato sauce at pH 4.2 (A and B) and pH 5.0 (C and D). HP process temperatures were 25, 40 and 60 °C for 10 min. Heat treatment was 80 °C for 10 min.

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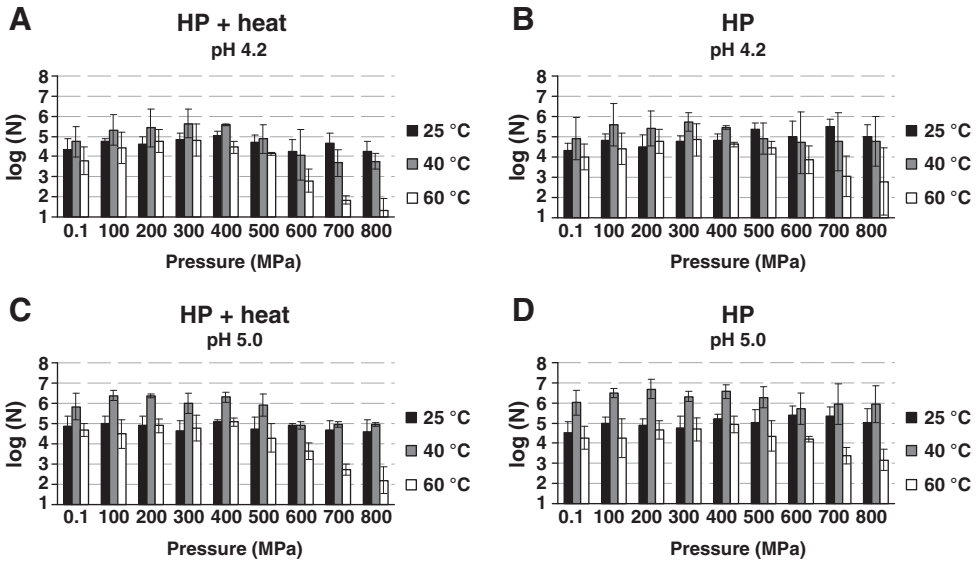

Fig. 3. Plate count (log N) of *B. coagulans* spores after HP and heat treatment (A and C) and after HP treatment only (B and D) in tomato sauce at pH 4.2 (A and B) and pH 5.0 (C and D). HP process temperatures were 25, 40 and 60 °C for 10 min. Heat treatment was 70 °C for 30 min.

being stimulated at low pH. These observations may indicate the existence of physiologically different germination pathways in these bacteria under the conditions of this experiment. The existence of a distinct low and high pressure germination pathway has been demonstrated in *B. subtilis* previously (Wuytack et al., 1998). A remarkable difference with *B. subtilis*, on the other hand, is the fact that germination can take place at low pH, since HP germination of *B. subtilis* spores is completely inhibited at pH<5.0 (Wuytack and Michiels, 2001). This may be related to the acidophilic or acidotolerant nature of both *A. acidoterrestris* and *B. coagulans*, which can grow down to about pH 2.5 (Yamazaki et al., 1996) and 4.0 (Mallidis et al., 1990) respectively. A few additional comments can be made about the behavior of *B. coagulans* spores, based on the data in Fig. 1. First, HP treatment at all pressures causes a slight but consistent increase in spore count at pH 5.0 and 7.0 (e.g. 0.8 log at 100 MPa in pH 7.0 buffer). This may indicate the existence of a small fraction of so-called superdormant spores which are activated by HP treatment. Second, resuspension of *B. coagulans* spores in low pH buffer slightly decreases the spore count (about 0.8 log). Since the spore suspensions contain little or no vegetative cells, and since *B. coagulans* is acidotolerant, this reduction is unlikely to represent cell death, and the most likely explanation is that an extra fraction of spores is induced to become superdormant at low pH. However, this was not further studied in this work.

In a second experiment, spore germination and inactivation was examined in tomato sauce at pH 4.2 and 5.0 at 40 °C but also at 25 and 60 °C. At 40 °C, a similar overall pattern was observed as in buffer for both bacteria, except that for *A. acidoterrestris* the effect of pH on germination was reversed since more germination was detected at pH 4.2 than at pH 5.0 in tomato sauce. In addition, germination levels were higher for both bacteria in tomato sauce than in buffer. This may be related to a cooperative effect between HP and physiological germinants present in the tomato sauce. A similar effect has been observed before upon HP treatment of *B. cereus* spores in milk (Van Opstal et al., 2004).

The HP treatments at different temperatures revealed a remarkable difference between germination at low (100–300 MPa) and high (500 – 800 MPa) pressure. High pressure germination, which occurred in both bacteria, showed a strong positive temperature dependence, whereas low pressure germination, which occurred only in *A. acidoterrestris* was little (pH 5.0) or not (pH 4.2) affected by temperature. This is a further indication that the high and low pressure germination proceeds via distinct mechanisms. Spore inactivation was absent or limited when the HP treatment was conducted at 25 or 40 °C for both bacteria, even under conditions which induced germination. However, at 60 °C, spore inactivation was observed under all the conditions which induced spore germination, and the degree of inactivation was almost equal to the degree of germination, indicating that spore inactivation takes place by pressure-induced germination followed by heat inactivation of the germinated spores. Similar observations have been made for spores of non-acidiphilic bacteria at neutral pH (Sale et al., 1970; Van Opstal et al., 2004). However, spores from neutrophilic bacteria do not germinate under HP at low pH, and consequently cannot be inactivated by HP treatment at 60 °C in acidic foods (Wuytack and Michiels, 2001). Our work shows for the first time that HP induces germination of spores from acidophilic or acidotolerant bacteria at low pH.

The lack of germination in *B. coagulans* at low pressure, even at 60 °C, may indicate that the low pressure pathway does not exist in this organism. Studies in *B. subtilis* have shown that low pressure germination proceeds through activation of the nutrient receptors, whereas high pressure germination bypasses these receptors (Wuytack et al., 2000). Thus, it could be that the germinant receptors in *B. coagulans* are not susceptible to HP activation.

The results of this work can be useful to optimize HP pasteurization processes for acidic foods in order to prevent spoilage by thermo-acidophilic sporeformers. In principle, two approaches are possible. The first is a two-step approach consisting of a mild 'cold' HP process to induce spore germination followed by the inactivation of the germinated spores, e.g. by a mild heat treatment. The advantage of such a process compared to a purely thermal process is that it causes a much lower thermal burden on the product, since HP germinated spores of *A. acidoterrestris* and *B. coagulans* can be inactivated at 70 – 80 °C (as shown in this work), while inactivation of intact spores requires processing at 90 – 110 °C (Ceviz et al., 2009; Palop et al., 1999;). Our results indicate that the two-step approach is only possible for *A. acidoterrestris*, since *B. coagulans* spores can not be germinated by mild cold HP treatment (Fig. 3A and C). For *A. acidoterrestris* in tomato sauce of pH 4.2, a 4 log reduction can be achieved by treatment at 200 MPa (25 °C) for 10 min, followed by heat treatment at 80 °C (Fig. 2A). Since germination of *A. acidoterrestris* was stimulated by lowering the pH of the tomato sauce, it is possible that even higher levels of germination, and thus also inactivation, can be achieved with a similar two-step process in more acidic products like apple juice.

The second approach to inactivate *A. acidoterrestris* and *B. coagulans* spores is a single-step HP process at elevated temperature. For

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A. acidoterrestris, HP treatment for 10 min at 60 °C caused 2.5 - 3.5 log inactivation in tomato sauce of pH 4.2, irrespective of the pressure used (Fig. 2B). However, it is likely that higher levels of inactivation can be achieved by further increasing the process temperature, as was demonstrated by Lee et al. (2002) for *A. acidoterrestris* spores in apple juice. For *B. coagulans*, HP processing at 60 °C required high pressures to induce germination and achieve inactivation. In the pH 4.2 tomato sauce, a 10 min treatment at 700 MPa (60 °C) resulted in a 2 log inactivation. For comparison, Roberts and Hoover (1996) reported 2 and 4.5 log inactivation of *B. coagulans* in McIlvaine buffer (pH 4.0) upon treatment at 400 MPa for 15 min at 45 and 70 °C respectively, indicating that the spores of some strains of *B. coagulans* can be germinated at lower pressures.

In conclusion, we demonstrated that HP treatment can induce germination of *A. acidoterrestris* and *B. coagulans* spores in acidic conditions. This phenomenon is probably linked to the acidophilic character of these bacteria, since germination of spores from the neutrophilic *B. subtilis* is inhibited at low pH. Further, HP treatments conducted at moderately elevated temperature (e.g. 60 °C) or followed by a moderate heat treatment can kill these acidothermophilic spores, and thus offer perspectives to reduce spoilage problems with acidic products caused by these bacteria.

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