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Adhesion behavior and removability of *Escherichia coli* on stainless steel surface

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ABSTRACT

Adhesion behavior of *Escherichia coli* from its suspension onto stainless steel (type 304) surface was studied, focusing on the effects of initial cell concentration of suspension, suspending medium, and roughness of stainless steel surface. The results demonstrated that the adhesion of *E. coli* was significantly enhanced by the cell concentration of contaminants. The adhesion was observed even in short contact periods of time, but it increased and reached a plateau within 3–4 h. The plateau value of surface cell density was roughly proportional to the cell concentration of the suspension. Rinsing experiments indicated that a relatively high percentage of cells were found to be irreversibly attached on the surface when peptone saline was used as a suspending medium. The adherent cells were however shown to be quite vulnerable to removal with water as a rinsing medium. Moreover surface roughness was found to affect the removability of adherent cells.

1. Introduction

Adhesion of microorganisms to food contact surfaces of processing equipment is a major concern from the viewpoints of controlling quality and safety of food products. If cleaning and sanitation are insufficient, microbial cells on equipment surface could survive to develop into a biofilm and cause cross-contamination leading to lowered shelf-life, food spoilage, and transmission of disease (Allison & Gilbert, 1992; Carpentier & Cerf, 1993; Sharma & Anand, 2002; Stepanović, Ćirković, Mijač, & Švabić-Vlahović, 2003). Marshall (1992) described biofilm formation as consisting of four distinct stages: the formation of a conditioning film, bacterial cell adhesion, growth and extracellular polymer production by the adhering cells, and maturation of the biofilm. The bacterial cell adhesion stage is generally believed to be reversible, at least initially, due to weak interactions between bacteria and substrate, including van der Waals, electrostatic, and hydrophobic interactions (Busscher & Van der Mei, 2006; Chmielewski & Frank, 2003). Subsequently, adhesion of microbial cell turns irreversible as a result of anchoring by appendages and/or production of extracellular polymers. Thus a comprehensive knowledge on the very initial stage of microbial adhesion is important to control the biofilm formation and the risk of microbial cross-contamination.

Among various types of materials used for food contact surfaces of processing equipment, stainless steel is most widely employed because of its mechanical strength, corrosion resistance, longevity, and ease of fabrication (Holah & Thorpe, 1990). So far, several studies on microbial adhesion to stainless steel surfaces have been conducted. Earlier reports about microbial adhesion include the following species: *Listeria monocytogenes* (Barnes, Lo, Adams, & Chamberlain, 1999; Helke, Somers, & Wong, 1993; Hilbert, Bagge-Ravn, Kold, & Gram, 2003), *Streptococcus thermophillus* (Boulangé-Petermann, Rault, & Bellon-Fontaine, 1997), *Staphylococcus aureus* (Barnes et al., 1999), *Staphylococcus marcescens* (Barnes et al., 1999), *Pseudomonas* sp. (Hilbert et al., 2003), *Candida lipolytica* (Hilbert et al., 2003). These works, however, are yet to clearly elucidate influences of different factors governing microbial adhesion. Moreover, they reported at times different tendencies of the influence for different microbial species. Thus systematic compilation of more adhesion data, especially for different species, is necessary to discuss the effects of various factors comprehensively. In this context, it is a problem that adhesion behaviors of several important microbial species have not been fully investigated. For example, very limited studies on adhesion behavior to stainless steel surface have been reported for *Escherichia coli*, which is an important species for judging hygienic status and contains pathogenic strains. Barnes et al. (1999) reported that the number of *E. coli* attached from its 10^8 CFU/ml suspension to a stainless steel (type 304) coupon after 2 h incubation at 20 °C was too small to asses any effect of experimental variables. In contrast, Ryu, Kim, Frank, and Beuchat (2004) reported that about 2×10^5 CFU/cm^2 of *E. coli* O157:H7 cells were found adherent on a stainless steel (type 304) coupon after incubation with 10^8 CFU/ml suspension at 4 °C for 24 h. Thus further information on adhesion behavior of *E. coli* is desired, preferably under conditions which enable comparison with those of other microbial species.

This study aims to provide adhesion data of *E. coli* from its suspension to stainless steel surfaces. The factors considered in this study include concentration of the cell suspension, suspending

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medium, and roughness of stainless steel surface. Additionally, the removability of adhered cells from the stainless steel surfaces with shear force application was also taken as a subject of this study. The adhesion behaviors observed for *E. coli* are discussed in comparison with those of *Staphylococcus epidermidis* observed under very similar conditions, which has been previously reported by the authors (Ortega, Hagiwara, Watanabe, & Sakiyama, 2008).

2. Materials and methods

2.1. Microbial strain

E. coli NBRC 3301, maintained as a glycerol stock at −80 °C, was inoculated in Trypticase soy broth (TSB) (Becton, Dickinson and Co., Maryland, USA) and grown at 37 °C for 18–20 h. The culture was then plated on Trypticase soy agar (TSA), which was made by the addition of 15 g/L agar to TSB. Following incubation at 37 °C for 48 h, the TSA plate was stored at 4 °C and used as a working stock.

2.2. Stainless steel surfaces

Three types of stainless steel (type 304) plates (50 × 50 × 3 mm) A, B, and C with different degrees of surface roughness was purchased from Toste (Osaka, Japan). The surface roughness qualities of the three types of the plate, as expressed in roughness parameter R_a are listed in Table 1. The values of R_a ranged from 0.04 for type A (the smoothest) to 1.37 for type C (the roughest). All the stainless steel plates were cleaned by soaking in an alkali detergent (SCAT 20-X, Dai-ichi Kogyo Seiyaku Co. Ltd., Kyoto, Japan) for 24 h, rinsed with water, dried in clean ventilated oven at 60 °C for 1 h, and stored in 70% ethanol. They were dried under UV light in a clean bench just before use in every experimental run.

2.3. Preparation of microbial suspension

For every run of adhesion experiment, *E. coli* cells from the working stock were sub-cultured in 5 ml TSB at 37 °C for 18–20 h. The stationary-phase microbial cells thus obtained were harvested by centrifugation at 3500 × *g* for 10 min and resuspended in peptone saline (1 g/L peptone and 8.5 g/L NaCl). The resuspended cells were then serially diluted in peptone saline to achieve the desired initial cell concentration. In some experiments, to study the effect of suspending medium on adhesion, phosphate buffered saline (PBS) and physiological saline (0.85% NaCl) were also used instead of peptone saline, with an additional centrifugation–resuspension procedure before dilution.

2.4. Adhesion experiment

A stainless steel plate was contaminated by soaking in 25 ml of the microbial suspension at 25 °C. The contaminated stainless steel plate was withdrawn after a designated period of exposure to the microbial suspension and rinsed twice with 20 ml of peptone saline to remove loosely adherent cells. Preliminary tests showed that further rinsing did not reduce the cells left on the surface (data not shown). The number of cells on the stainless steel plate was enumerated as described in Section 2.5. Change in the cell concentration of the microbial suspension during the exposure period was also monitored by colony counting on TSA.

Table 1
Surface roughness of each type of stainless steel plate.

Plate type	A	B	C
Average roughness, R_a (μm)	0.04	0.14	1.37

2.5. Enumeration of microbial cells adherent to stainless steel surface

The microbial cells adherent to a unit area of the stainless steel surface (surface cell density) was enumerated by swab-vortex method as follows. The entire surface of the stainless steel plate was swabbed twice with a sterile cotton swab. The cotton tip was then cut off and soaked in 1 mL of peptone saline and subjected to vigorous vortex-mixing for 1 min. The cell suspension thus obtained was adequately diluted if necessary and plated on TSA. The number of *E. coli* cells recovered by swabbing (X_1) was determined from colony count on the TSA plate after incubation at 37 °C for 18–20 h. To confirm the number of *E. coli* cells still remaining on the stainless steel plate after swabbing (X_2), the plate was put in contact with TSA for 1 min. The number of colonies that emerged on the TSA after incubation at 37 °C for 48 h was taken as X_2. The surface density of *E. coli* cell was calculated from the sum of X_1 and X_2. In general, X_2 accounted for less than 4% of the sum ($X_1 + X_2$).

2.6. Removability of adherent cells

To study the removability of cells adherent to the stainless steel surface, the artificially contaminated plates obtained as described in Section 2.4 were rinsed with shear force application using either sterile distilled water or peptone saline solution as a rinsing medium. A 2-L capacity stainless steel vessel (135 mm in diameter, 145 mm in height) containing 1 L of a rinsing medium was placed in a water bath kept at 25 °C. The contaminated plate and a 3-blade impeller were placed in the rinsing medium such that the distance between the plate and the center of impeller was 45 mm. After the rinsing medium was stirred at 2000 rpm for a designated period of time, the number of cells remaining on the plate surface was enumerated as described in Section 2.5.

2.7. Data analysis

All experiments were done in triplicate. Values of the surface cell density in CFU/cm^2 were converted to $\log_{10}$ values for statistical analysis by Student's *t*-test or analysis of variance (ANOVA) followed by Tukey's multiple comparison test. Statistical significance was set at *P*-value less than 0.05.

3. Results

3.1. Adhesion as affected by initial concentration of cell suspension

Fig. 1 shows adhesion courses of *E. coli* cells suspended in peptone saline onto type A stainless steel surface (R_a = 0.04 μm) at 25 °C. Suspensions with the initial cell concentration of approximately 10^2, 10^3, and 10^4 CFU/ml were employed to simulate low to medium degrees of microbial contamination. For each microbial suspension tested, adhesion onto the stainless steel surface occurred at a low level within 0.5 h of exposure to the cell suspension. Surface cell density attained a plateau value and remained approximately constant after 3–4 h of exposure for all the test concentrations, though adhesion seemed to become slightly slower with decrease in the cell concentration of suspension. Results also showed that the cell concentration of suspension remained approximately constant during 6 h for each experimental run, indicating that no distinct growth of initially inoculated cells occurred under the conditions tested. Thus, the increase in surface cell density observed within 3–4 h of exposure can be ascribed to adhesion

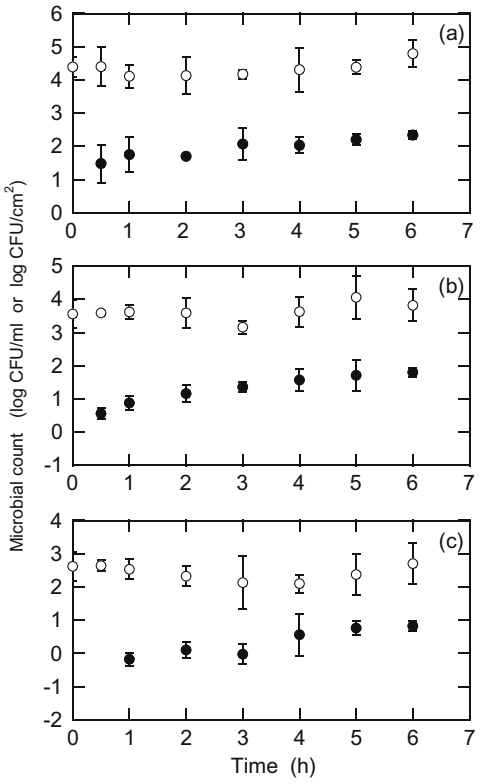

Fig. 1. Viable cell numbers of *E. coli* in suspension (○) (log CFU/ml) and on type A stainless steel plate (●) (log CFU/cm^2) at varying levels of initial cell suspension concentration: (a) 10^4 CFU/ml, (b) 10^3 CFU/ml, and (c) 10^2 CFU/ml. Each value is a mean of three tests in duplicate.

and not to growth. The plateau value of surface cell density was roughly proportional to the suspension concentration, and the final number of cells adherent to the surface was about 2–6% of the cell number in the corresponding suspension.

3.2. Adhesion as affected by suspending medium

Fig. 2 compares adhesion behaviors of *E. coli* cells suspended in peptone saline, PBS, and physiological saline. The microbial suspensions were put in contact with type A stainless steel plates (R_a = 0.04 μm) at 25 °C for 3 h. The levels of adhesion of cells suspended in PBS and physiological saline were shown to be slightly lower compared to that in peptone saline, particularly in higher concentration of cell suspensions (10^3–10^4 CFU/ml). However, except for 10^3 CFU/ml suspension, statistical significance ($P < 0.05$) was not found among the three types of suspending medium. This suggests that the presence of organic components has only a very weak influence on the adhesion of *E. coli* onto stainless steel surface.

3.3. Removability of adherent cells

Removability of the cells adherent to type A stainless steel surface (R_a = 0.04 μm) was studied under shear force application by whirlpool rinsing. The stainless steel surface was preliminarily contaminated by soaking in 10^4 CFU/ml microbial suspension in peptone saline at 25 °C for 3 h. Fig. 3 shows the surface cell density of *E. coli* remaining on the surface after whirlpool rinsing for 0–15 min. The rinsing medium was either peptone saline solution (with the same composition as the suspending medium used for preliminary contamination) or distilled water. When peptone saline was used as the rinsing medium, the surface cell density decreased gradually with rinsing time, and was reduced to 35% of the initial surface cell density after 15 min of rinsing. In contrast, upon whirlpool rinsing with water only for 5 min, the number of viable cells remaining on the surface was significantly reduced to 5% of the initial surface cell density. Further rinsing with water up to 15 min slightly decreased the number of remaining cells. The difference in the surface cell density between the two cases was proven statistically significant ($P < 0.05$) at every rinsing time except 0 min. Viability tests for *E. coli* cells suspended in distilled water showed no lethal effect of its low osmotic pressure on the microbial cells within 15 min (data not shown). This suggested that the significant reduction of surface cell density by whirlpool water rinsing was not due to lethal effect of low osmotic pressure of water.

3.4. Adhesion and removability as affected by surface roughness

Fig. 4 compares the surface densities of *E. coli* cell adherent to stainless steel surfaces with varying surface roughness (R_a = 0.04–1.37 μm) before and after whirlpool water rinsing for 15 min. Statistical analyses indicated no significant difference in cell adhesion on all types of stainless steel surfaces tested. After whirlpool water rinsing, however, surface cell density was significantly higher on the stainless steel plate of type C (R_a = 1.37 μm) than on the plates of type A and type B ($R_a \leqslant 0.14$ μm). Thus the roughness of stainless steel surface affected the removability of adherent *E. coli* cell though the removal reached 95–99% of adherent cells in all whirlpool rinsing experiments.

4. Discussion

Microbial adhesion to stainless steel surface is probably governed by various physical, chemical, and biochemical factors. The

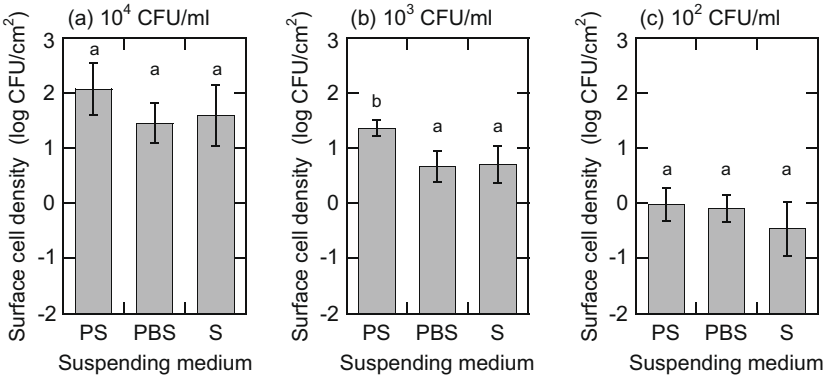

Fig. 2. Comparison of surface cell densities of *E. coli* on type A stainless steel plate from cells suspended in different suspending media. Each stainless steel plate was exposed for 3 h to cells suspended in peptone saline (PS), phosphate buffered saline (PBS), or physiological saline (S). Each value is a mean of three tests in duplicate.

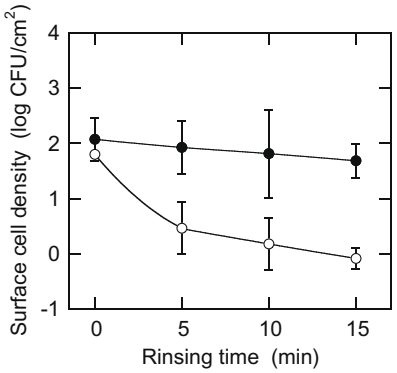

Fig. 3. Surface cell density on type A stainless steel plate after whirlpool rinsing as an index of removability of adherent *E. coli* cells. Artificially contaminated stainless steel plates were rinsed with shear force application (2000 rpm) using peptone saline or water for 0–15 min. Each value is a mean of three tests in duplicate.

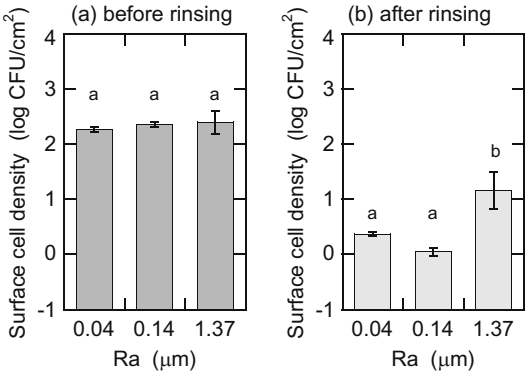

Fig. 4. Comparison of surface cell densities of *E. coli* on stainless steel plates of varying surface roughness before and after whirlpool water rinsing (2000 rpm, 15 min). Each value is a mean of three tests in duplicate.

focus of this study is on cell suspension concentration, suspending medium, and surface roughness.

Course data of bacterial adhesion over a period of time have scarcely been reported in literature, though adhesion data of several microbial species at a fixed period of time from 5 s to 2 h of exposure to various surfaces are available (Chmielewski & Frank, 2003; Gilbert, Evans, Duguid, & Brown, 1991; Hood & Zottola, 1997; Mafu, Roy, Goulet, & Magny, 1990; Wong, Chung, & Yu, 2002). In our previous work, the authors showed that the adhesion of *S. epidermidis* to stainless steel surface took approximately 3 h of exposure to cell suspension to reach a plateau of surface cell density (Ortega et al., 2008). The present study similarly showed that adhesion of *E. coli* to stainless steel surface also took 3–4 h of exposure to reach a plateau of surface cell density (Fig. 1). Although mechanism of the increase in surface cell density in the initial 3–4 h period is not clear at present, such period of time may be required for the cells to make their attachment firm enough to withstand the mild rinsing employed in the enumeration procedure of surface cell density. It seemed that *E. coli* took a slightly longer time to reach the plateau adhesion than *S. epidermidis*. This might be related to difference in mobility between the microbial species as affected by the presence or absence of cellular appendages such as flagella.

The surface cell density at plateau adhesion was roughly proportional to the cell concentration of suspension. Such result of concentration dependence may often suggest reversible adhesion. However, only about 65% of the adherent cells from suspension in peptone saline were detached from the surface by 15-min whirlpool rinsing with peptone saline as the rinsing medium (Fig. 3). This result indicated that some portion of the cells from the suspension already irreversibly adhered to the stainless steel surface despite the lack of visible evidence of exopolysaccharide substance production on the surface. This was also the case for *S. epidermidis*, though reversibility of adhesion of *S. epidermidis* seemed slightly higher than that of *E. coli* since the percentage of detached cells by whirlpool rinsing was about 87% for *S. epidermidis* (Ortega et al., 2008). This may be related to different roles of exopolysaccharides in biofilm formation reported for the two species. Danese, Pratt, and Kolter (2000) has shown that colanic acid, an exopolysaccharide of *E. coil* K-12, is required not for initial attachment to an abiotic surface but rather for establishing the complex three-dimensional structure of an *E. coli* biofilm. In contrast, initial attachment of *S. epidermidis* requires production of capsular polysaccharide/adhesin (McKenney et al., 1998).

As for the influence of suspending medium on the adhesion of *E. coli* onto stainless steel surface, the presence of organic components was suggested to have only a very weak influence, if any (Fig. 2). In contrast, our previous work (Ortega et al., 2008) showed that the adhesion of *S. epidermidis* was significantly enhanced with peptone saline as the suspending medium relative to the cases with PBS and physiological saline. Thus, the influence of organic components on bacterial cell adhesion is concluded to be species-dependent.

It is generally supposed that, when microorganisms and substrate surface are in aqueous environment containing organic molecules, the surface may first become covered with a layer of the organic molecules prior to adhesion of microorganisms (Bos, Van der Mei, & Busscher, 1999). Adhesion of organic molecules to substrate surface can occur within seconds of exposure to an aqueous environment (Chmielewski & Frank, 2003). Based on this well-accepted assumption, the interaction between organic molecules on the surface and microbial cells is a key to determine the effect on the microbial adhesion. So far several studies have reported the effects of suspending media and/or surface pre-conditioning on microbial adhesion to stainless steel surface. Their results include both enhancing and suppressing effects. Helke et al. (1993) reported lower numbers of *L. monocytogenes* and *Salmonella typhimurium* adherent to stainless steel surface when cells were suspended in whole milk, skim milk, or diluted milk compared to PBS. Barnes et al. (1999) also found reduced adhesion of *S. aureus*, *L. monocytogenes*, and *Serratia marcescens* to stainless steel coupons pre-treated with skim milk. On the other hand, the use of lactose and non-casein protein solutions as a suspending medium was reported to increase the adhesion of *Moraxella*-like sp. and *Pseudomonas* spp. onto stainless steel surfaces compared to cells suspended in quarter strength Ringer's solution (Speers & Gilmour, 1985). Flint, Palmer, Bloemen, Brooks, and Crawford (2001) found that milk-fouled surfaces attracted 10–100 times more vegetative cells and spores of *Bacillus stearothermophilus* compared to clean stainless steel surface. To comprehensively discuss the results mentioned above, compatibility between pre-conditioning organic molecule and bacterial cell surface will be a key concept. Physicochemical properties of bacterial cell surfaces vary with species (Chmielewski & Frank, 2003), and such variability in turn affects the compatibility with the pre-conditioning organic molecules. Furthermore, the compatibility depends also on the type of pre-conditioning organic molecules (McEldowney & Fletcher, 1987). Therefore, further compilation and systematic analysis of adhesion data for microbial species with specified organic components are needed for better understanding of the effect of organic molecules.

Removability of adherent *E. coli* cells was shown to vary depending on the type of rinsing medium (Fig. 3). After 15-min whirlpool rinsing, the surface cell density showed a decrease of around 65% with peptone saline solution as a rinsing medium,

whereas a greater decrease (99%) was shown with water. The significantly higher removability with water may be attributed to the inherent cell surface characteristics of *E. coli*. Based on results of hydrophobic interaction chromatography, *E. coli* is considered to be a highly hydrophilic microorganism (Gilbert et al., 1991; Strevett & Chen, 2003), which could then be expected to have higher affinity with water compared with peptone saline as rinsing medium. In the case of *S. epidermidis* as previously reported (Ortega et al., 2008), no significant difference in removability was observed between peptone saline solution and water used as a rinsing medium. Such different trends in adhesion and removal of the two species suggest the importance of studies on the strength of interaction between microbial cells and stainless steel surface with or without pre-conditioning by organic substances.

As shown in Fig. 4, adhesion of *E. coli* cells was not significantly influenced by the roughness of the stainless steel surface, while their retention through the whirlpool rinsing was significantly higher for the roughest surface of stainless steel (R_a = 1.37 μm). The adhesion was evaluated after mild rinsing with peptone saline for a short period, whereas the retention was evaluated after shear force application (2000 rpm) in water for 15 min. Thus the different retention trends indicate different firmness of adhesion and/or different resistance against shear force applied. Although the retention was at a relatively low level, percentage of *E. coli* cells adherent firmly to the surface was slightly but significantly higher on the rough surface (R_a = 1.37 μm) than on smoother surface ($R_a \leqslant 0.14$ μm). From a hygienic point of view, this indicates superiority of smooth surface. As far as the results obtained in this study, R_a value of 0.14 μm seems sufficient for the lowest level of microbial retention under shear force application. The high retention of *E. coli* cells after whirlpool water rinsing may be attributed to possible entrapment of microbial cells in crevices of the surface, because deep crevices or polish lines can provide refuge to the adherent cells from shear force. Microorganisms concealed in the cracks and crevices of the substrate surface may not be efficiently removed during cleaning and disinfecting treatments and could potentially be a source of cross-contamination of food products during processing (Hilbert et al., 2003).

As for the effect of roughness of stainless steel surface to microbial adhesion or removal, opposing observations have been reported in literature. Hilbert et al. (2003) reported that surface roughness did not significantly affect the attachment to and removal from stainless steel surface in the range of R_a value from 0.01 to 0.9 μm for *Pseudomonas* sp., *L. monocytogenes*, and *C. lipolytica*. Boulangé-Petermann et al. (1997) found no clear relationship between the roughness parameter and the number of viable *S. thermophillus* adherent to the surface for stainless steel surfaces having R_a values between 0.015 and 1.04 μm. Flint, Brooks, and Bremer (2000) also showed that the adhesion of thermo-resistant streptococci was almost independent from surface roughness (R_a = 0.5–3.3 μm). On the other hand, Barnes et al. (1999) observed greater number of adherent *S. aureus* on 2B stainless finish (R_a = 0.412 μm) compared to the No. 8 mirror finish (R_a = 0.035 μm). Our previous work (Ortega et al., 2008) also showed increased adhesion and decreased removability of *S. epidermidis* for a rough stainless steel surface (R_a = 1.37 μm) compared with smoother surface ($R_a \leqslant 0.14$ μm). Furthermore, earlier works by Leclercq-Perlat and Lalande (1994) and by Wirtanen, Ahola, and Mattila-Sandholm (1995) demonstrated a positive correlation between cleanability and increased surface smoothness in the removal of biofilms. The effect of surface roughness might depend on the microbial species, possibly due to difference in adhesion manner and/or cell surface characteristics.

In conclusion, this work has demonstrated that adhesion of *E. coli* from its suspension to stainless steel surface was significantly enhanced by the cell concentration of contaminants. The adhesion was observed even in short contact periods of time but it increased and reached a plateau within 3–4 h. A relatively high percentage of *E. coli* cells were found to be irreversibly attached on the surface. The adherent cells were however shown to be quite vulnerable to removal with water as a rinsing medium. Moreover surface roughness was found to affect the removability of adherent cells. This study provides valuable information for better understanding of the adhesion behavior of *E. coli* on stainless steel surface, though further study is necessary to fully elucidate the mechanism of adhesion.

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