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Influence of different cleaning and sanitisation procedures on the removal of adhered *Bacillus cereus* spores

Maria Cecília E. Ribeiro*, Meg da Silva Fernandes, Arnaldo Yoshiteru Kuaye, Mirna L. Gigante

Department of Food Technology, Faculty of Food Engineering, University of Campinas, UNICAMP, 13082-862, Campinas, S.P, Brazil

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ABSTRACT

The effectiveness of nine different sanitation procedures for the removal and control of adhered *Bacillus cereus* spores isolated from the dairy industry was evaluated. Four sets of experiments were produced by varying the conditioning matrix (water and UHT whole milk) and the inoculation medium of the pool of *B. cereus* spores (water and UHT whole milk), resulting in $E_{water/water}$, $E_{water/milk}$, $E_{milk/water}$, and $E_{milk/milk}$. The experiments were repeated three times and evaluated by ANOVA and Tukey's test. Sanitisation with sodium hypochlorite was more effective than peracetic acid for removal of adhesion, especially when associated with a cleaning procedure, with counts below the limit of detection (<0.7 log cfu cm^{-2}). The experiments formed when the pool of spores was inoculated on water ($E_{water/water}$ and $E_{milk/water}$) presented greater resistance against the sanitation procedures studied. These results highlight the importance of the water quality used in the industries for sanitation of equipment.

1. Introduction

Bacillus cereus is considered to be the fourth major agent responsible for foodborne diseases (Tran et al., 2011) and is a natural contaminant of raw milk (Bartoszewicz, Hansen, & Swiecicka, 2008). Because it is a thermoresistant spore-forming organism, *B. cereus* has been associated with contamination of dairy products, since they are able to survive most of the heat treatments commonly used in industry (Granum & Lund, 1997). Due to the risk to public health and its association with the deterioration of milk and dairy products, several studies have investigated the occurrence of *B. cereus* in raw milk, pasteurised milk, ultra high temperature (UHT) milk, and yoghurt (Banykó & Vyletělová, 2009; Bartoszewicz et al., 2008; Blel, Legentilhomme, Legrand, Bénézech, & Gentil-Lelièvre, 2008; Chitov, Dispan, & Kasinrerk, 2008; De Jonghe et al., 2010; Faille et al., 2014; Rather, Aulakh, Gill, Verma, & Rao, 2011).

B. cereus may be present in biofilms formed in milk and dairy processing environments, due to the accumulation of organic and inorganic matter on a surface, along with the colonisation of bacteria or their spores. During the heat treatment, proteins and minerals in milk can form deposits on heat exchangers and evaporators (Bremer, Seale, Flint, & Palmer, 2009), leading to the adhesion and biofilm formation. After this event, the detachment of individual cells or the release of biofilm fragments in subsequent processing may lead to contamination of the final product (Burgess, Brooks, Rakonjac, Walker, & Flint, 2009; Salustiano et al., 2010; Scott, Brooks, Rakonjac, Walker, & Flint, 2007; Svensson, Eneroth, Brendehaug, Molin, & Christiansson, 2000). Different *B. cereus* strains are able to adhere and form biofilms in milk processing plants (Fernandes, Fujimoto, Schneid, Kabuki, & Kuaye, 2014; Malek, 2012; Peña et al., 2014; Salustiano et al., 2010; Shi & Zhu, 2009; Wijman, De Leeuw, Moezelaar, Zwietering, & Abee, 2007).

Both vegetative cells and spores are able to interact with stainless steel surfaces and with compounds from milk incrustations (Flint, Palmer, Bloemen, Brooks, & Crawford, 2001; Parkar, Flint, Palmer, & Brooks, 2001; Seale, Flint, McQuillan, & Bremer, 2008). However, spores adhere more easily to the stainless steel surface, mainly due to their hydrophobic properties (Ryu & Beuchat, 2005). The spores adhered and present in biofilms become even more resistant to sanitation procedures and can recontaminate the processed food. Additionally, under favourable environmental conditions, the spores can germinate by converting to vegetative cells, thus causing deterioration or foodborne illness (Elhariry, 2011).

Our research group has studied *B. cereus* vegetative cells isolated from the ricotta processing environment. Among the 42 isolates, four

* Corresponding author. Tel.: +55 19 35213993.
E-mail address: cissaribeiro@gmail.com (M.C.E. Ribeiro).

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have a marked virulence profile (enterotoxin genes for NHE and HBL, beta-haemolytic activity and multiple antibiotic resistance ampicillin, penicillin and trimethoprim) and were able to form biofilm at different temperatures (25 and 39 °C) on stainless steel surface, using a culture medium to simulate the ricotta processing (80% whey and 20% whole milk). The results have shown an increase in the counts of *B. cereus* spores during biofilm maturation, for both conditions studied (Fernandes et al., 2014). In addition, in a subsequent study (Ribeiro, Fernandes, Kuaye, Jimenez-Flores, & Gigante, 2017), the preconditioning of the stainless steel surface with whole milk, skim milk, and water affected the adhesion of *B. cereus* spores isolated from ricotta processing. The higher spore adhesion was observed after the stainless steel surface preconditioning with whole milk. In addition, these strains were able to form multi-species biofilms with *Enterococcus faecium* and *Enterococcus faecalis*, with the presence of *B. cereus* spores (Fernandes et al., 2017). Those authors reported that the biofilm formation by *B. cereus* strains isolated from industry depends on the environment in which they are exposed, which emphasises the importance of a specific hygiene plan according to the biofilm formation to prevent its formation or to eliminate the biofilms formed.

One of the main strategies used in biofilm control is sanitation, which consists of the combination of detergents and sanitisers (Forsythe, 2013). The most used sanitisers in the dairy industry are peracetic acid, quaternary ammonium compounds, and sodium hypochlorite (Morente et al., 2013). However, the effectiveness of the products available in the market is based on tests performed with planktonic cells, which may be physiologically different from the cells present in biofilms, which may imply the lower effectiveness of the products (Hood & Zottola, 1995). The presence of bacterial spores in biofilms makes the sanitation procedures less effective (Pompermayer & Gaylarde, 2000). The multi-species biofilms formed by *E. faecium, E. faecalis*, and *B. cereus* spores were more efficiently removed using the anionic surfactant, followed by acid detergent and sanitiser. Peracetic acid was the most effective for the removal of multi-species biofilms when using only sanitisers, whereas sodium hypochlorite was the least effective (Fernandes et al., 2017).

Considering the complexity of the adhesion and biofilm structure of *B. cereus* spores and the effect of the preconditioning of stainless steel surface on adhered *B. cereus* spores (Ribeiro et al., 2017), it is important to understand the cleaning and sanitisation procedure to control the formation and removal of biofilms from dairy plants, thus guaranteeing the microbiological quality and safety of the products. In this context, the objective of this study was to evaluate the effectiveness of cleaning and sanitisation procedures against a pool of adhered *B. cereus* spores under different conditions, using a combination of sanitation procedures with anionic surfactant detergent, acid detergent, peracetic acid, and sodium hypochlorite as sanitising agents.

2. Material and methods

2.1. Origin of isolates and preparation of spore suspension

Four *Bacillus cereus* strains were used, which encode the HBL and NHE enterotoxins responsible for the diarrhoeal syndrome and resistance to different antibiotics previously isolated from the ricotta processing environment (Fernandes et al., 2014). Strains encoded as 164, 167, 174, and 176 were maintained in BHI broth (Brain Heart Infusion; Difco, Sparks, MD, USA) containing 20% glycerol at −80 °C. The *B. cereus* spores were produced according to the methodology described by Samapundo, Heyndrickx, Xhaferi, de Baenst, and Devlieghere (2014), with modifications. The *B. cereus* strains were separately activated in TSB broth (Difco, Sparks, MD, USA) at 35 ± 1 °C for 24 h, streaked for isolation on AK agar # 2 sporulation medium. The other steps were performed as previously described by Ribeiro et al. (2017). Thus, the spores from each strain were resuspended in 30 mL of sterile refrigerated ultra pure water and stored at 4 °C until use. Spore counting was performed by pour plate method on TSA agar (Difco) supplemented with 0.2% (w/v) soluble starch, and the plates were incubated at 35 ± 1 °C for 24–48 h (Rueckert, Ronimus, & Morgan, 2005).

For the adhesion on a stainless steel surface, 1 mL of each suspension of *B. cereus* spores was added into a sterile test tube. The mixture of four strains (pool) was serially diluted in peptone water (0.1%, w/v) until reaching the final concentration of approximately 10^5 cfu mL^{-1} in the culture media used in the experiments.

2.2. Adhesion of Bacillus cereus spores on the stainless steel surface

The coupons used in the experiments (AISI 304#4 stainless steel, 0.366 μm roughness, square, 10 mm × 10 mm × 1 mm) were previously cleaned according to Ribeiro et al. (2017). A small hole was made in the edge of each coupon in which a polyamide yarn was placed. The steps of cleaning consisted of immersion in distilled water, followed of sodium hydroxide and nitric acid immersion, and sterilisation at 121 °C for 15 min. After that, *B. cereus* spores were subjected to four different adhesion conditions as previously reported in another study (Ribeiro et al., 2017). Thus, the coupons were aseptically immersed in sterile universal bottles containing 60 mL of each matrix (sterile ultra pure water or UHT whole milk from Shefa LTDA, Amparo, SP, Brazil) so that the coupons would not touch the edges of the bottles (Fig. 1). The bottles were then kept under agitation at 200 rpm at 55 °C for 20 h (Lab-Line Orbit Environ – Shaker 3527, LabLine Instruments, Melrose Park, IL, EUA). After 20 h, the coupons were removed from the conditioning matrices (water or UHT whole milk) and placed in sterile flasks containing water or whole milk (60 mL) inoculated with the pool of *B. cereus* spores, with a final concentration of approximately 10^5 cfu mL^{-1}. The flasks were kept under agitation at 200 rpm at 55 °C for 24 h (LabLine Orbit Environ - Shaker 3527, Lab-Line Instruments, Melrose Park, IL, USA). After 24 h, for each condition of adhesion ($E_{water/water}$, $E_{water/milk}$, $E_{milk/water}$, and $E_{milk/milk}$),

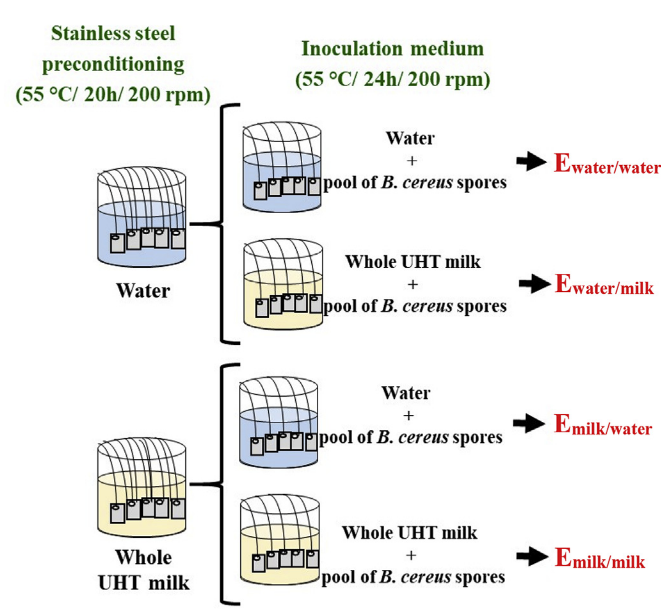

Fig. 1. Adhesion of *B. cereus* spores isolated from the dairy industry.

a coupon was removed from the culture medium and immediately submitted to different cleaning and sanitisation procedures.

2.3. Sanitation procedures for the removal of adhered B. cereus spores

The coupons submitted to the four different adhesion conditions were subjected to each of the nine different cleaning and sanitisation procedures as described in Table 1. The sanitation procedures followed the methodology described by Fernandes, Kabuki, and Kuaye (2015), with modifications. For the cleaning steps, anionic surfactant detergent based on sodium alkylbenzene sulfonate (Start Química, Lima & Pergher Ind. Com. And Rep. LTDA, Uberlândia, MG) at 3.0% (v/v) pH 6.0, and nitric acid detergent (Start Química) at 0.4% (v/v), pH 1.8. For the sanitisation steps, 0.2% sodium hypochlorite (v/v, 200 mg L^{-1}) of total chlorine residual (TCR), pH 7.0 (Start Química) and 0.2% peracetic acid (v/v, 300 mg L^{-1}), pH 2.8 (Start Química). The detergents and the sanitisers were used according to the manufacturers' instructions.

All solutions were prepared in sterile flasks prior to the experiments, and 10 mL of each solution (detergent or sanitiser) was transferred to sterile test tubes. The steps of pre-rinsing, intermediate rinsing, and final rinsing were performed in sterile test tubes containing 10 mL of sterile ultra pure water. After the cleaning and sanitising procedures using acid detergent, sodium hypochlorite, and peracetic acid, the coupons were transferred to tubes containing 10 mL peptone water (0.1%, w/v) supplemented with a neutralising agent (sodium thiosulphate, 1% w/v). For the cleaning procedure using the anionic surfactant, the coupons were transferred directly to tubes containing only 10 mL of 0.1% (w/v) peptone water. Then, the coupons were vortexed for two minutes to remove the sessile cells (Andrade, Bridgeman, & Zottola, 1998). For each adhesion condition, a coupon was used as a control of the initial *B. cereus* population, which was not subjected to the cleaning and sanitisation procedures (negative control).

2.4. Determination of the number of adhered cells

The number of cells adhered after the cleaning and sanitising procedures was determined using the plate counting technique. After vortexing, the solutions together with the coupons were subjected to a heat treatment of 80 ± 1 °C for 12 min. Serial dilutions in peptone water were then performed for spore counting, followed by plating in TSA agar (Difco) supplemented with 0.2% (w/v) soluble starch and incubation at 35 ± 1 °C for 24–48 h (Rueckert et al., 2005). The results were expressed in colony forming units per cm^2 (cfu cm^{-2}), representing the remaining *B. cereus* spores counts in each coupon after the sanitation procedures, and the negative control coupon count was used to calculate the number of decimal reductions.

2.5. Statistical analysis and experimental design

The experimental design was performed in randomised blocks and the experiments were repeated three times. The effect of the cleaning and sanitising procedures (described in Table 1) on the

Table 1
Sanitation procedures for the removal of adhered *B. cereus* spores.[a]

Cleaning and sanitisation procedures	Steps	Conditions
Anionic tensioactive cleaning	1. Pre-rinse	1 min, RT
	2. Anionic tensioactive detergent	7 min, 40 °C
	3. Final rinse	1 min, RT
Anionic tensioactive + acid cleaning	1. Pre-rinse	1 min, RT
	2. Anionic tensioactive detergent	7 min, 40 °C
	3. Rinse	1 min, RT
	4. Acid detergent	10 min, 40 °C
	5. Final rinse	1 min, RT
Acid + Anionic tensioactive cleaning	1. Pre-rinse	1 min, RT
	2. Acid detergent	10 min, 40 °C
	3. Rinse	1 min, RT
	4. Anionic tensioactive detergent	7 min, 40 °C
	5. Final rinse	1 min, RT
Peracetic acid sanitisation	1. Pre-rinse	1 min, RT
	2. Sanitiser (peracetic acid)	10 min, RT
Sodium hypochlorite sanitisation	1. Pre-rinse	1 min, RT
	2. Sanitiser (sodium hypochlorite)	10 min, RT
Anionic tensioactive cleaning + peracetic acid sanitisation	1. Pre-rinse	1 min, RT
	2. Anionic tensioactive detergent	7 min, 40 °C
	3. Rinse	1 min, RT
	4. Sanitiser (peracetic acid)	10 min, RT
Anionic tensioactive cleaning + sodium hypochlorite sanitisation	1. Pre-rinse	1 min, RT
	2. Anionic tensioactive detergent	7 min, 40 °C
	3. Rinse	1 min, RT
	4. Sanitiser (sodium hypochlorite)	10 min, RT
Anionic tensioactive + acid cleaning + peracetic acid sanitisation	1. Pre-rinse	1 min, RT
	2. Anionic tensioactive detergent	7 min, 40 °C
	3. Rinse	1 min, RT
	4. Acid detergent	10 min, 40 °C
	5. Rinse	1 min, RT
	6. Sanitiser (peracetic acid)	10 min, RT
Anionic tensioactive + acid cleaning + sodium hypochlorite sanitisation	1. Pre-rinse	1 min, RT
	2. Anionic tensioactive detergent	7 min, 40 °C
	3. Rinse	1 min, RT
	4. Acid detergent	10 min, 40 °C
	5. Rinse	1 min, RT
	6. Sanitiser (sodium hypochlorite)	10 min, RT

[a] Adapted from Fernandes et al. (2015). All steps involved constant agitation at 340 rpm; RT, room temperature.

removal of the pool of adhered *B. cereus* spores on the stainless steel surface was evaluated separately for each condition ($E_{water/water}$, $E_{water/milk}$, $E_{milk/water}$, and $E_{milk/milk}$), using analysis of variance (ANOVA) and Tukey's test for comparison of means at a significance level of 5%.

3. Results and discussion

Adhered *B. cereus* spores used as an initial reference (negative control coupon) for the evaluation of the sanitation procedures differed according to the different conditions for adhesion (Table 2). In addition, the effectiveness of the different cleaning and sanitisation procedures was affected not only by the type of experiment (adhesion condition) but also by the sanitation procedure (Fig. 2). Regardless of the conditioning steps, a higher adhesion was observed when the pool of *B. cereus* spores was inoculated in water (Table 2) ($E_{water/water}$ and $E_{milk/water}$), and the experiments showed higher resistance to spore removal when subjected to the different sanitation procedures (Fig. 2a,c). Although different sanitation treatments led to a reduction in the number of spores from the $E_{water/water}$ (Fig. 2a), only the complete CIP procedure with anionic detergent and acid detergent followed by sanitation with sodium hypochlorite resulted in total removal, showing that the *B. cereus* spores counts on stainless steel surfaces were below the limit of detection (<0.7 log cfu cm^{-2}). The high hydrophobicity of *B. cereus* spores suspension isolated from the dairy industry (Ribeiro et al., 2017) and the hydrophobic character of the exosporium of some strains (Ankolekar & Labbé, 2010; Faille et al., 2002, 2010) favoured the strong adhesion of the spore to the stainless steel surface, and the consequent resistance of biofilms to removal, once the hydrophobic interactions are considered the most strong among the non-covalent interactions (Harimawan, Zhong, Lim, & Ting, 2013).

Similar resistance to sanitation procedures was observed for the $E_{milk/water}$. As can be seen in Fig. 2c, cleaning with the anionic surfactant followed by sanitisation with sodium hypochlorite, in the absence of acid-cleaning, was also effective to remove the $E_{milk/water}$, resulting in *B. cereus* spores counts on the stainless steel surfaces below the limit of detection (<0.7 log cfu cm^{-2}).

An opposite behaviour was observed for both the spore adhesion, as well as the removal, when the pool of *B. cereus* spores was inoculated in milk, regardless of the conditioning matrix. In this case, lower spore adhesion was observed (Table 2) and the conditions ($E_{water/milk}$ and $E_{milk/milk}$) showed less resistance to removal when submitted to different sanitation procedures (Fig. 2b,d). The lower adhesion of *B. cereus* spores in the presence of whole milk was previously observed by our research group (Ribeiro et al., 2017), which suggested that when whole milk is used as the conditioning matrix, the *B. cereus* spores and milk fat, both hydrophobic, compete for binding sites on the stainless steel surface, thus leading to the spore adhesion to a lower surface, reducing the number of sites for adhesion (Barnes, Lo, Adams, & Chamberlain, 1999). The presence of whole milk as a conditioning film (Fig. 3) may have altered the physicochemical properties of the surface and the surface/spore interaction, resulting in poor adhesion (Dickson & Koohmaraie, 1989), which is easier to remove when compared with different sanitation procedures.

Almost all sanitation procedures evaluated in the present study were effective to remove both the $E_{water/milk}$ and $E_{milk/milk}$, as shown in Fig. 2b,d, resulting in *B. cereus* spores counts on the stainless steel surface below the limit of detection (<0.7 log cfu cm^{-2}), except for the sanitation with peracetic acid that was exclusively used for the removal of $E_{water/milk}$. In this case, although a significant reduction of spore counts from 2.73 to 2.0 log cfu cm^{-2} was observed, there was no effective adhesion removal.

The removal of adhesion and biofilms formed by spore-forming bacteria is quite complex, especially when dealing with *B. cereus* spores, which are more hydrophobic than other *Bacillus* spp. (Granum & Lund, 1997) and may exhibit high resistance, being able to germinate and form biofilm after being recovered from a milk powder silo that underwent alkaline cleaning (Shaheen, Svensson, Andersson, Christiansson, & Salkinoja-Salonen, 2010). In this study, we observed that the conditions formed when the pool of *B. cereus* spores were inoculated in water ($E_{water/water}$ and $E_{milk/water}$) were much more resistant to removal when compared with the conditions formed when the spores were inoculated in whole milk ($E_{water/milk}$ and $E_{milk/milk}$), that is, the spores were less resistant when protected by the milk matrix. This behaviour may be due to the use of the anionic surfactant detergent, once it decreases the surface tension of water, allowing the contact between the residues and the cleaning agents, thus contributing to the weakening of the bonds between the surface and the residue (Salustiano et al., 2010). Thus, the anionic surfactant detergent was not effective in removing the spores that were not protected by the milk matrix ($E_{water/water}$ and $E_{milk/water}$); however, it may be effective when the spores were protected by the milk matrix ($E_{water/milk}$ and $E_{milk/milk}$), by weakening the protein bonds and acting in milk fat emulsification.

The anionic surfactant is able to remove the milk constituents (fats, proteins, and carbohydrates), while the acid detergent has the ability to solubilise the minerals involved in the biofilm-surface interactions or to break bonds, promoting the release of the remaining cells strongly adhered to the stainless steel surface (Fernandes et al., 2015).

The complete CIP procedure using anionic surfactant detergent and acid detergent followed by sanitisation with sodium hypochlorite was the only sanitation condition that was able to remove all the conditions formed by the pool of *B. cereus* spores (Fig. 2), resulting in *B. cereus* counts on stainless steel surfaces below the limit of detection (<0.7 log cfu cm^{-2}), for all experiments. These results suggest that sodium hypochlorite was more effective than peracetic acid as a sanitiser for the removal of almost all conditions from *B cereus* spores, except for the $E_{milk/milk}$. The peracetic acid was not able to remove the $E_{water/water}$ even after using an anionic surfactant and acid detergent (Fig. 2a). In addition, the sanitisation with peracetic acid was not effective to remove the $E_{water/milk}$. The lower effectiveness of peracetic acid to remove adhered *B. cereus* spores from the stainless steel surface when compared with sodium hypochlorite was previously reported by other authors (Ryu & Beuchat, 2005), the ineffectiveness of peracetic acid to remove

Table 2
Counts (log cfu cm^{-2}) of adhered *B. cereus* spores (negative control coupons) after 24 h.[a]

Experiments	Adhesion procedure	Spores count
$E_{water/water}$	Conditioning of coupons with water (20h), followed by immersion at water inoculated with the pool of *B. cereus* spores (24h)	$3.62^a \pm 0.24$
$E_{water/milk}$	Conditioning of coupons with water (20h), followed by immersion at whole milk inoculated with the pool of *B. cereus* spores (24h)	$2.73^b \pm 0.12$
$E_{milk/water}$	Conditioning of coupons with whole milk (20h), followed by immersion at water inoculated with the pool of *B. cereus* spores (24h)	$3.59^a \pm 0.28$
$E_{milk/milk}$	Conditioning of coupons with whole milk (20h), followed by immersion at whole milk inoculated with the pool of *B. cereus* spores (24h)	$2.63^b \pm 0.12$

[a] In all cases, conditioning and immersion were at 200 rpm at 55 °C. Values (log cfu cm^{-2}) are the average ± standard deviation (n = 3); values followed by different superscript letters are significantly different ($P < 0.05$).

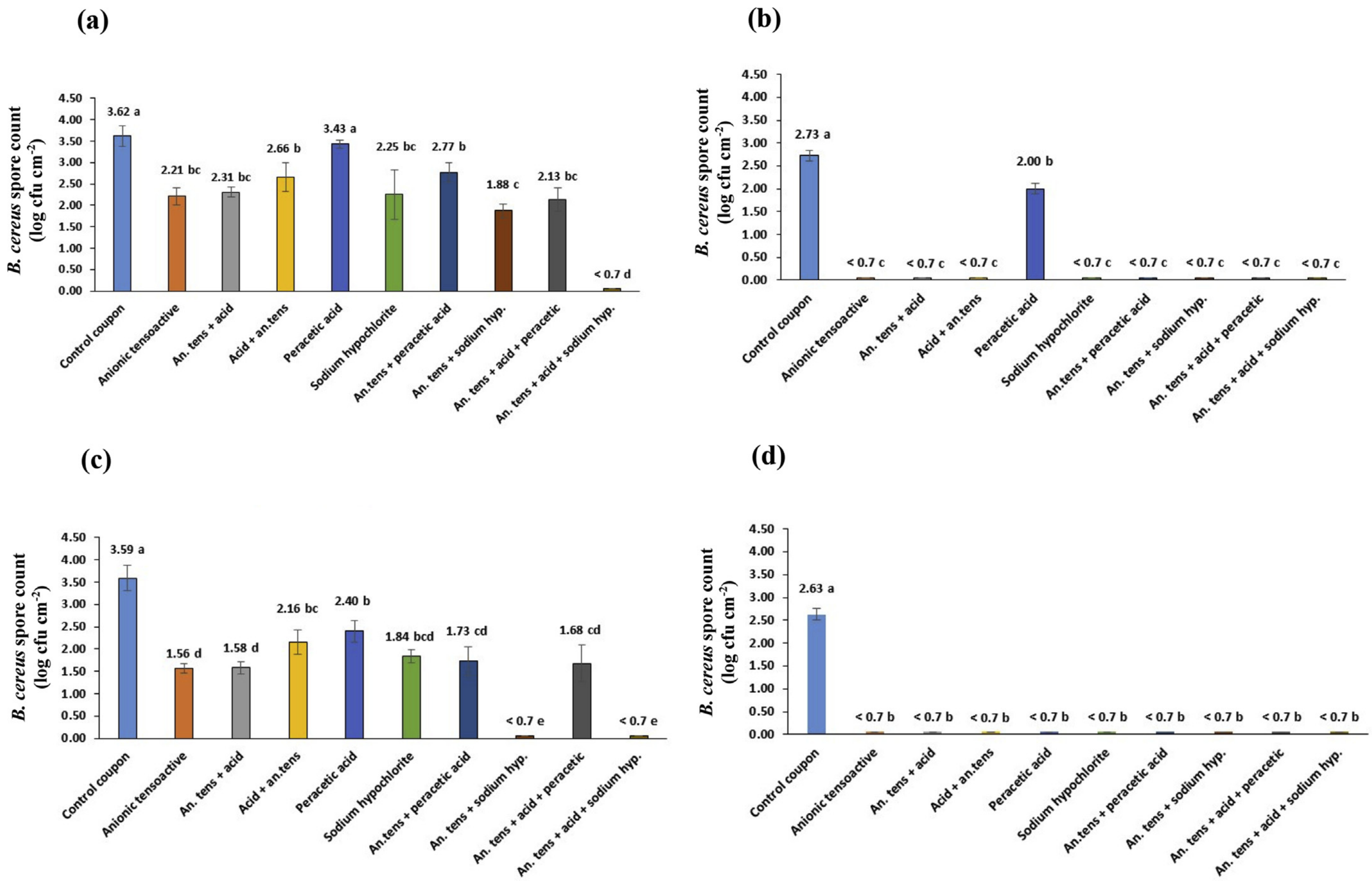

Fig. 2. Effect of different sanitation procedures on the removal of adhered *B. cereus* spores. (a) $E_{water/water}$, (b) $E_{water/milk}$, (c) $E_{milk/water}$, (d) $E_{milk/milk}$. [a,b] For each experiment of adhesion, different lowercase letters differ significantly by Tukey's test ($P < 0.05$). [§]Method detection limit was 0.7 log cfu cm^{-2}; standard deviation not established, n = 3.

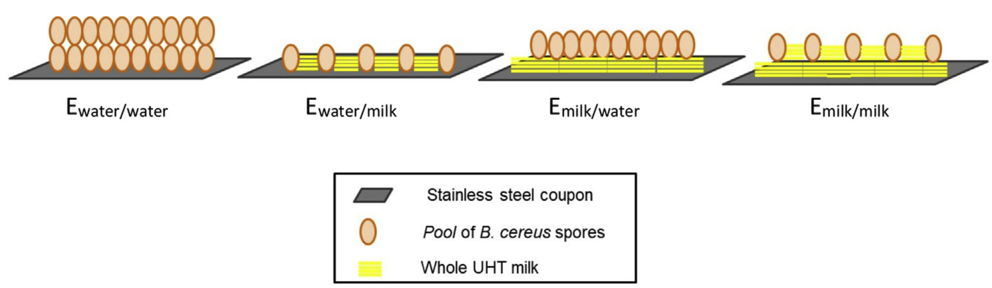

Fig. 3. Schematic representation of stainless steel surface after the adhesion of pool of *B. cereus* spores.

some biofilms may be due to the aldehydes can stabilise the biofilm, rather than degrade them (Srey, Jahid, & Ha, 2013).

These results once again reveal the complexity of the issue, since peracetic acid, when compared with sodium hypochlorite, was the most effective sanitiser for the removal of multi-species biofilms composed by the same *B. cereus* strains evaluated in this study and by *E. faecium* and *E. faecalis* (Fernandes et al., 2017). Biofilm resistance to sanitising agents is due to the inefficient control of biofilms by the conventional sanitising processes, which reduce the number of microorganisms and/or spores, but are not able to remove them completely (Malek, 2012). Bacteria can survive and produce biofilms on stainless steel surface even after CIP procedures (Shi & Zhu, 2009). The great resistance of the cells present in the biofilms may be due to the low diffusion rate of the chemical agents through the biofilm layers, which may be 60–80% lower, thus making it difficult to reach the deeper layers (Królasik, Zakowska, Krępska, & Klimek, 2010). It is believed that the conditions used in the sanitation procedures resulting in the partial spore removal, as observed in our study, especially for the $E_{water/water}$ (Fig. 2a) and the $E_{milk/water}$ (Fig. 2c), can affect the physical properties of the spores, including the hydrophobicity, and modify the ability to adhere to stainless steel surface after releasing from the biofilms (Faille et al., 2010). This behaviour was observed for *Geobacillus* sp. spores, which presented six times higher hydrophobicity and higher adhesion ability on stainless steel surface after the treatment with sodium hydroxide (1%, w/v) when compared with spores that did not undergo this treatment. Using transmission electron microscopy, the authors related the higher hydrophobicity to the partial hydrolysis of the spore layer, which resulted in the release of more hydrophobic groups (Seale, Bremer, Flint, & McQuillan, 2010).

4. Conclusions

All simulated conditions of adhered *B. cereus* spores revealed counts (log cfu cm^{-2}) that can be critical for contamination during the processing of dairy products when considering the total area of the equipment used. Plate heat exchangers, including pasteurisers and evaporator sections, are among the most critical areas for adhesion and biofilm formation due to their large surface area. The effectiveness of the sanitation procedures varied according to the condition formed. In general, when the pool of *B. cereus* spores was inoculated in milk matrix ($E_{water/milk}$ and $E_{milk/milk}$), the adhesion were much less resistant to removal than the adhesion formed when the spores were inoculated in water matrix ($E_{water/water}$ and $E_{milk/water}$). In addition, the sanitising sodium hypochlorite was more effective than peracetic acid for removal of all conditions. The partial spore removal during the cleaning procedures may result in contamination of the final product, when released from the biofilm in the processing flow, thus affecting the product's quality and microbiological safety, once these strains have the genes related to the HBL and NHE enterotoxins responsible for diarrhoeal syndrome, besides exhibiting resistance to multiple antibiotics.

The higher resistance observed in the $E_{water/water}$ for most of the cleaning and sanitising procedures evaluated in this study suggests the need to evaluate the water used in the hygiene of dairy processing plants, once the *B. cereus* spores can adhere and form biofilms even in conditions with no nutrient availability. In addition, most of the surfaces are not dried after the sanitising procedures, and the presence of contaminated water or pre-existing contamination on the poorly sanitised surface in contact with the accumulated water can be the first step in the formation of new biofilms, thus the wet surfaces should be avoided after performing the sanitation procedures.

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