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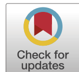

The effect of citric acid, heat, and high-pressure on the morphology and physiology of *Bacillus* spores: Simulating high-pressure processing of complementary foods

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ABSTRACT

To ensure the safety and quality of infant complementary foods (CFs), a combination of citric acid (CA), heat (H), and high-pressure (HP) treatments can be used. The impact of these treatments on the physiological state and morphology of spores of *Bacillus* spp. was studied. *B. amyloliquefaciens* TMW 2.479 Fad 82 spores were suspended in CA and subjected to H at 100 °C for 25 min, followed by HP at 600 MPa for 3 and 4 min at 9 °C. Spore germination rate for all the treatments was below 10 % and all the treatments except CA affected the spore's morphology. However, H treatment alone or combined with HP and CA disrupted the internal membrane (IM) and enhanced the damage to the *B. amyloliquefaciens* spores Cortex (Cx). Acidification of the *B. amyloliquefaciens* spores with CA led to the permeability of the spore's IM but with intact Cx. The combined treatments of CA, H, and HP significantly affected the integrity of the spore's intracellular structure. This study elucidates how the sequential application of CA, H, and HP can affect the morphology and physiology of *Bacillus* spores and improves the safety and quality of infant CFs.

1. Introduction

Complementary foods (CFs) supplements essential nutrients that are important for the dietary development of infants from the age of 6 months (Malik, Krishnaswamy, & Mustapha, 2021; World Health Organization, 2023). The introduction age of commercially processed foods varies, starting as young as 6 months in South Africa and even earlier in parts of South Asia (Manikam et al., 2018; Sayed & Schönfeldt, 2018). These foods may harbour spoilage and pathogenic microbes, including spore formers (Engel et al., 2022; Ismail et al., 2021; Mario, Belay, & Amare, 2024; Teng et al., 2024; Theurich, Humphreys, Gosselin, & McCool-Myers, 2019). Studies have identified pathogens like *Cronobacter, Staphylococcus aureus, Salmonella, and Bacillus cereus* in CFs, with *B. cereus* being highly prevalent in ready-to-eat products in China (Teng et al., 2024). Other *B.* species such as *B. subtilis, B. licheniformis, B. circulans, B. macerans*, and *B. polymyxa* have also been isolated from commercial infant foods and chilled vegetable-based purees (Carlin et al., 2000; Sadek, Abdel-Rahman, Azab, Darwesh, & Hassan, 2018). Despite the rarity of foodborne illnesses outbreaks linked to CF consumption, Azemi et al. (2013) identified bacteria like *Salmonella, Escherichia coli*, and spore formers such as *Clostridium difficile, C. botulinum*, and *B. cereus* as primary agents causing acute infectious diarrhoea in infants treated in a paediatric clinic. The recent WHO guidelines for complementary feeding emphasize safe preparation practices for CFs (WHO, 2023), highlighting the need for processing techniques that maintain CF safety and nutritional integrity required to maintain children's health (Malik et al., 2021).

High-pressure processing (HPP) has been adopted by the infant food industry to produce safe, fresh-tasting CFs while preserving nutritional qualities (Balasubramaniam, Martínez-Monteagudo, & Gupta, 2015; Pasdar et al., 2024). Industrial HPP applies isostatic pressure between 400 and 600 MPa for durations of 1.5–6 min at temperatures under 45 °C (Koutsoumanis et al., 2022). Under these industrial HPP conditions, pathogens like *E. coli, Salmonella, Listeria monocytogenes*, and *Cronobacter sakazakii* are effectively inactivated by 1–6 log CFU/g in beverages (Liao et al., 2023) and >5 log CFU/g in human milk (Liang et al., 2023). However, these HPP parameters are ineffective against *Bacillus* spores, showing <1 log CFU/mL inactivation in beverages and human milk (Liang et al., 2023; Liao et al., 2023), failing to meet the ≥5 log CFU/g industrial requirement (Georget et al., 2015). Researchers have explored high-pressure (HP) based hurdle interventions to deactivate resistant spores (Ozkan, Subasi, Capanoglu, & Esatbeyoglu, 2022; Pou &

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Raghavan, 2020; Roobab et al., 2022). The effectiveness of these methods depends on the combination of treatments used with HP (Cho & Chung, 2020; Delbrück, Zhang, Heydenreich, & Mathys, 2021; Setlow & Christie, 2021). Pressure-assisted thermal processing (PATP) or HP thermal processing (HPTP) at pressures above 600 MPa and temperatures above 90 °C for 5 min can achieve >5 log CFU/g reduction in buffer and food matrices (Al-ghamdi, Sonar, Patel, & Albahr, 2020; Ratphitagsanti, De Lamo-Castellvi, Balasubramaniam, & Yousef, 2010). However, these technologies are not yet successfully available in the food industry, probably due to technical limitations on the HPP equipment (Balasubramaniam et al., 2015; Georget et al., 2015). Combining heat (H), weak organic acids, and HP has shown promise in reducing the resistance of *B. amyloliquefaciens* spores (Ratphitagsanti et al., 2010). While this study applied PATP at 700 MPa and 105 °C for up to 5 min, our study aims to apply sequential HPP parameters used in the baby food industry (suspending citric acid (CA) first, followed by H, then HP) to produce HP-processed CFs. The process of spore inactivation by HP involves germination or structural and physiological damage (Delbrück et al., 2021; Rao et al., 2016; Wang, Xia, & Li, 2017). This affects the spore's functionality through structural and functional modifications (Mathys, Chapman, Bull, Heinz, & Knorr, 2007), impacting metabolic dormancy, protective layers, DNA protection, and resistance to processing treatments (Georget et al., 2015; Kanaan et al., 2022). In the germination-inactivation strategy, HP or food additives can trigger spore germination, resulting in viable cells that are more vulnerable to further processing conditions (Delbrück et al., 2021; Lyu et al., 2023; Mújica-Paz et al., 2011). However, if the germinated spores are not eliminated by an additional treatment step, this could lead to spoilage and food safety issues (Aldrete-Tapia & Torres, 2020; Setlow et al., 2017; Wells-Bennik et al., 2016), particularly for refrigerated CFs during shelf-life. This presents a significant challenge that the infant food industry needs to address. However, not all spores are activated to germinate by HP (Margosch, Gänzle, Ehrmann, & Vogel, 2004; Wells-Bennik et al., 2016). Damage to the *Bacillus* spore structure, particularly the spore cortex (Cx) and inner membrane (IM), can compromises spore resistance (Rao et al., 2016; Wang et al., 2017). The spore Cx, which is a thick peptidoglycan layer, encloses the less permeable internal membrane (IM) (Driks, 2002, 2003 & 2004). The Cx is responsible for the structural integrity of the spores, while the IM protects the genetic materials and the essential spore components (Aldrete-Tapia & Torres, 2020; Delbrück et al., 2021). Therefore, depending on the treatments applied, activation or suppression of spore germination is crucial to effectively inactivate spores (Delbrück et al., 2021) and ensure safety of CFs. Studies have shown that HPP alone (300 MPa for 20 min) had no effect on *B. cereus* spore structure, while combining HP with slightly acidic electrolyzed water (SAEW) damaged spore morphology and distorted the Cx and IM (Wang et al., 2017). However, no studies have analysed how CA, H, and HP applied in sequence affect germination, physiology, and intracellular structure of *Bacillus* spores. Understanding the mechanism of spore inactivation involving damage to spore morphology and physiology is essential for destabilising spore functionality (Rao et al., 2016). The unpredictable response of *Bacillus* spores to processing treatments raises concerns regarding the production of HP-processed CFs (Lyu et al., 2023; Zhang, Delbrück, Off, Benke, & Mathys, 2020). This study aims to investigate the effects of sequentially applied CA, H, and HP on *Bacillus* spore morphology and physiological state during CF processing. The goal is to develop effective HP-based inactivation strategies that target *Bacillus* spores, enhance the quality and safety of CFs, and ultimately improve infant health outcomes. Understanding the interactions and effects of these treatments on spores will inform the development of better *Bacillus* spore inactivation methods, ensuring the safety of CFs (Brul et al., 2011; Zhang et al., 2020).

2. Materials and methods

2.1. *B. amyloliquefaciens* spore preparation

B. amyloliquefaciens TMW 2.479 Fad 82 which produce spores that are resistant to heat and pressure (Margosch et al., 2006) was collected from the Department of Agricultural, Food and Nutritional Science, University of Alberta, Edmonton, Alberta, Canada, was grown according to the method of Ratphitagsanti et al. (2010) with some modifications. Sporulation was monitored by phase contrast microscopy. Centrifugation at 8000×g for 15 min at 4 °C was used to harvest the spores (Centrifuge model Z366K, Hermle Labortechnik, Germany), washed five times with sterile double distilled water (DDW) and heated at 80 °C for 30 min. Spore pellets were resuspended in sterile DDW to obtain a stock culture of approximately 10^9 spores/mL and stored at 4 °C.

2.2. Spore preparation for the different treatments

As described by Ratphitagsanti et al. (2010), aliquots (0.2 mL) of *B. amyloliquefaciens* spore suspension and 1.8 mL of sterile CA solution (0.1 M; pH 5, and DDW; pH 6.8) were prepared to obtain approximately 10^8 CFU/mL. The spore suspensions were aseptically transferred into sterile vacuum bags (5 × 2.5 cm polyethylene bags, 01-002-57, Fisher Scientific, Pittsburgh, Pennsylvania, USA). The bags were heat sealed, and all inoculated vacuum bags were stored for up to 2 h in an ice–water bath (4 °C) before treatment, as indicated in Table 1. Spores of *B. amyloliquefaciens* suspension were treated by first suspending the spores in CA at pH of 5, followed by H at 100 °C for 25 min, and then HPP at 600 MPa; 9 °C for 3- and 4-min holding time, to simulate the South African food industry processing conditions for HP processed CFs such as the chilled infant purees (SAHPP, 2024).

2.3. Estimation of *B. amyloliquefaciens* spore germination under citric acid, heat and high-pressure processing conditions

B. amyloliquefaciens spore germination was estimated as described by Ratphitagsanti et al. (2010) with some modifications. After treatments (Table 1), 4-mL spore suspension for each treatment was divided into two equal portions to estimate spore count with and without heat shock and 2 mL of the treated spore suspension plated on TSAYE (Oxoid) was incubated at 37 °C for 48 h. Another 2 mL of the treated spore suspensions were heat shocked at 80 °C for 20 min to kill germinated spores and enumerated as described. Heat sensitivity was used as a criterion for spore germination (Black et al., 2008; Van Opstal, Bagamboula, Vanmuysen, Wuytack, & Michiels, 2004; Wuytack, Boven, & Michiels, 1998, pp. 3220–3224).

The percentage of spore germination in treated spores (Table 1) was estimated using Eq. (1) from the knowledge of the total surviving

Table 1
Citric acid, heat and high-pressure processing treatment conditions of *B. amyloliquefaciens* TMW 2.479 Fad 82 spores.

	Processing combinations
A[a]	Untreated spores (Control)
B	Acidification only (CA)
C	Heat treatment only at 100 °C for 25 min (H)
D	Acidification before heat treatment (CA/H)
E	High pressure processing for 3 min (HPP3)
F	Acidification before HPP3 (CA/HPP3)
G	Heat treatment before HPP3 (H/HPP3)
H	Acidification before heat treatment, followed by HPP3 (CA/H/HPP3)
I	High pressure processing for 4 min (HPP4)
J	Acidification before HPP3 (CA/HPP4)
K	Heat treatment before HPP4 (H/HPP4)
L	Acidification before heat treatment, followed by HPP4 (CA/H/HPP4)

[a] Letters A – L represents treatments (samples) used in this study under the different processing combinations.

population after these treatments (Ntreatment). In addition to the number of dormant spores that survived the treatments, heat shock (HS) (Ntreatment + HS) at 80 °C for 20 min was followed.

$$\% \, Spore \, Germination = \frac{[Ntreatment - (Ntreatment + HS)]}{Ntreatment} \times 100 \quad (1)$$

2.4. Flow cytometry analysis of heated and high-pressure treated B. amyloliquefaciens spore in citric acid

All CA, H and HPP treated samples and combinations were snap-frozen after treatment and stored at −20 °C until flow cytometry (FC) analysis (Reineke, Schlumbach, Baier, Mathys, & Knorr, 2013). An Accuri C6 (BD Accuri Cytometer Inc., USA) equipped with a 15-mW air-cooled 488 nm argon-ion laser and a 633 nm red diode laser was used for this analysis, with double-distilled water used as the sheath fluid (Coulter Corporation, Miami, FL, USA). Staining of the samples was done by the double staining method (Mathys et al., 2007; Reineke, Schlumbach, et al., 2013), using dyes of propidium iodide (PI) (Invitrogen by Thermo Fisher Scientific, Oregon, USA) and SYTO16 (Invitrogen by Thermo Fisher Scientific, Oregon, USA) added to 1 mL of each sample to make a final concentration of 15 μM and 0.5 μM respectively. The final stained suspension concentration was approximately 10^7 CFU/mL, and samples were well-mixed with the fluorescent dyes and incubated in the dark at room temperature for 20 min before analysis. When bound to nucleic acids, the fluorescence excitation maximum for PI is 535 nm, and the emission maximum is 617 nm. The maximum values of SYTO16 for the absorption and fluorescence emissions of the complex with DNA observation were at 488 and 518 nm. Although both fluorescent dyes can stain DNA, the membrane-impermeant PI indicates membrane damage (Mathys et al., 2007), and the SYTO16 acts as an indicator for Cx damage or spore germination (Black et al., 2005; Reineke, Schlumbach, et al., 2013). Data acquisition was on a logarithmic scale set at a nominal flow rate of 15,000 events s^{-1}. Spores were analysed at a nominal data flow rate of 12 mL/min. We used FlowJo software (version 10.8.0) to analyse the flow cytometry data. The gating strategy applied the control, staining untreated spore with either PI or SYTO16. We based the live gate on untreated spores and as negative controls, ensuring that all spores remained alive and not stained with PI or SYTO16.

Spores that were unstained by the red (PI) and green (SYTO16) fluorescence were PI and SYTO16 negative, respectively and were represented by PI negative (PIN) and SYTO negative (SYN) on the histogram, indicating intact IM and Cx respectively. On the other hand, spores stained by PI and SYTO16 were PI and SYTO16 positive, respectively and were represented by PI positive (PIP) and SYTO positive (SYP), indicating damage to the IM and Cx, respectively.

2.5. Scanning electron microscopy (SEM) photograph of B. amyloliquefaciens spores treated with citric acid, heat and high-pressure

The method used by Wang et al. (2017) with some modifications, was used to analyse the effect of the treatments (Table 1) on the morphology of B. amyloliquefaciens spores. The spore suspensions were centrifuged at 4000×g for 15 min at 4 °C, and the supernatant removed. The spores' pellets extracted were centrifuged for a second time at 3000×g for another 3 min, and the pellets were extracted. The pellets were washed with 0.075 M phosphate buffer (PB) for 10 min before removing the PB supernatant. The pellets were pre-fixed with 2.5 % glutaraldehyde (GA) solution for 1 h. The GA solution was removed, and the pellets were washed 3 times in 0.1 M PB (pH 7.2). After 1 h of resuspension and fixing in 1 % osmium tetraoxide solution, the spore samples were rewashed three times in PB solution for 10 min each. After the PB solution was removed, pellets were dehydrated in 30 %, 50 %, 70 %, 90 % and 100 % series of ethanol for 10 min each, with dehydration in 100 % ethanol being repeated three times. Spore samples were left in 100 % ethanol for 30 min. Following dehydration, spore cells were covered in 50:50 mixture of hexamethyldisilazane (HMDS) and 100 % ethanol for 1 h. Upon the removal of the HMDS-ethanol mixture, 1–2 drops of 100 % HMDS were added to the spore cells, sprayed on slides, and allowed to dry overnight. Spore cells were then coated with carbon before mounting on the SEM (Zeiss 540 Ultra PLUS FEG; Zeiss Microscopy, Durban, South Africa).

2.6. Transmission electron microscopy (TEM) photograph of B. amyloliquefaciens spores treated with citric acid, heat and high-pressure

For the pretreatment of the B. amyloliquefaciens spores for the TEM analysis, fixation and dehydration of the spores were done according to the SEM analysis. After dehydration of the spores, the TEM analysis was conducted as described by Wang et al. (2017) and the spores placed on the copper wire mesh were observed and images collected using TEM (Jeol JEM 21000 200 kV, Tokyo, Japan).

2.7. Data analysis

All experiments were conducted in duplicate. Analysis of variance (ANOVA) was used to statistically analyse the effect of the different treatments (CA, H, and HPP for 3- and 4-min holding time) time) on the spore germination counts and percentage damage to the IM and Cx of B. amyloliquefaciens spores using GraphPad Prism (version 9.0.0). Posthoc analysis was carried out by Tukey's multiple comparison test to determine significant differences between the treatments ($P \leq 0.05$).

3. Results

3.1. Effect of citric acid, heat and high-pressure on B. amyloliquefaciens spore germination

B. amyloliquefaciens spore germination for all the treatments was below 10 %, ranging between 0.5 and 10 %, depending on the treatment combination (Fig. 1). Sequential processing of B. amyloliquefaciens spores with H/HPP3 and CA/H/HPP3 induced the lowest spore germination at 0.5 and 0.64 % respectively and compared to HPP3 alone, these treatments significantly ($p \leq 0.05$) affected the spore germination (Fig. 1). The application of only HPP3 or HPP4 induced 4 and 3 % spore

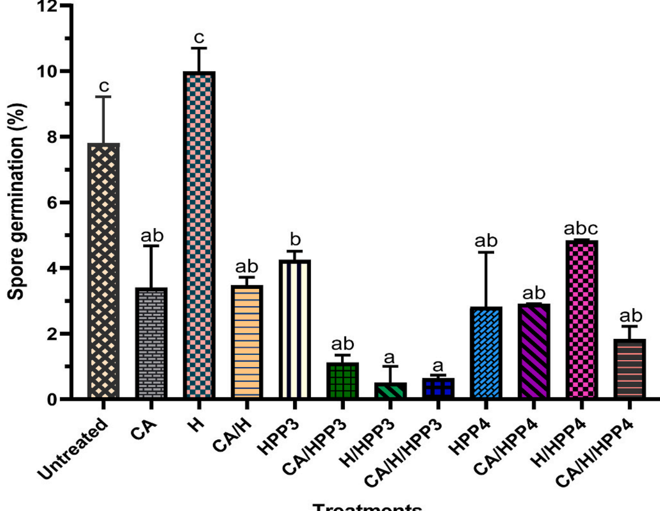

Fig. 1. Effect of Citric Acid (CA), Heat (H) and High-Pressure processing (HPP) at 600 MPa and 9 °C for 3 (HPP3) and 4 min (HPP4) on *B. amyloliquefaciens* spore germination (%). All data on the bar chart are presented in mean ± SD, (n = 2). Different letters a-c on the bar chart indicates statistically significant differences between all the treatments ($p \leq 0.05$).

germination respectively. H treatment on the other hand, significantly ($p \leq 0.05$) affected the germination of the spores with the highest spore germination of 10 %. In essence, out of all the treatments used in this study (Table 1) the spores that were treated with only H significantly ($p \leq 0.05$) germinated the spores after heat shock at 80 °C for 30 min. Although H/HPP4 induced the spore germination by 5 %, this outcome was similar to the HPP4, CA/HPP4, CA/H/HPP4 treatments with 3, 3 and 2 % spore germination respectively (Fig. 1).

3.2. Effect of citric acid, heat and high-pressure on the internal membrane of B. amyloliquefaciens spore

In comparison to the untreated B. amyloliquefaciens spores, CA (65 %), H (75 %), and combinations of CA/H (64 %), CA/HPP3 (61 %), H/HPP3 (81 %), CA/HPP4 (66 %), H/HPP4 (72 %), CA/H/HPP3 (64 %) and CA/H/HPP4 (65 %) significantly ($p \leq 0.05$) affected the spore's IM (Figs. 2 and 3). On one hand, the fluorescence histogram (Fig. 2) showed that the application of these treatments increased the red (PI) fluorescence, due to a significant ($p \leq 0.05$) increase in the spore's IM permeability. On the other hand, the HPP3 (17 %) and HPP4 (16%) treatments did not affect IM permeability (Figs. 2 and 3) of the B. amyloliquefaciens spores compared to the untreated spores with an intact IM (1.36 %), and a greater IM integrity (Fig. 2) for untreated spores and HP treated spores compared to other treatments applied during this study (Table 1).

3.3. Effect of citric acid, heat and high-pressure on the cortex of B. amyloliquefaciens spores

Treatment of the B. amyloliquefaciens spores with CA, HPP3 and HPP4 alone or when combined showed greater signs of intact Cx integrity. SYTO16 fluorescence was undetectable on the fluorescence histogram of these spores (Fig. 4A, B, 4E, 4F, 4I, and 4J). The level of Cx damage was below 8 % for these sets of treatments, which was comparable to the intact Cx of the untreated spores (Fig. 5). Nonetheless, combining CA and/or HPP3 and HPP4 with H significantly ($p \leq 0.05$) affected the spore's Cx, as shown by an increase in SYTO16 fluorescence

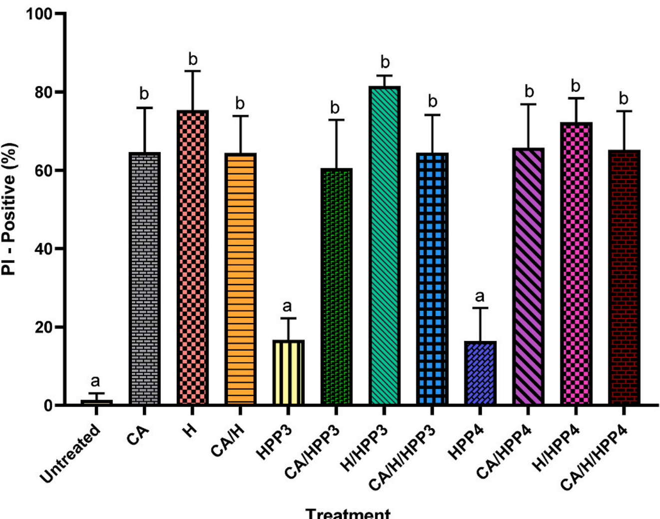

Fig. 3. Effect of citric acid (CA), heat (H) and high-pressure processing (HPP) at 600 MPa and 9 °C for 3 (HPP3) and 4 min (HPP4) on the permeability of *B. amyloliquefaciens* internal membrane. All data on the bar chart are presented in mean ± SD, (n = 2). Different letters a-c on the bar chart indicates statistically significant differences between all the treatments ($p \leq 0.05$).

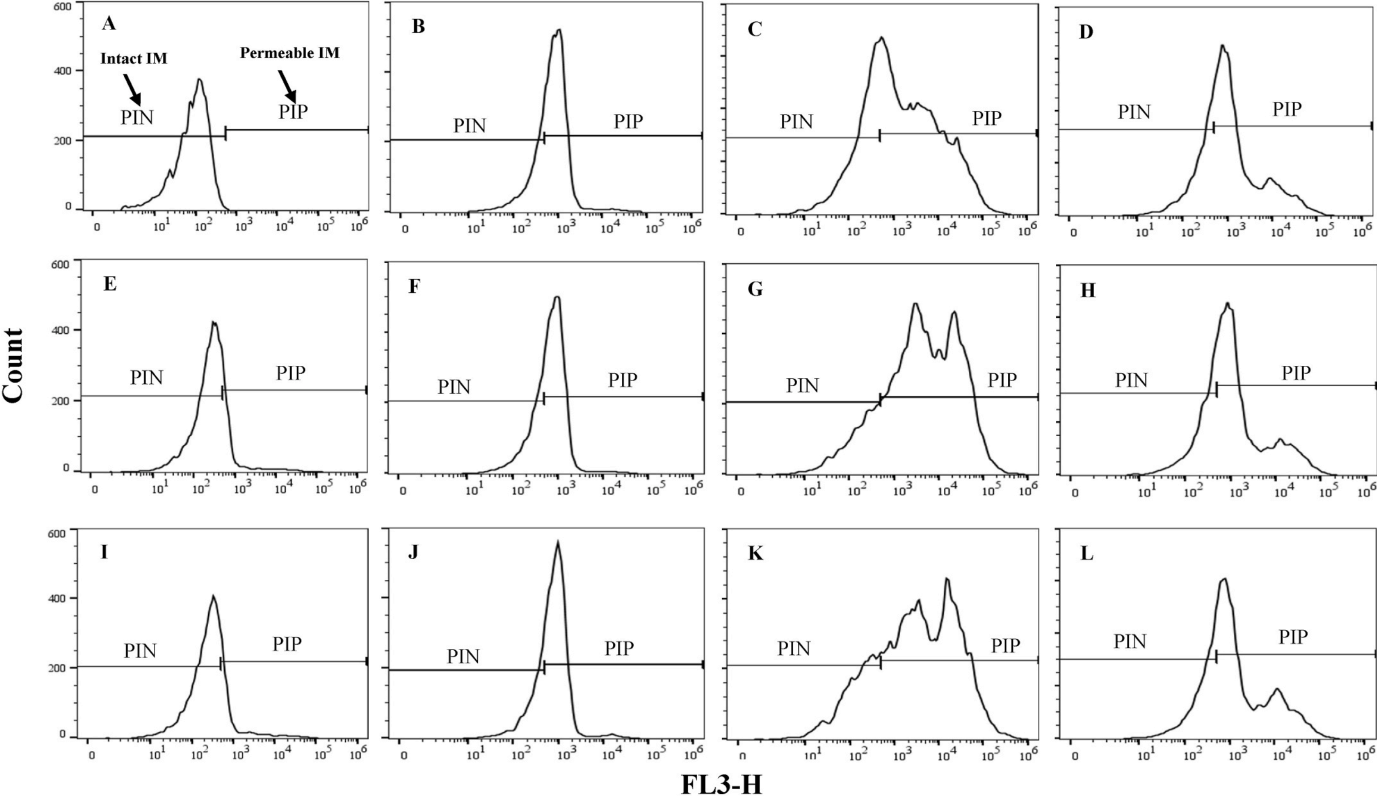

Fig. 2. Flow cytometry histogram of the effect of citric acid (CA), heat (H) and high-pressure processing (HPP) at 600 MPa and 9 °C for 3 (HPP3) and 4 min (HPP4) on the Internal Membrane (IM) of *B. amyloliquefaciens* spore after staining with Propidium iodide (PI). **A** – (Untreated spores), **B** (CA), **C** (H), **D** (CA/H), **E** (HPP3), **F** (CA/HPP3), **G** (H/HPP3), **H** (CA/H/HPP3), **I** (HPP4), **J** (CA/HPP4), **K** (H/HPP4), **L** (CA/H/HPP4). PIP – (Propidium Iodide stain positive), PIN (Propidium Iodide stain negative).

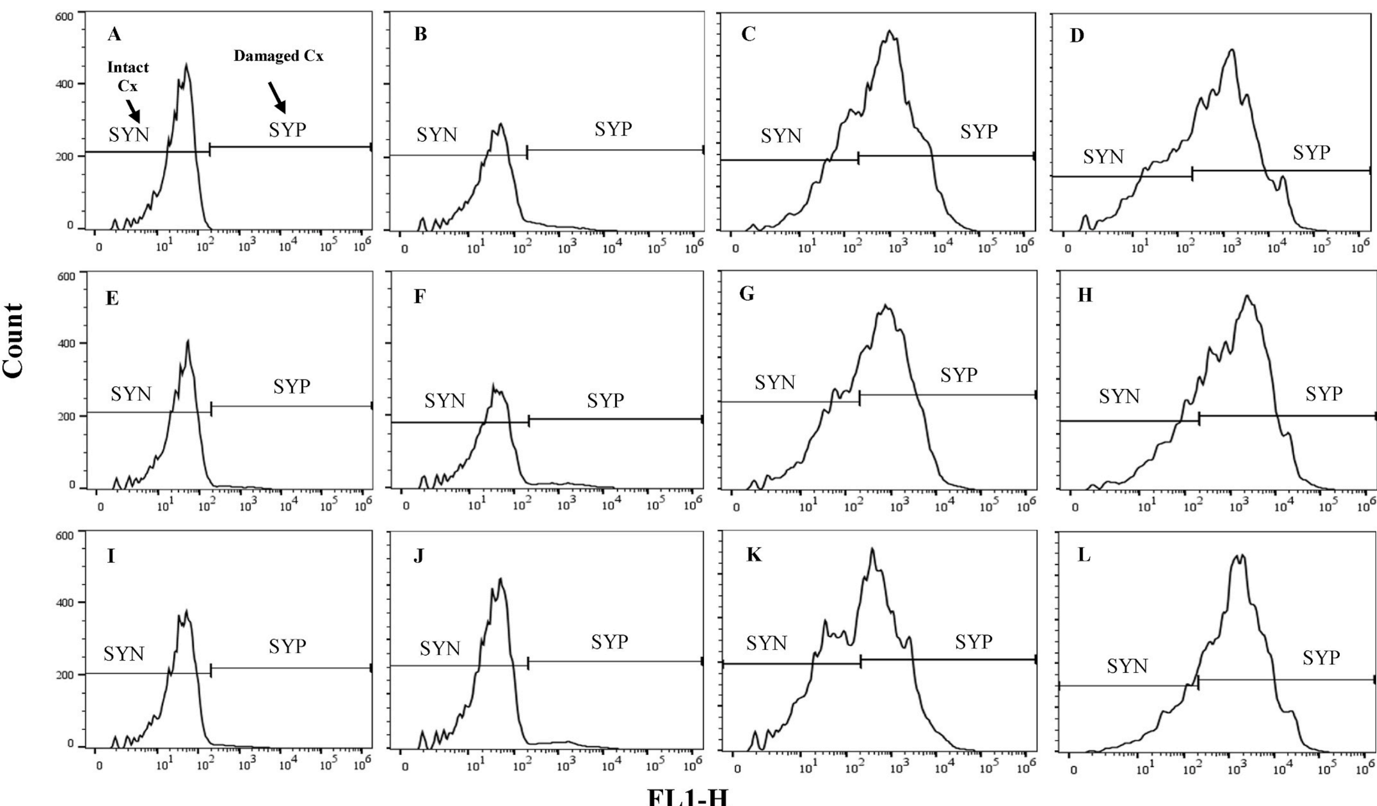

Fig. 4. Flow cytometry histogram of the effect of citric acid (CA), heat (H) and high-pressure processing (HPP) at 600 MPa and 9 °C for 3 (HPP3) and 4 min (HPP4) on the Cortex (Cx) of *B. amyloliquefaciens* spore after staining with SYTO16. **A** – (Untreated spores), **B** (CA), **C** (H), **D** (CA/H), **E** (HPP3), **F** (CA/HPP3), **G** (H/HPP3), **H** (CA/H/HPP3), **I** (HPP4), **J** (CA/HPP4), **K** (H/HPP4), **L** (CA/H/HPP4). SYP – (SYTO16 stain positive), SYN (SYTO16 stain negative).

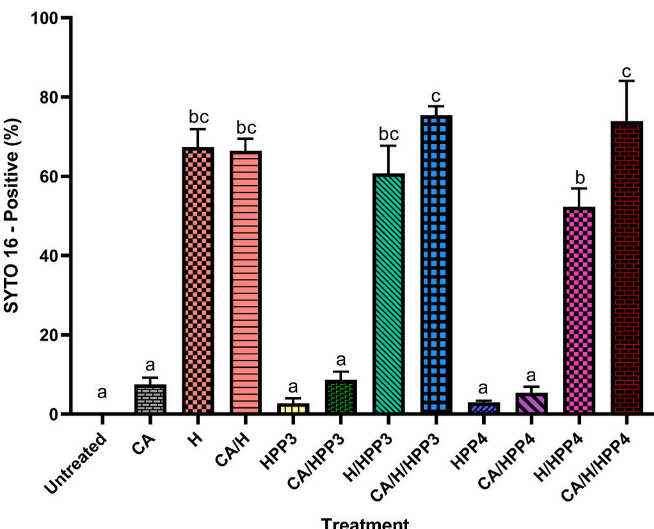

Fig. 5. Effect of citric acid (CA), heat (H) and high-pressure processing (HPP) at 600 MPa and 9 °C for 3 (HPP3) and 4 min (HPP4) on *B. amyloliquefaciens* cortex (Cx) (n = 2).

in the spore's fluorescence histogram (Fig. 4C, D, 4G, 4H, 4K and 4L). The introduction of H to the CA treatment significantly ($p \leq 0.05$) affected the spore's Cx (66 %) (Fig. 5). Similarly, when combined with H, the HPP3 and HPP4 treatments significantly ($p \leq 0.05$) affected the spore's Cx by 61 % and 52 %, respectively.

When combined with CA and HP, H treatment was a contributing factor to the spores' Cx damage (67 %) (Fig. 5). However, the most significant ($p \leq 0.05$) damage occurred when CA and H were combined with both HPP3 and HPP4. Combining the three treatments, CA/H/HPP3 (75 %) and CA/H/HPP4 (74 %), was more effective in damaging the B. amyloliquefaciens spores Cx than the H, CA/H, H/HPP3 or H/HPP4 combinations (Fig. 5).

3.4. Effect of citric acid, heat and high-pressure on the structure and morphology of B. amyloliquefaciens spores

The surface morphology of the untreated B. amyloliquefaciens spores (Fig. 6A) was similar to the spore suspended in CA (6 B). However, spores subjected to H (Fig. 6C) or treated with HPP3 or HPP4 (Fig. 6E and I) appeared slightly crushed and dented. A combination of CA/HPP3 and CA/HPP4 also resulted in a similar effect (Fig. 6F and J), where the spores were crushed and dented. B. amyloliquefaciens spores suspended in CA/H (Fig. 6D) or treated with HPP4 (Fig. 6I) had embedded holes. When the three treatments were combined, the CA/H/HPP3 and CA/H/HPP4 treated spores were visibly dented and crushed (Fig. 6H and L).

CA-only treatment disrupted the IM of the B. amyloliquefaciens spores (Fig. 7B). In contrast, H treatment compromised the integrity of both the spores' IM and Cx (Fig. 7C). Compared to the untreated spores with an intact IM and Cx (Fig. 7A), the spores suspended in CA showed an enlarged core of the B. amyloliquefaciens spores with an indistinguishable IM, still, with an intact Cx (Fig. 7B). The spores subjected to H treatment also had an indistinguishable IM but experienced a loss of spores' core material and damaged Cx (Fig. 7C), although the TEM micrograph showed that this impact was less severe compared to the effect exerted by CA/H combination. B. amyloliquefaciens spores treated with CA/H were indistinguishable, the spores were completely ruptured with loss of the spores' core material and a damaged Cx (Fig. 7D). When H and HP, regardless of the holding time, were combined, the spores ruptured, making the IM indistinguishable (Fig. 7G–K). Neither HPP3 nor HPP4 caused structural damage to the B.

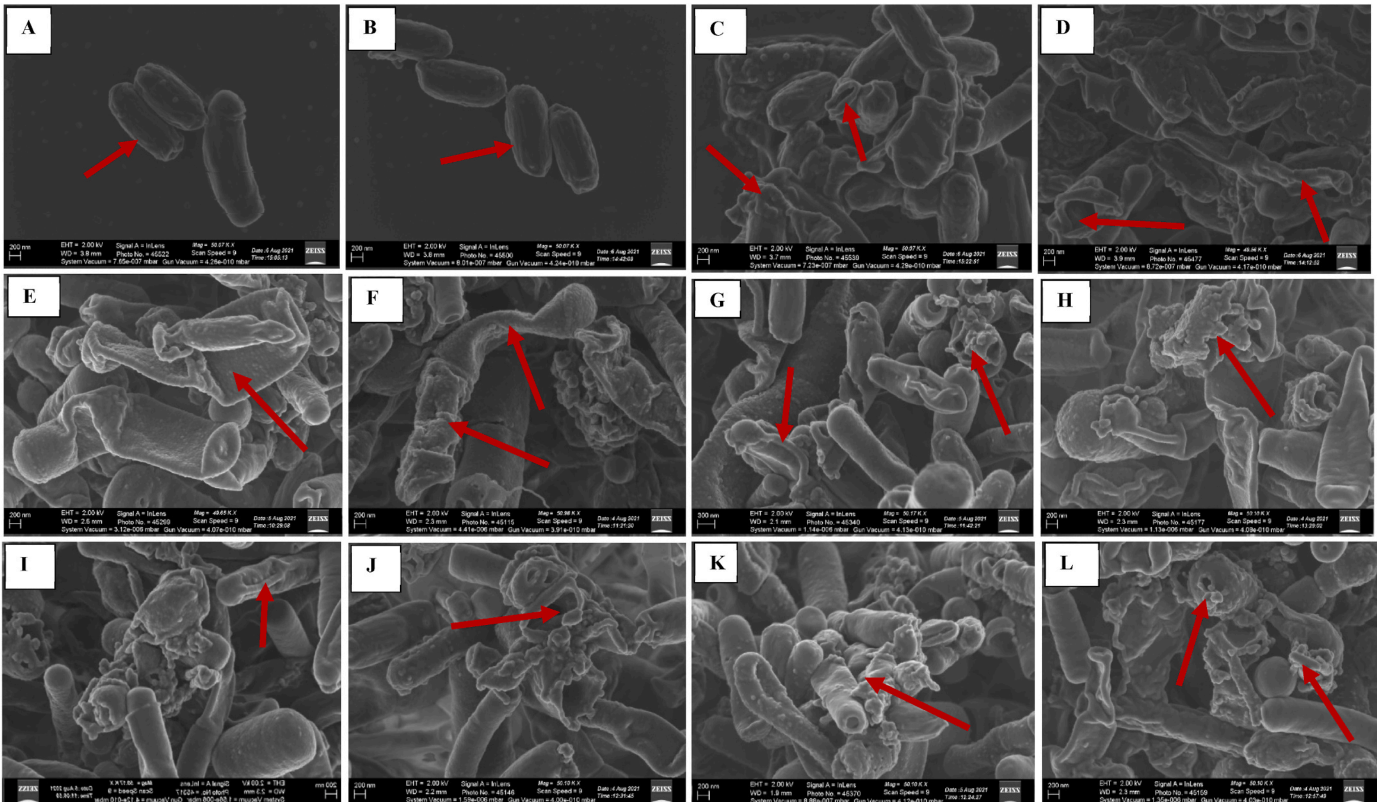

Fig. 6. Scanning electron microscope (SEM) images of *B. amyloliquefaciens* spores treated with citric acid (CA), heat (H) and high-pressure processing (HPP) at 600 MPa and 9 °C for 3 (HPP3) and 4 min (HPP4). **A** – (Untreated spores), **B** (CA), **C** (H), **D** (CA/H), **E** (HPP3), **F** (CA/HPP3), **G** (H/HPP3), **H** (CA/H/HPP3), **I** (HPP4), **J** (CA/HPP4), **K** (H/HPP4), **L** (CA/H/HPP4).

amyloliquefaciens spores. Combining HPP3 and HPP4 with CA led to an indistinguishable IM with loss of the spores' core material. At the same time, the Cx remained intact (Fig. 7F–J). Combining all three treatments (CA/H/HPP3 and CA/H/HPP4) caused severe rupturing of B. amyloliquefaciens spores and loss of their core material (Fig. 7).

4. Discussion

Inactivation of endospores can be effectively achieved using treatments that suppress or activate spore germination. Spore activation involves applying treatments such as mild temperatures ≤80 °C (Reineke & Mathys, 2020) and moderate HP (mHP) or very high-pressure vHP at 50–300 MPa or 600 MPa and above respectively (Lyu et al., 2023; Heydenreich et al., 2024), initiating germination. The spores become more susceptible to subsequent heat or HP interventions (Black et al., 2007), in a method termed the germination-inactivation strategy (Delbrück et al., 2021; Heydenreich et al., 2024). Conversely, spore inactivation by suppressing germination may induce physiological and morphological damage that either injures the spores directly or renders them inactive (Rao et al., 2016). In this study, all treatments resulted in less than 10 % germination of *B. amyloliquefaciens* spores, significantly lower than the 44–75 % germination when *B. amyloliquefaciens* TMW 2.479 spores were treated with PATP (700 MPa at 105 °C for 2 min) combined with CA (Ratphitagsanti et al., 2010). We propose that the sequential combination of CA, H, HPP3, and HPP4 in our study achieves spore inactivation primarily by suppressing germination rather than utilizing the germination-inactivation strategy. Supporting this, the flow cytometry histogram and the percentage PI/SYTO 16 bar chart displayed an absence of predominantly SYTO16-stained *B. amyloliquefaciens* spores. In flow cytometry, germinated spores are stained with SYTO16 but remain negative for PI (Mathys et al., 2007).

HP has been reported to stimulate spore germination at vHP of above 600 MPa (Delbrück et al., 2021; Lyu et al., 2023), the HPP parameters in this study did not sufficiently promote *B. amyloliquefaciens* spore germination, even when combined with CA and H. Nevertheless, HPP3 and HPP4 treatments alone induced minimal germination of 4 % and 3 %, respectively. Notably, extending the HPP duration to 4 min (HPP4) or sequentially combining treatments (CA/HPP4, H/HPP4, CA/H/HPP4) did not significantly affect spore germination. Nonetheless, when HPP3 was applied following H and CA treatments (H/HPP3 and CA/H/HPP3), germination was significantly reduced to 0.50 % and 0.64 %, respectively. This suggests that applying H (10 %) or CA/H (3 %) before HPP3 effectively inactivated germinated spores, as prior H and CA/H treatments likely increased susceptibility of the spores to HPP3 (Delbrück et al., 2021; Heydenreich et al., 2024). This implies that using HPP3 as an additional step can effectively eliminate spores germinated by prior treatments. HP affects spores weakened by H and CA/H treatments by altering membrane fluidity, thus suppressing germination (Nguyen Thi Minh et al., 2010; Delbrück et al., 2021).

Similar to our study, Liao et al. (2023) reported 2 % *B. coagulans* spore germination after high pressure (HP 600 MPa at 25 °C for 2.5 min) treatment in coffee (pH 5) while Ratphitagsanti, Ahn, Balasubramaniam, and Yousef (2009) noted that HP treatment alone at 600–700 MPa and 105 °C for up to 5 min did not induce adequate *B. amyloliquefaciens* spore germination. Lowering the treatment temperature to 35 °C resulted in similar spore germination effects (0.3 log CFU/mL) (Ratphitagsanti et al., 2010). In another study, Wang et al. (2017) observed maximum germination in *B. cereus* spores treated with H at 121 °C for 20 min, while Ratphitagsanti et al. (2010) reported approximately 0.4 log CFU/mL spore activation when treated with H at 105 °C for up to 5 min. Conversely, a two-cycle HP treatment of 200 MPa followed by SAEW (pH 6.08) and 500 MPa resulted in high spore germination (Wang et al., 2017), unlike our findings with less than 10 % spore germination where CA, H, HPP3, and HPP4 were applied

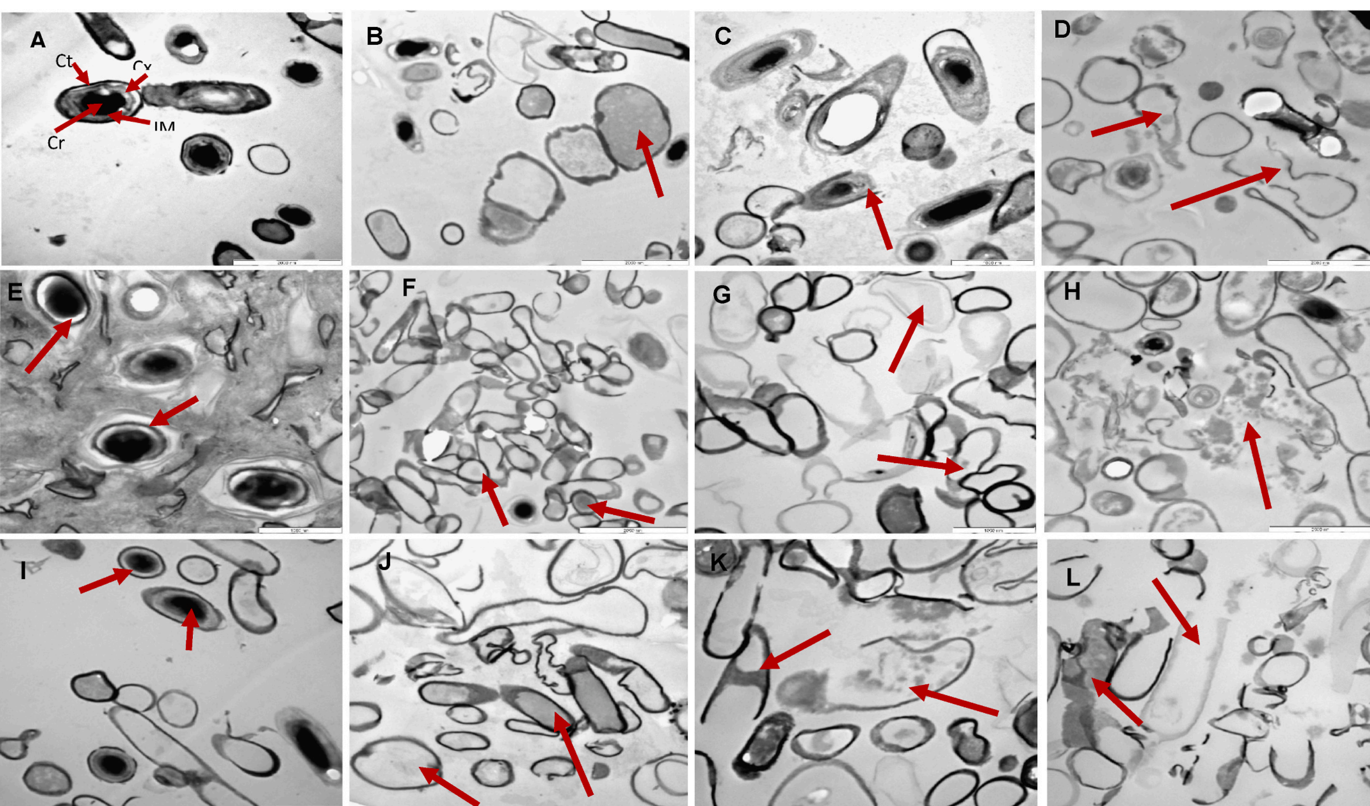

Fig. 7. Transmission electron microscope (TEM) images of *B. amyloliquefaciens* spores treated with citric acid (CA), heat (H) and high-pressure processing (HPP) at 600 MPa and 9 °C for 3 (HPP3) and 4 min (HPP4). **A** – (Untreated spores), **B** (CA), **C** (H), **D** (CA/H), **E** (HPP3), **F** (CA/HPP3), **G** (H/HPP3), **H** (CA/H/HPP3), **I** (HPP4), **J** (CA/HPP4), **K** (H/HPP4), **L** (CA/H/HPP4).

sequentially. The high spore germination observed was attributed to a 90 % release of DPA. The difference in spore germination outcomes between these studies might be due to the mHP used compared to vHP, the HP duration, and the lower acid medium (pH 5) applied in our study (Black et al., 2007; Delbrück et al., 2021). *Bacillus* spp. displays notable heterogeneity in spore germination under HP conditions (Delbrück et al., 2021; Lyu et al., 2023; Zhang et al., 2020). Nonetheless, spores incapable of germination are effectively considered dead (Setlow & Christie, 2021). The low level of *Bacillus* spore germination observed in our study is favourable for the quality and safety of commercial foods. Higher germination rates could potentially compromise the effectiveness of HP in inactivating germinated spores, posing risks of spoilage or safety concerns (Wells-Bennik et al., 2016). Therefore, assessing how specific HPP techniques affect H and pressure-resistant *B. amyloliquefaciens* spores is crucial for the baby food industry.

The treatments applied in this study affected the IM and Cx of the *B. amyloliquefaciens* spores. Physical or chemical agents cause morphological and physiological changes to bacteria spores (Rao et al., 2016; Rivalain et al., 2010; Wang et al., 2017), depending on the spore type and the type of treatment applied (Reineke & Mathys, 2020). CA and H alone or combined significantly affected the permeability of the IM of the *B. amyloliquefaciens* spores, while H treatment alone affected the Cx in addition to the IM permeability of the spores. This was confirmed by the TEM micrograph showing a significant distortion of the spore's internal structure when CA was combined with H. This combination of treatments also impacted the morphology of the spores, although the spore morphology for CA only treated spores were intact. The susceptibility of the *B. amyloliquefaciens* spores to a combination of CA, and H is due to the ability of the CA to diffuse into the bacteria spore's core as observed by the disruption of the spore's IM, as seen in the TEM micrograph. The CA lowers the intracellular pH of the spores and acidifying the cytosol (Beilen, Mattos, Hellingwerf, & Brul, 2014; Ter Beek et al., 2015). Although CA does not affect spore's Cx, which can be attributed to the inherent structural and chemical properties of the spore and its protective layers, combination of the CA and H treatment jointly disrupts the delicate balance needed for the spore integrity. H treatment denatures spore surface enzymes and disrupts the chemical bonds within the peptidoglycan matrix of the Cx, leading to a breakdown of the spore structural integrity (Setlow & Christie, 2021). H treatment can also cause permeabilisation of the spore's IM by denaturing membrane proteins (Coleman, Chen, Li, Cowan, & Setlow, 2007; Setlow, 2006) and the subsequent hydration of the spore core, which affects the spore structure stabilisation (Delbrück et al., 2021; Reineke, Ellinger, et al., 2013; Reineke & Mathys, 2020). This process leads to the mobilisation of proteins and a consequent reduction in thermal resistance, which may eventually result in spore denaturation (Reineke & Mathys, 2020). Setlow (2006) also suggested that spores treated with organic solvents inactivate spores at high temperatures by rupturing the IM permeability barrier.

Combining H with HP also caused damage to the IM and Cx of the *B. amyloliquefaciens* spores, while combination of CA with HPP3 or HPP4 only affected the IM of the spores, although we found that applying only HPP3 or HPP4 affected the spore's morphology, leading to dents and shrinking of the spores with HPP4 having a higher impact by creating holes on the spore surface. Regardless of the holding time, the HP treatments had no effect on the spore's intercellular structure. Similarly, a combination of HP and SAEW significantly deformed the structure and morphology of *B. cereus* spores (Wang et al., 2017), although the *B. cereus* spore Cx was affected by this combination as opposed to the outcome of our study. This could be attributed to the two-cycle HPP (200 MPa + SAEW + 500 MPa) strategy applied. The concurrent permeability of the spore's IM and hydrolysis of the spore's Cx is a function of the wet H, and not HP (Reineke, Schlumbach, et al., 2013) in our combination. The adverse effect on the spores when combining HP

and H is noted when the pressure applied is ≥ 800 MPa (Reineke, Schlumbach, et al., 2013). When HP and H were combined as a treatment on B. amyloliquefaciens spores in a similar study by Ahn and Balasubramaniam (2007), it caused a damage to the spore's IM and Cx, which was associated with degradation of the spores. It was also reported that pressure >550 MPa and high temperature concurrently in High Pressure Thermal Sterilisation (HPTS) affected the IM of B. subtilis spore repair (Reineke, Schlumbach, et al., 2013), although, these damaged spores can be persistently present until they recover through membrane repair, phospholipids synthesis, ribosomal RNA synthesis, DNA synthesis and protein repairs (Reineke, Schlumbach, et al., 2013).

A combination of the three treatments used in this study (CA/H/HPP3 and CA/H/HPP4) significantly caused the Cx of *B. amyloliquefaciens* spores to be hydrolysed. We showed that this treatment combination significantly distorted the integrity of the spore's structure by denting the spore's surface morphology and creating holes, while permeabilising and completely damaging the spores. Therefore, we propose that the application of the sequential CA/H/HPP treatment on CFs would supress germination, while damaging the spore's morphology and intracellular structure. The presence of CA would prevent the spore outgrowth, as low pH inhibits spore germination (van Melis, Almeida, Kort, Groot, & Abee, 2012). Rao et al. (2016) who subjected *B. subtilis* spores to high pressure CO_2 (HPCD) processing at 20 MPa, and 84−86 °C for 30 min reported that this treatment combination inactivated 95% of the spores by causing damage to the IM and Cx of the *B. subtilis* spore without spore germination. Direct access to and damage of the spore's DNA, IM and proteins in the core that are essential for spore metabolism precede spore inactivation (Rao et al., 2016; Setlow & Christie, 2021). However, the exact mechanism by which the combined treatment of B. amyloliquefaciens spores by CA/H/HPP acts on and damages the IM and Cx remains unclear. It was suggested that damage to the Bacillus spore's IM by a similar treatment combination resulted from permeabilisation and extraction of cellular constituents, structural modifications of the cell membrane, and damage to essential spore proteins and enzymes (Rao et al., 2016; Setlow & Christie, 2021). This damage was also suggested to have led to plasma membrane leakage, impairing energy metabolism and at the same time have the potential to damage essential proteins that are crucial for spore germination and outgrowth in the IM, inducing lethal effects on spore structures (Delbrück et al., 2021; Mathys et al., 2007).

5. Conclusions

In producing CFs, sequentially applying CA, H, and HPP as hurdle technology is an effective food quality and safety measure. This combination of treatments causes the distortion of *Bacillus* spore's functionality by impacting the physiology and degrading the morphology of the spores, while supressing the spore from germinating. Although it might appear that the effect of HP treatment step at the end of the CFs processing is inconsequential in relation to the spore internal structure integrity, HPP for 3 min contributes by suppressing *B. amyloliquefaciens* spore germinated by H and CA/H combination. From a food safety perspective, suppression of spore germination is essential for the application of these CA/H/HPP treatment combinations to produce CFs. By elucidating the mechanisms by which acidification, H, and HP affects Bacillus spores, these findings have significant food safety and quality implications for using HPP in the food processing industry, particularly providing a basis for controlling spores in CFs. These results can support validation studies and may aid in the future establishment of safe harbours for the HPP industry.

CRediT authorship contribution statement

Basirat Arinola Olaonipekun: Writing – original draft, Software, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Elna Maria Buys:** Writing – review & editing, Supervision, Project administration, Conceptualization.

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Declaration of competing interest

The authors declare no conflict of interest.

Data availability

Data will be made available on request.

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