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Controlling *Bacillus cereus* adherence to stainless steel with different cleaning and sanitizing procedures used in dairy plants

[*Controle do processo de adesão de* Bacillus cereus *ao aço inoxidável após diferentes procedimentos de limpeza e sanitização usados na indústria de laticínios*]

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ABSTRACT

Bacillus cereus *adherence to stainless steel used to milk contact surfaces was observed, depending on the cleaning and sanitizing procedures applied and the physicochemical properties of the surfaces. Numbers of surviving* B. cereus *after hygiene procedures were affected by temperature, the concentrations of both alkaline and acid washes, and the pH of the chlorine solution. The adhesion of* B. cereus *to the stainless steel was not thermodynamically favorable, and the adherence of this microorganism occurred in lower number, in accordance to the thermodynamic aspect of adhesion.*

Keywords: Bacillus cereus*, equipment surface, dairy industry, hygiene procedures*

RESUMO

Foi observada a adesão de *Bacillus cereus* em superfície de aço inoxidável em contato com o leite, segundo o procedimento de limpeza, a sanitização e as propriedades físico-químicas da superfície. O número de *B. cereus* viáveis, após os procedimentos de higienização, foi afetado pela temperatura, pela concentração das soluções alcalinas e ácidas e pelo pH da solução clorada. A adesão de *B. cereus* em aço inoxidável não foi termodinamicamente favorável, e ocorreu pouca adesão desse microrganismo, de acordo com os aspectos termodinâmicos da adesão.

Palavras-chave: *Bacillus cereus*, equipamento, indústria do leite, higienização

INTRODUCTION

Adhesion processes and biofilm formation can occur on milk contact surfaces post-pasteurization (Chmielewski and Frank, 2003; Ryu and Beuchat, 2005). The biological activity of *Bacillus cereus* leads to undesirable alterations of milk proteins and lipids by proteases and lipases, decreasing the shelf life of the product (Fromm and Boor, 2004). Consumption of food containing more than 10^5 viable *B. cereus* cells/g has been implicated in outbreaks of emetic and diarrheic toxicity. In the bacterial adherence to surfaces, physicochemical properties such as hydrophobicity, electrical charge, and roughness of the surfaces should be taken in account.

Correctly applied cleaning and sanitizing procedures can prevent and control the bacterial adhesion process on equipment surfaces (Griffith, 2005; Lelieveld et al., 2005). Hygiene procedures should be detailed enough to obtain effective removal of organic and inorganic residues, as well as microorganisms from the milk contact surfaces by using cleaners and sanitizer agents correctly. The establishment of

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Sanitation Standard Operating Procedures (SSOP) is very important in controlling the presence of *B. cereus* on milk contact surfaces (Lelieveld et al., 2005; Andrade et al., 2008). The alkaline agents are responsible for fatty acids saponification and for making proteins residues soluble in water by changing of electrical charges of amino acid in high pH. At pH 12 and 60^{o}C of cleaning solution the protein residues reach 100% of solubility. Complexant agents, for example, sodium ethylene diamine tetraacetic acid(EDTA-Na) are able to sequestrate mineral residues such as calcium present in the hard water and in the milk, preventing milk deposit formation on the stainless steel surfaces. Acid solutions, such as prepared with acid nitric, prevent mineral incrustation on the surfaces. Tense active agents such as sodium dodecyl benzene sulphonate decrease the interfacial tension of water improving the contact between residues and cleaners.

The aim of this work was to evaluate the adhesion of *B. cereus* on stainless steel surfaces AISI 304, #4, by different Sanitation Standard Operations Procedures (SSOP). Also, some physicochemical properties of the stainless steel and *B. cereus* surfaces were studied.

MATERIAL AND METHODS

The study of *B. cereus* adherence and its control with hygiene procedures was conducted according to the methodology proposed by Gilmour et al. (1993). One milliliter of *B. cereus* (RIBO 1 222-173-S4) suspension, activated three times in BHI containing approximately 1.0x10^{8}cfu/mL of bacteria at 32^{o}C for 24h, was added in 100mL of whole pasteurized milk. This ribogroup was previously found on four surfaces and also in the milk, indicating the role of the equipment surfaces as reservoirs of *B. cereus* (Salustiano et al., 2009)

The inoculated milk was used to fill four sections of stainless steel pipe (AISI 304, # 4) with an internal diameter of 3.1cm and a length of 33cm, which was sealed with rubber caps and sterilized at 121^{o}C for 15min. The pipe sections were previously conditioned with raw milk before use with the following procedure: the tubes were conditioned by scrubbing with sodium carbonate (0.75g/100mL) and sodium sulfite (0.05g/100mL) at ambient temperature (20-25°C), rinsed with cold water, and autoclaved at 121°C for 15min. To complete the conditioning process, the tubes were filled with raw milk, incubated at 22°C for 6h, washed, and sterilized. This procedure was repeated three times.

The pipe sections were placed on a flat surface and, from a central point, rolled three times longitudinally up and down. The pipes were also rolled three times laterally from left to right, and then manually stacked three times in an upright manner. This entire procedure was performed six times across a square template (46cm long) delimited on the bench surface of the laboratory. In sequence, the pipes sections were incubated at 4^{o}C for 6h to examine bacterial adherence to the stainless steel. This temperature and time simulated the milk storage after pasteurization and the filling of the packages. After the adherence step, the pipe sections were submitted to different cleaning and sanitizing procedures (Table 1). The chosen hygiene procedures represent real situations found in the routine of the dairy plants.

The adhesion process of *B. cereus* and SSOP procedures (Andrade et al., 2008) were performed for five consecutive days over a period of five weeks, with five repetitions per week. After the fifth day, the number of cells remaining on the surfaces of the pipes after the SSOP was determined as follows: initially, sessile cells were removed by filling in the pipe sections with 100mL of a quarter-strength Ringer solution – sodium chloride (2.25g/L), potassium chloride (0.105g/L), anhydrous calcium chloride (0.12g/L), sodium bicarbonate (0.05g/L), and sodium thiosulfate (0.25g/L); then the pipes were manually stacked six times and the Ringer solution was discharged. In sequence, pipe sections were filled with 100mL Ringer solution and the attached cells were removed from the walls of the tubes using a squeegee sampler, that consisted of a metal rod to which a 3.25cm diameter, and 3mm thick neoprene rubber disc was attached, with a 3mm diameter drilled hole in each quadrant to allow the passage of rinse solution. The squeegee samplers were individually sterilized and ready for use. The top closures were removed from the pipes and the rod samplers were pushed to the bottom of the pipes. The rods were then drawn up the pipe section until the washer reached 2cm from the top. This procedure was repeated six times and then the rods were removed. The top of the pipe was closed again and the Ringer solution remained in contact with the surface for a minute. Finally, the pipe sections were stacked again. Aliquots from the Ringer solution were inoculated in the Plate Count Agar (Merck - São Paulo, Brazil) at 32°C for 24h, according to the methodology of Evancho et al. (2001). The results were expressed as cfu/cm^2.

Table 1. Sanitation Standard Operating Procedures for the pipe sections with *Bacillus cereus* RIBO 1 222-173-S4

Procedure	Sanitation Standard Operating Procedures
Control	i) Disposal of the inoculums; ii) a pre-washing with distilled water at 25°C; iii) an alkaline washing with sodium hydroxide (10g/L), EDTA (0.4g/L), and sodium dodecyl sulphonate (0.1g/L), pH 13, at 60°C; iv) a rinsing with distilled water at 40°C; an acid washing with HNO_3 (10mL/L), pH 1.5, at 25ºC; rinsing with distilled water at 25ºC, and finally, sanitization with 100mg/L of free available chlorine (FAC), prepared from sodium dichloroisocyanurate, with a pH 7.0-7.5, 25ºC. Note: After treating the test tubes with the alkaline, acidic, and sanitizing solutions, the tubes were submitted to the adherence procedures, as previously mentioned.
Procedure A	Procedure A followed the steps of the control, with the following modifications: the sodium dodecyl sulphonate was excluded from the alkaline formulation and sanitization was performed with FAC (50mg/L[1]).
Procedure B	Procedure B followed the steps of the control with the following modifications: use caustic alkalinity (5g/L), pH 12.8, at 25°C, and use of a sanitizing solution containing 50mg/L, with pH adjusted to 10.
Procedure C	Procedure C followed the steps of the control with the following modifications: i) a washing with distilled water at 50°C, ii) use an alkaline wash (5g/L), pH 12.8, at 25°C, iii) the acid washing was excluded and replaced with a sanitizing solution containing 50mg/L with a pH adjusted to 10.

Surface hydrophobicity was determined based on contact angle measurements of the stainless steel and *B. cereus* surfaces with water, formamide, and α-bromonaphtalene and the surfaces using a goniometer (Kruss®, Germany). From the hydrophobicity, the interfacial tension (γ), the total free energy of interaction (ΔG_{sws}^{TOT}), and global free energy of adhesion between stainless steel and *B. cereus* ($\Delta G_{adhesion}$) were determined by appropriate formulas (van Oss, 1994).

The total free energy of interaction among molecules of the surface(s) immersed in water (w) was determined by the sum of the apolar and polar free energy of interaction, ΔG_{sws}^{LW} and ΔG_{sws}^{AB}, respectively.

$$\Delta G_{sws}^{TOT} = \Delta G_{sws}^{LW} + \Delta G_{sws}^{AB} \text{ (Equation 1)}$$

$$\Delta G_{sws}^{LW} = -2x\sqrt{\gamma_s^{LW} - \gamma_w^{LW}} \text{ (Equation 2)}$$

$$\Delta G_{sws}^{AB} = -4\left(\sqrt{\gamma_s^+\gamma_s^-} + \sqrt{\gamma_w^+\gamma_w^-} - \sqrt{\gamma_s^+\gamma_w^-} - \sqrt{\gamma_w^+\gamma_s^-}\right) \text{ (Equation 3)}$$

When $\Delta G_{sws}^{TOT} > 0$, the surface is considered hydrophilic, conversely, $\Delta G_{sws}^{TOT} < 0$ the surface is hydrophobic.

From the values of the components of the interfacial tensions, it is possible to determine the $\Delta G_{adhesion}$ between two surfaces (microbial cells (b) and food processing surfaces (s)):

$$\gamma_{bs} = \gamma_{bs}^{LW} + \gamma_{bs}^{AB}$$

$$\gamma_{bs}^{LW} = \gamma_b^{LW} + \gamma_s^{LW} - 2\sqrt{\gamma_b^{LW}\gamma_s^{LW}}$$

$$\gamma_{bs}^{AB} = 2\left(\sqrt{\gamma_b^+\gamma_b^-} + \sqrt{\gamma_s^+\gamma_s^-} - \sqrt{\gamma_b^+\gamma_s^-} - \sqrt{\gamma_b^-\gamma_s^+}\right)$$

As the free energy is related to the interfacial tension, then $\Delta G_{adhesion}$ can be represented by the following:

$$\Delta G_{adhesion} = \Delta G_{bls}^{LW} + \Delta G_{bls}^{AB}$$

$$\Delta G_{bls}^{LW} = \gamma_{bs}^{LW} - \gamma_{bl}^{LW} - \gamma_{sl}^{LW}$$

$$\Delta G_{bls}^{AB} = \gamma_{bs}^{AB} - \gamma_{bl}^{AB} - \gamma_{sl}^{AB}$$

in which γ_{bs} is the interfacial tension between the bacterial surfaces and the adhesion surface; γ_{bl} is the interfacial tension between the bacterial surfaces and the liquid; and γ_{sl} is the interfacial tension between the adhesion surfaces and the liquid.

The $\Delta G_{adhesion}$ values allow for evaluation of the thermodynamics of the adhesion process: if $\Delta G_{adhesion} <0$, the process is favorable; if $\Delta G_{adhesion} >0$, the process is unfavorable.

The roughness was evaluated using Atomic Force Microscopy (AFM) (Universal SPM System Ntegra Prima/NT-MDT) and a perfilometer (Ambios Technology, XP1) in the Nanoscopy Laboratory at the Departamento de Física at the Universidade Federal de Viçosa, Minas Gerais, Brazil.

The electrical charge of the ribotype was determined based in the % of cells retention in the ionic charge resins (anionic and cationic) by using of electrostatic interaction chromatography. The analyses of electric charge were conducted with the use of SP Sepharose XL and Q Sepharose XL columns (GE Healthcare®).

The numbers of cfu/cm^2 after different cleaning and sanitizing procedures on *B. cereus* RIBO 1 222-173-S4 adherence to pipe surfaces (stainless steel AISI 304, #4) were compared with APHA recommendation which is 2cfu/cm^2 (Evancho et al., 2001) The results concerning physicochemical properties of the surfaces were discussed by descriptive analysis.

RESULTS AND DISCUSSION

The conditions of the stainless steel surfaces adhered with *B. cereus* for five weeks were dependent on the cleaner and sanitizer agents used in the SSOP. Procedure C was less effective, since after three repetitions, the number of *B. cereus* was higher than 2cfu/cm^2 (Table 2). The recommended value for mesophilic aerobic microorganisms by Evancho et al. (2001) was adopted in this research as a limit for *B. cereus* counts on surfaces to be considered in good hygienic condition for food processing. The probable causes of poor sanitation were the following: a) the low concentration of the alkaline solutions, b) the alkaline solution without the addition of complex agents or caustic active agents, c) the absence of acid washing, and d) the high pH of the chlorine solution.

Table 2. Number of *Bacillus cereus* (cfu/cm^2) adhered to the stainless steel AISI 304, #4, after different cleaning and sanitizing procedures

Procedure	Repetition				
	1	2	3	4	5
Control*	<0.85	0.85	<0.85	<0.85	<0.85
A*	<0.85	<0.85	<0.85	<0.85	0.85
B*	<0.85	1.70	0.85	<0.85	0.85
C*	2.51	0.85	2.98	<0.85	2.13

*Procedures performed according to Table 1.

Procedure B showed the number of adhered *B. cereus* to be a little higher than the numbers found in procedure A and the control. However, the microbial counts after procedure B were lower than the specification (2cfu/cm^2). The bacterial removal by procedure A and the control were much closer. In this experiment, it seemed that the absence of the tense active agent and a chlorine solution concentration lower than generally recommended did not affect the outcome of the procedures. However, it is important to mention the role of the tense and active agents in cleaning formulations, which have the effect of decreasing the surface tension of water and improving the contact between the detergent and food residues (Andrade et al., 2008).

The effectiveness of the hygiene treatments was reduced when the concentration (Procedure B) and temperature (Procedure C) of the alkaline solution was decreased, thus showing the importance of controlling these factors for the prevention of microbial contamination of the surfaces. The removal of food residues from the surfaces by cleaning agents before sanitization is fundamental for microbiological control.

Peng et al. (2001) showed that the fibrils of *Bacillus cereus* were responsible for the adherence of the microorganism to stainless steel, and they were not removed without previous cleaning with detergents. Peng et al. (2002) observed a higher reduction in the numbers of microorganisms on the surfaces that was due to the actions of the alkaline detergent. The steps of acid washing and sanitization did show a significant reduction in the microbial population of *B. cereus* remaining on the surfaces. However, the importance of the acid detergent and sanitizers should be stressed. The acid solutions are very important in controlling and preventing mineral deposits on the equipment surfaces, which are responsible for the corrosion process and support points where biofilms can start. The sanitizer solutions are responsible for reducing microorganisms to levels considered safe for food processing and limiting food spoilage by eliminating these pathogenic microorganisms. Therefore, the hygiene procedures should be conducted in two basic steps: cleaning by using detergents to remove organic and inorganic food residues, and sanitizing by the use of physical or chemical agents to control microorganisms.

The cleaning and sanitizing procedures are affected by several surface physicochemical properties of the stainless steel and the microorganisms. Among them, surface hydrophobicity, roughness, and electrical charge are included. Some physicochemical analysis of the stainless steel and B. *cereus* evaluated in this study are showed in Table 3. The roughness of the surfaces of stainless steel (Ra=0,22µm) can be considered smooth. Generally, when the mean roughness (Ra) is approximately of the bacterial size (about 1.0µm), the adherence process is favored. The studied strain of *B. cereus* was considered hydrophilic while the stainless steel was considered hydrophobic. The adhesion was not thermodynamically favorable ($\Delta G_{adhesion}$=2.55mJ/m^2) between the stainless steel and the studied strain of *B. cereus*. Thus, the interaction between them was not favored by thermodynamic aspect of adhesion.

Table 3. Some physicochemical characteristics of stainless steel and *B. cereus*

Physicochemical characteristics	Stainless steel	*B. cereus*
Mean roughness-Perfilometry (Ra)	0.22µm	-
Mean roughness- Atomic Force Microscopy (Ra)	83.35nm	-
Electrical anionic resin (% of retention)	-	89.82
Electrical cationic resin (% of retention)	-	90.96
Contact angle with water (θ_A)	70.77	24.52
Interfacial tension (mJ/m^2)	40.83	56.52
Total free energy of interaction (ΔG_{sws}^{TOT}) (mJ/m^2)	-28.73	19.81
Global free energy of adhesion ($\Delta G_{adhesion}$) between stainless steel and *B.cereus* (mJ/m^2)	2,55	

The dairy industry should carefully evaluate its SSPO, focusing on the steps of the pre-rinse with water, the use of the detergents (alkaline and acid), the post-rinse with water, and the use of sanitizers. The SSPO should include adequate information about the concentration, time, and temperature of the cleaning and sanitizing applications.

Finally, the quality of surfaces for food processing should be periodically monitored to detect deviations from the proposed specifications, permitting corrective action to be taken when necessary. Despite of physicochemical properties of the stainless steel AISI 304, #4, and *B. cereus* RIBO 1 222-173-S4 were not favored to adhesion process, after the different hygiene procedures, a number ranging 0.85cfu/cm^2 to 2.98cfu/cm^2 was counted on the stainless steel. Then, it can be concluded that the adherence of *B. cereus* occurred in lower number, in accordance to the thermodynamic aspect of adhesion.

CONCLUSIONS

The number of *B. cereus* RIBO 1 222-173-S4 found on stainless steel surface AISI 304 # 4, reached 2.98cfu/cm^2. The number of this microorganism on the surface was dependent on the cleaning and sanitization procedures, being affected by composition of the detergent and temperature applied. The control treatment that used an alkaline detergent at 80 ºC (sodium hydroxide, EDTA, and sodium dodecyl sulphonate), an acid detergent (nitric acid), and a chemical sanitizer (sodium hypochlorite) was the best in the control of the adhesion of *B. cereus* on the surface, reaching <0.85cfu/cm^2. The adherence of this microorganism occurred in lower number, in accordance to the physical and chemical aspects of adhesion, once the $\Delta G_{adhesion}$ was 2.55mJ/m^2, being not thermodynamically favorable.

Equipment surfaces play an important role in the recontamination of pasteurized milk, especially if hygiene procedures are not correctly applied. To control *B. cereus*, the dairy industry must emphasize how to conduct the cleaning and sanitizing procedures, with details about the procedure, the control of pH, the concentrations of reagents to be used, and the temperatures and exposure times of the solutions. Also, it is important to establish a monitoring process for assessing corrective actions that can be conducted when necessary.

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