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Full Length Research Paper

Influence of bacterial species on adhesion to stainless steel

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To assess the adherence of bacteria in stainless steel used in food processing, we developed an experimental model of a milk circulation line equipped with coupons T, 90° (elbow) and cylindrical-shaped constructed AISI 304 stainless steel. We evaluated the adherence of *Enterococcus faecium*, *Pseudomonas aeruginosa* ATCC 15442 and *Bacillus cereus* NCTC 11145 in vegetative and spore forms, before and after milk circulation in the model. The micro-organisms were activated (35°C/12 h) in MRS to *E. faecium* and *B. cereus* and nutrient broth for *P. aeruginosa* and were used to inoculate milk so as to obtain a count of 1.0 x 10^6 CFU/ml and placed within the test coupons in order to fill them and subsequently incubation was performed at 18°C/12 h. It was concluded that percentages of adherent bacteria before milk circulation were significantly different ($p<0.05$) that is 13.6% for the *B. cereus* spores that adhered, 6.0% for the *P. aeruginosa*, 1.28% for vegetative and spore forms of *B. cereus* and 0.31% for *E. faecium*. There was no significant difference ($p\geq0.05$) between the micro-organisms that remained attached after the milk circulation. There was significant difference in removing bacteria, among coupons proof. No coupon T removal was higher than in cylindrical, probably due to the higher turbulent flow, whereas in the later, there is greater tendency of bacteria still attached.

Key words: Milk, adhesion, bacterium, biofilm.

INTRODUCTION

Industries have rendered increasingly larger amounts of food to meet the needs of the market. With the increase in processing capacity, several problems have emerged. One is the post-processing loss or reduced shelf life of food due to the contamination of food with micro-organisms attached to the production line. Since these microorganisms have adhered and biofilm formed on the surface of food processing, their removal or destruction will be much more difficult. Hence, the need for a good cleanup process which can remove microorganisms with

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efficiency. Microbial adhesion and subsequent biofilm formation occur in many fields of industrial and medical applications (Flemming, 2002; Von Eiff et al., 2005). About 99% of all bacteria in natural environments reside in biofilms that consist of microorganisms adhering to a surfaces with the aid of extracellular polymeric substances produced by the micro-organisms themselves (Jung et al., 2013). Bacteria adhering in dairy plant are resistant to the cleaning process with 200 ppm of chlorine for 30 min and contaminate subsequent batches of cheese (Somers et al., 2001). The production of exopolysaccharides increases when a bacterium adherent to a surface and is influenced by time and type of carbohydrate present in the food (Jung et al., 2013). It should be considered that milk is a complex substrate for microorganisms, the formation of biofilms is rapid since for instance, Gram negative bacteria easily multiply in milk residues after improper cleaning of milk equipment. Various factors affect the bacterial adhesion process and the formation of biofilms, including genotypic, thermodynamic and environmental factors (Renier et al., 2010). The genetic makeup of the organism, including the presence of flagella and fimbria, determines its ability to produce exopolysaccharides that assist in adhesion (Folson, 2006). Hydrophobicity and electric charge play an important role in the process of bacterial adhesion (Boks et al., 2008). Time, temperature, pH, the presence of alcoholic substances and the flow of cleaning solutions on the surfaces also actively participate in the process. The established biofilm matrices enable incorporation of pathogens like *Listeria monocytogenes*, which can cause a continuous contamination of food processing plants or another plant (Blackman and Frank, 1996). This microorganism is frequently found in raw milk and non-pasteurized raw milk products and as part of a biofilm community in milk meters and bulk milk tanks (Weiler et al., 2013). In a study performed to evaluate the adherence capacity of *Escherichia coli* O157: H7 stainless steel and high density polyethylene, researchers found that even at 4 or 12°C, this micro-organism can adhere and multiply on the surface of both surfaces studied (Dourou et al., 2011). In an experiment to induce the formation of biofilm FCM 40 of *Salmonella* in various materials used in the poultry industry in India, it was observed that after incubation at 28°C/48 h in tryptone broth inoculated with this bacteria, the biofilm had scores of 1.20 x 10^7, 4.96 x 10^6 and 2.23 x 10^5 CFU/cm^2 in high density polyethylene, concrete and stainless steel, respectively (Joseph et al., 2001). These authors also assert that this micro-organism cell in biofilms are more resistant to the action of chlorine to the planktonic cells and high density polyethylene was not possible to inactivate all the cells even with a 100 ppm treatment of Cl_2 for 25 min. Various micro-organisms have been studied with respect to their ability to form biofilm on surfaces in food processing, including *Staphylococcus aureus, Yersinia enterocolitica, Listeria monocytogenes* and *Campylobacter jejuni* (Weiler et al., 2013; Furukawa et al., 2010). The objective of this study was to assess the adherence of bacteria in stainless steel and its resistance to removal by the flow of food in a milk circulation model system.

MATERIALS AND METHODS

Microorganisms and culture media

The microorganisms used in this study were *Pseudomonas aeruginosa* ATCC 15442, *Bacillus cereus* NCTC11145 and *Enterococcus faecium*. The culture media used was Lactobacilli MRS broth for *B. cereus* and *E. faecium* and nutrient broth for *P. aeruginosa*.

Activation and inoculation of the microorganisms

The microorganisms were activated in 10 mL of culture media and then incubated at 36°C for 12 h. After this period, the cultures were subcultured in the same media and incubated at 35°C for 10 h. After activation, 400 mL of sterilized milk was inoculated (121°C/15 min) with *P. aeruginosa*, *B. cereus* or *E. faecium*, alternatively. The cultures were inoculated to obtain approximately 1.0 x 10^6 CFU/mL. The count of microorganisms was done using a pour-plate technique with standard plate count agar (PCA) to verify the number of bacteria in the milk. In the specific case of *B. cereus*, the plating was made for total cell count and spores count. To count spores, the suspension composed of milk and *B. cereus* was heated at 70°C/30 min with subsequent plating.

Description of equipment and experimental model

To determine the ability of the three bacterial cultures to adhere to steel surfaces, we used a stainless steel milk processing circuit (Figure 1), which constituted a ¾-inch-diameter (internal) pipe with a total length of 5.8 m. The milk circulated through the pipe from a tank of 25 L that served as a reservoir. At specific points in the pipe test coupons of 90° (Elbow), T-coupons and cylindrical-shaped coupons made of a stainless steel were installed. The internal surface area of the test coupons was as follows: 108.06 cm^2 for the T-shaped coupons, 52.74 cm^2 for the 90° (Elbow) coupons and 84.69 cm^2 for the cylindrical coupons.

To allow for adhesion, the stainless steel test coupons were removed from the equipment, filled with inoculated milk and closed with cap. The quantities used were 27 mL of milk in the elbow coupon (1A and 1B), 57 mL in the T (3A and 3B) and 49 mL in the cylindrical coupon (2A and 2B) followed by incubation at 18°C for 12 h. After this period, milk samples from the coupons were plated as described previously and the remainder discarded. Sterile milk was added to the test coupons, maintained inside for two minutes and then discarded to remove planktonic cells and/or spores that had reversibly adhered. The test coupons 1A, 2A and 3A were filled with a 2% sodium citrate solution (20 mL in the elbow coupon, 30 mL in the cylindrical coupon and 40 mL in the T-shaped coupon) and washed with manual agitation for 15 min. The wash solution was then plated to determine the number of bacteria that adhered to the test coupons before milk circulation. The sodium citrate solution was discarded and the three coupons (1A, 2A and 4A) were treated for five minutes with a sodium hypochlorite solution at pH 7.5 containing 300 mg/L of free residual chlorine. The coupons were then washed three times in distilled water and subsequently

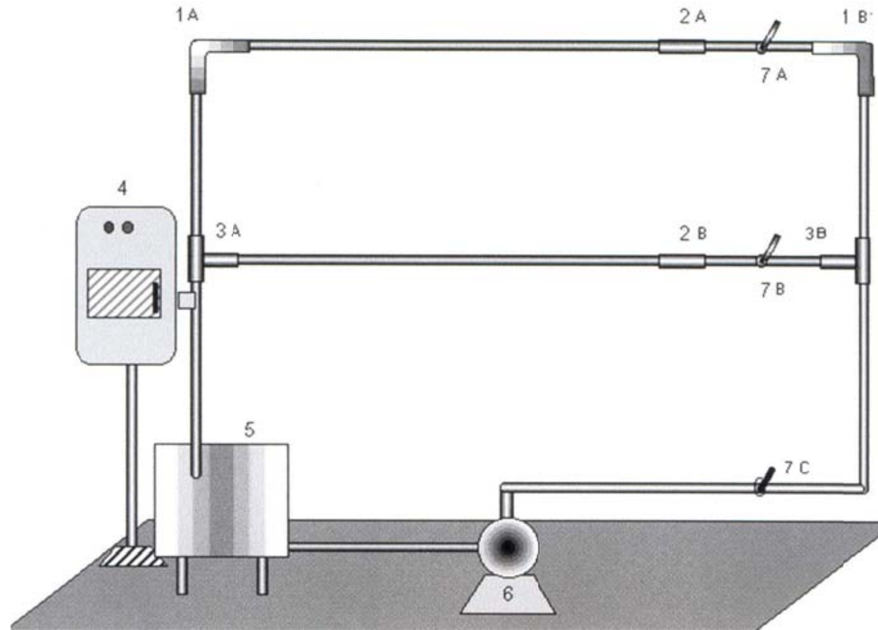

Figure 1. Model of the milk processing line (1 - 90° (Elbow) test coupon; 2 - cylindrical test coupon; 3– T test coupon; 4 – on/off control; 5 - milk reservoir; 6 - centrifugal pump; 7– butterfly valves).

used in the circuit model. The six test coupons were connected to the equipment and 10 L of sterilized milk (121°C for 15 min) was added to the reservoir to circulate for 10 min at 1 m/s at an average temperature of 15°C.

To determine the flow rate at which a velocity of 1.0 m/s was achieved, we used the following calculation:

$$X = \frac{Y\ (m/s)}{\pi\ (R)^2}$$

where: Y = the desired velocity; R = the pipe radius.

The coupons 1B, 2B and 3B, unwashed, were removed from the system and filled with citrate for 2 min with subsequent disposal of the liquid. After that, the coupons were rinsed with a sodium citrate solution as described above. The wash solutions were plated after adequate dilution. Plating, in duplicate, was performed using plate count agar and the incubation was at 35°C for 48 h. The experiment was done in three repetitions.

The experiment was conducted using a split-plot design in which four bacterial forms are used as main treatments and three types of test coupons as secondary treatments. Each experiment was performed in triplicate. Statistical analysis was performed using the number of decimal reductions in the population of microorganisms before (DR_A) and after (DR_B) milk circulation. To determine DR_A, the following calculation was done:

$$DR_A = \log (N_0 \times 133) - \log (N_1 \times 245{,}5)$$

Where: N_0 = total number of bacteria (planktonic) inside the coupon after 12 h of incubation; N_1 = number of adhered bacteria inside of the coupon after 12 h of incubation.

The number of sessile cells (N_1) was obtained by washing coupons 1A, 2A and 3A and plating 1 mL aliquot of sodium citrate solution used to wash the test coupons. The value obtained was multiplied by the total wash volume used in the coupon. To obtain N_2, coupons 1B, 2B and 3B were also washed after milk circulation at the desired velocity. To determine DR_B, the following calculation was done:

$$DR_B = \log N_1 - \log N_2$$

where: N_2 = number of bacteria that remained adherent to the coupon after milk circulation. Therefore, adhesion was considered to be greater for bacteria that had a lower decimal reduction. For comparisons of interest, we performed a Tukey test at 5% probability ($P<0.05$).

RESULTS AND DISCUSSION

The data showed that among the bacteria evaluated, *E. faecium* had the greatest ability to multiply at 18°C in milk (Table 1). We observed that this microorganism increased by about 2 logarithmic cycles in 12 h. *P. aeruginosa* increased by 0.9 logarithmic cycles, whereas *B. cereus* (spores and vegetative cells) increased by 0.4 logarithmic cycles.

We observed a small increase in the number of *B. cereus* spores from 7.8×10^2 CFU/mL at the moment of inoculation to 1.4×10^3 CFU/mL after 12 h.

If we consider that the total area of the three coupons was 245.5 cm^2 and the number of *P. aeruginosa* before the milk circulation was 2.4×10^5 CFU/cm^2 (Table 2), we will come to the conclusion that the total number of adhered cells was 5.9×10^7 CFU. Considering also that there was 7.3×10^6 CFU/ml of milk in the group of coupons which had a total volume of 133 ml milk, we conclude that total number of bacteria in milk at 12 h was

Table 1. Number of colony forming units (CFU/mL) in milk immediately after inoculation and after 12 h of incubation at 18°C. Average of three replicates.

Time	*Enterococcus faecium*	*Pseudomonas aeruginosa*	*Bacillus cereus (spores + vegetative*	*Bacillus cereus* (spores)
	CFU/mL			
Initial	2.4 x 10^6	9.3 x 10^5	1.2 x 10^6	7.8 x 10^2
12 h (N_0)	2.1 x 10^8	7.3 x 10^6	3.0 x 10^6	1.4 x 10^3

Table 2. Number of adherent cells in the pipe (test coupons) before and after the circulation of milk at 1 m/s for 10 min.

Adhesion	*Enterococcus faecium*	*Pseudomona s aeruginosa*	*Bacillus cereus (esporos + vegetativas*	*Bacillus cereus* (esporos)
	CFU/cm^2			
Before milk circulation (N_1)	6.5x10^5	2.4x10^5	3.9 x 10^4	1.9 x 10^2
After milk circulation (N_2)	3.3x10^4	1.7x10^4	9.0x10^2	7.8x10^0

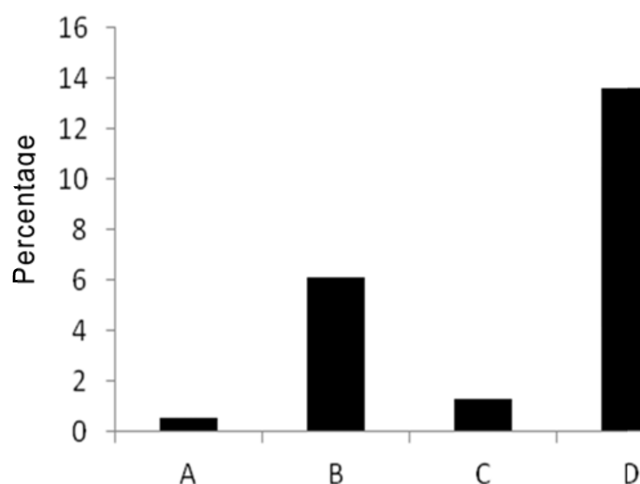

Figure 2. Percent adhesion of bacteria in coupons, before the milk flow. Calculated in relation to number of bacteria (CFU/mL) in the coupon after 12 h in stainless steel at 18°C and number of adherent cells (N_1). *Enterococcus faecium* (A), *Pseudomonas aeruginosa* (B), *Bacillus cereus* vegetative cells and spores (C) and (D) spores of *B. cereus.*

Table 3. Decimal reductions of adhered cells in relation to planktonics microorganisms in test coupons upon 12 h (DR_A= Log N_0 x 133 – Log N_1 x 245,5) of incubation at 18°C.

Microorganisms	DR_A
Bacillus cereus (spores)	0.62^A
Pseudomonas aeruginosa	1.22^B
Bacillus cereus (spores and vegetative)	1.69^C
Enterococcus faecium	2.24^D

*Averages followed by the same letter do not differ significantly at Tukey test (5%).

9.7 x10^8 CFU; thus 6.0% of these bacteria were adhered.

When evaluating bacterial adhesion of *E. faecium* after 12 h (6.5x10^5 CFU/cm^2), in relation to the planktonics cells in milk (2.1 x 10^8 CFU/mL), we observed 0.31% (Figure 2) of the adhered cells; *B. cereus* vegetative and spore forms 1.28% and spores of *B. cereus* (13.6%). This result is consistent with studies of Suarez (1991); these studies observed that, in several species of psychrotrophic microorganisms isolated from milk, Gram negative species had an increased ability to adhere to stainless steel, rubber and glass surfaces than Gram positive species.

It is interesting to note the high percentage of adhesion obtained with spores, which reached 13.6%, about 10 times higher than the adhesion of vegetative cells and spores. According Ronner et al (1990), some spores are highly hydrophobic which facilitates their adhesion to surfaces. In a study involving five species of bacteria that produce spores, it was observed that the spore of *B. cereus* presented the greater adhesion capacity, about 45% in hydrophobic surface, whereas *Bacillus licheniformis* has better adhesion on hydrophilic surface. The study also showed that the adhesion ability of the spore form is much higher than vegetative form of the same microorganism.

In Table 3, it is shown that *B. cereus* showed the largest adhesion capacity, in other words, the lowest decimal reduction. These spores likely adhere to surface of equipment then germinate and compromise the quality of the milk. The following are ranked in ascending order according to their decimal reduction: *P. aeruginosa, B. cereus*, including spores and vegetative cells, and *E. faecium.* Based on analysis of variance of the decimal reduction and Tukey test, it is concluded that there is a significant difference ($p<0.05$) between the types of bacteria used in relation to the ability of adhesion.

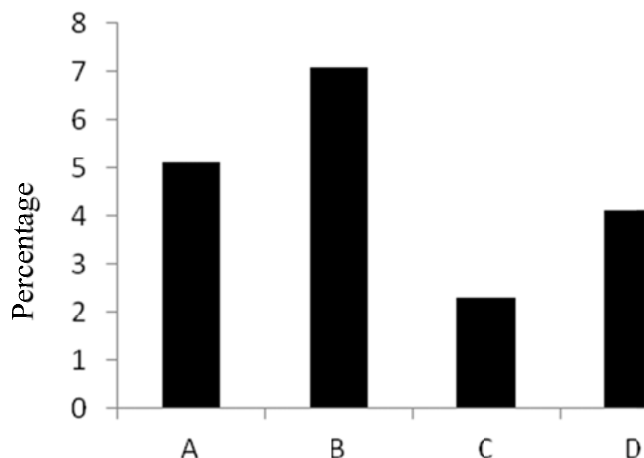

Figure 3. Percent adhesion of bacteria in coupons, after milk circulation. Calculated in relation to number of bacteria adhered before milk circulation (N_1) and after circulation (N_2). *Enterococcus faecium* (A), *Pseudomonas aeruginosa* (B), *Bacillus cereus* vegetative cells and spores (C) and (D) spores of *B. cereus*

Table 4. Decimal reductions (DR_A= Log N_0 – Log N_1) of adhered cells after milk circulation model at a velocity of 1 m/s for 10 min at 15°C.

Microorganisms	DR_A
Pseudomonas aeruginosa	1.15^A
Enterococcus faecium	1.28^A
Bacillus cereus (spores)	1.39^A
Bacillus cereus (spores and vegetative)	1.63^A

*Averages followed by the same letter do not differ significantly at Tukey test (5%).

After milk circulation (Figure 3), we observed that 5% of *E. faecium* cells remained adhered. We calculated 6.5 x 10^5 CFU/cm^2 of *E. faecium* before milk circulation, where this number was reduced to 3.3 x 10^4 CFU/cm^2 after circulation. Our results also indicated that 7.1% of the *P. aeruginosa* cells remained adherent in the circuit model. This percentage, calculated based on the number of adherent cells before milk circulation, represented 1.7 x 10^4 CFU/cm^2 of the surface. The number of micro-organisms here is still large enough to cause milk conta-mination because the lipases produced by *Pseudomonas* sp. are extremely resistant to thermal heat treatment at 110°C for 10 min (Robinson, 1990).

Our results also indicated that 7.0% of the *P. aeruginosa* cells remained adherent in the circuit model. The number of microorganisms here is still large enough to cause milk contamination because the lipases produced by *Pseudomonas* sp. are extremely resistant to thermal heat treatment at 110°C for 10 min (Robinson, 1990).

There was 1.28% adhesion of *B. cereus* spores and vegetative cells before milk circulation, and 2.4% of the cells that adhered resisted the milk flow. However, it should be noted that although the spores had an increased ability to adhere to surfaces (13.6%) in foods, 4.1% of the adhered spores resisted the flow of milk. This may be because spores do not have the ability to produce polymeric substances that facilitate their adhesion to coupons. Consequently, the spores only remain adherent by forces, such as electrostatic attraction, which reduces their counts after milk circulation to levels well below the initial counts.

The decimal reduction, for different microorganisms after milk circulation is shown in Table 4. The analysis of variance (Table 5) performed for the results showed that there were no significant differences ($p \geq 0.05$) in adhesion between the different types of bacteria. However, there was a difference with respect to the removal of cells from the different types of coupons.

The interaction of microorganisms with the different coupons was not significant (Table 5). This analysis was intended to determine whether a particular bacterium adhered at a higher percentage to one particular type of coupon, whereas another species could have a higher percentage of adhesion to a second type of coupon.

The highest rate of bacterial removal occurred in the T-shaped coupon (Figure 4), whereas the lowest rate of removal occurred in the cylindrical coupon. This difference was significant at level of 5% (Table 6). We did not observe a significant difference in Tukey test ($p \geq 0.05$) in the removal of bacteria from the cylindrical and elbow or T and elbow coupons.

It is possible that the turbulence in cylindrical pipes is lower than in pipes with contouring formats, such as in elbow and T-shaped pipes. Therefore, the shear caused by the fluid on the walls of the cylindrical test coupons is lower, and this can hamper the removal of micro-organisms from surfaces.

Conclusion

We observed a significant difference ($p<0.05$) in the ability of the three microorganisms to adhere to stainless steel before milk circulation. Thus, 13.6% of *B. cereus* spores adhered, the vegetative and spore forms of *B. cereus* together showed 1.28% of adhesion, *P. aeruginosa* and *E. faecium* had 6.0 and 0.31% respectively. This demonstrates that *B. cereus* spores have an increased adhesion ability as compared to the other species evaluated.

After milk circulation, there were no significant differences in the number of bacteria that remained adherent ($p \geq 0.05$). However, there were differences in the removal of adherent cells between different types of coupons test. The removal was higher in T coupon and the coupon lower cylindrical presenting statistically

Table 5. Summary of the analysis of variance of decimal reductions in the different microorganisms on the different test coupons after using the milk circulation model at a velocity of 1 m/s for 10 min at 15°C.

S.V.	D.F.	S.S.	M.S.	F	$F_{5\%}$
Bacterium	3	0.7837613	0.2612537	2.2119ns	4.07
residue (a)	8	0.9448708	0.1181088		
Coupon	2	0.4470955	0.2235478	6.30*	3.63
Coupon*inoculation	6	0.4788803	0.07981338	2.25ns	2.74
Residue (b)	16	0.5674057	0.03546286		
TOTAL	35	3.222013			

*Significant at 5% significance by the F-test; ns Not significant at 5% significance by the F-test.

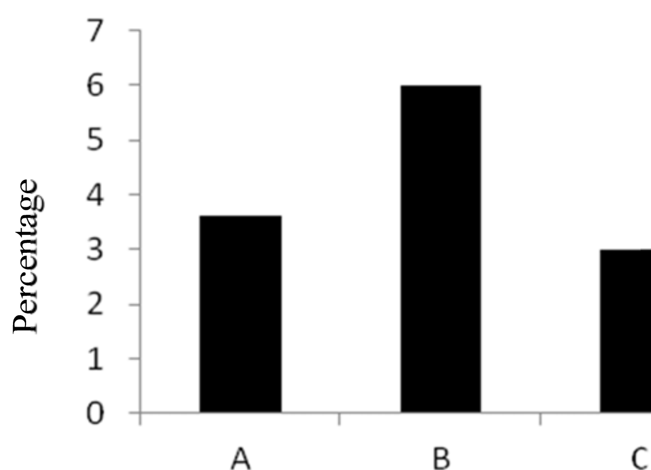

Figure 4. Percent adhesion of bacteria in coupons, after milk circulation. Elbow coupon (A), cylindrical coupon (B), T-coupon (C).

Table 6. Averages of decimal reductions in the microbial populations for the different test coupons after milk circulation at 1 m/s for 10 minutes at 15°C.

Coupon type	DR^{B}
T	1.5496^{A}
Elbow	1.4713^{AB}
Cylindrical	1.2840^{B}

*Averages followed by the same letter do not differ significantly at Tukey test (5%).

significant difference (p<0.05). We concluded that it is necessary to avoid T-shaped points in the pipes through which foods pose a barrier to bacterial adhesion.

Conflict of Interests

The authors have not declared any conflict of interests.

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