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The effects of stainless steel finish on *Salmonella* Typhimurium attachment, biofilm formation and sensitivity to chlorine

Dov B. Schlisselberg, Sima Yaron*

Faculty of Biotechnology and Food Engineering, Technion – Israel Institute of Technology, Haifa 32000, Israel

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ABSTRACT

Bacterial colonization and biofilm formation on stainless steel (SS) surfaces can be sources for cross contamination in food processing facilities, possessing a great threat to public health and food quality. Here the aim was to demonstrate the influence of surface finish of AISI 316 SS on colonization, biofilm formation and susceptibility of *Salmonella* Typhimurium to disinfection.

Initial attachment of *S.* Typhimurium on surfaces of SS was four times lower, when surface was polished by Bright-Alum (BA) or Electropolishing (EP), as compared to Mechanical Sanded (MS) or the untreated surface (NT). The correlation between roughness and initial bacterial attachment couldn't account on its own to explain differences seen. Biofilms with similar thickness (15–18 μm) were developed on all surfaces 1-day post inoculation, whereas EP was the least covered surface (23%). Following 5-days, biofilm thickness was lowest on EP and MS (30 μm) and highest on NT (62 μm) surfaces. An analysis of surface composition suggested a link between surface chemistry and biofilm development, where the higher concentrations of metal ions in EP and MS surfaces correlated with limited biofilm formation. Interestingly, disinfection of biofilms with chlorine was up to 130 times more effective on the EP surface (0.005% surviving) than on the other surfaces. Overall these results suggest that surface finish should be considered carefully in a food processing plant.

1. Introduction

Foodborne illness is a serious health problem worldwide. A report of the Centers for Disease Control and Prevention (CDC) estimated that 76 million cases of foodborne illness occur each year causing 325,000 hospitalizations and 5000 deaths in the United States alone despite of intensive efforts that have been made to improve the hygienic conditions during food production (Nyachuba, 2010). One known causative for food contamination is the attachment of bacteria to food processing surfaces as these bacteria can move to the food or its package and lead to product contamination at different stages of processing. In the plant facility, surfaces are exposed to microorganisms, including spoilage and pathogenic microorganisms, even in controlled environments, through raw materials, wash water, air or food handlers.

Attachment of cells to processing surfaces may be the first step in the formation of biofilms (Lewis, 2001; Steenackers et al., 2012). Biofilms are notoriously difficult to eradicate and, thus, are capable of surviving in the plant environment for very long periods of time (Branda et al., 2005; Brooks and Flint, 2008; Steenackers et al., 2012; Vestby et al., 2009). In the food industry biofilms create a persistent source of microorganisms, leading to serious hygienic and safety problems and also to economic losses due to food spoilage and reduced shelf-life (Shi and Zhu, 2009). The attachment of *Salmonella enterica* to food surfaces had been the first published report on biofilms of foodborne pathogens (Duguid et al., 1966), years before the term "biofilm" was established. Since 1966, many reports documented biofilm formation on various food processing plants, some examples given are *S. enterica*, *Listeria monocytogenes* and *Escherichia coli* in beef processing plants (Rivera-Betancourt et al., 2004), *Bacillus cereus*, *Shigella* spp., *E. coli* and *Staphylococcus aureus* in dairy processing plants (Sharma and Anand, 2002a, 2002b) and *S. enterica*, *Pseudomonas* spp., *Vibrio* spp. and others in the fish industry (Gunduz and Tuncel, 2006; Vestby et al., 2009).

Bacteria are able to form biofilms on many abiotic surfaces such as plastic, rubber, glass, cement and stainless steel (Joseph et al., 2001; Kim and Wei, 2009; Morita et al., 2011). Stainless steel is an ideal material for fabricating surfaces and machinery equipment due to its physicochemical stability and high resistance to corrosion (Holah and Thorpe, 1990; Shi and Zhu, 2009). Nevertheless, while many reports can be found regarding problems caused by biofilms in food processing plants (Gunduz and Tuncel, 2006; Sharma and

* Corresponding author. Tel.: +972 4 8292940; fax: +972 4 8293399.
E-mail address: simay@tx.technion.ac.il (S. Yaron).

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Anand, 2002a, 2002b; Shi and Zhu, 2009; Vestby et al., 2009), factors and surface properties that affect biofilm formation on stainless steel are not yet fully explored. A good example for this was given by Arnold and Bailey (2000). They found that the natural bacterial population of chicken carcass could form biofilms on several stainless steel surface finishes. Moreover, following bacterial colonization and biofilm formation, cells of *Salmonella* in a biofilm were found to survive approximately one year in dry conditions on stainless steel bolt threads (Morita et al., 2011).

In the food industry various strategies are applied in order to control microbial attachment and biofilm formation. Most strategies rely on hygienic plant lay-out and disinfection with different chemicals. These chemicals often contain peroxides, chloramines or hypochlorites. The Food and Drug Administration allows the use of sodium hypochlorite as a disinfecting agent for the food contact surfaces of food processing equipment in concentrations up to 200 ppm (Code of Federal Regulations). Since the liberation of chlorine can cause pitting and thus result in damaging of the passive oxide layer, the duration, concentration and temperature are carefully monitored to minimize potential threat to the surface or equipment (British Stainless Steel Association, 2001). Once bacterial biofilms are formed, their removal has been proven to be extremely difficult. For example, it was found that biofilms of *S. enterica* serovar Typhimurium formed on different surfaces were resistant to sodium hypochlorite (Lapidot et al., 2006; Scher et al., 2005).

To summarize, biofilms are developed easily on abiotic environments. Once a biofilm is formed, it can survive for very long periods of time and so it may pose a threat to product safety. The development of microbial resistance together with the well-known resistance of biofilms and today's demand to decrease the use of antimicrobials opened the way to screening for novel strategies such as special design of equipment or careful selection of the physical properties of the used material (Giaouris et al., 2012). In our study, fluorescence image-based optical detection and microbial enumeration were applied to examine the *S.* Typhimurium attachment and biofilm formation capabilities over several days and biofilm' susceptibility to chlorination with four types of stainless steel finishes: bright annealed, mechanical sanded, electro-polished and a not-treated control.

2. Materials and methods

2.1. Preparation of metal coupons

The flat, stainless steel coupons (type AISI 316) used in this study were all supplied by LIMAT Metal Surface Treatments Ltd (Kibbutz Givat Haim Meuhad, Israel). All coupons had a total surface area of 8 cm^2. We examined four steel finishes which were all prepared by LIMAT MST Ltd.: Mechanically brushed polished (MS) stainless steel, also known as nr. 4, was achieved by mechanically hand brushing of the metal surface; Bright annealed (BA) stainless steel, also known as 2R, is the result of cold rolling. Controlled conditions of an inert atmosphere or vacuum were used to reduce oxidation and thus a bright surface was retained. The electro-polished (EP) finish is achieved by immersion in an electrolyte and subjecting to a direct electrical current, thus removing metal film from the steel sheet. Controls were untreated coupons (NT). All coupons were sonicated for 30 min in distilled water before immersion in the bacterial suspensions as described previously (Arnold and Bailey, 2000).

2.2. Analysis of surface topography using atomic force microscopy

Surface roughness was evaluated using atomic force microscope (AFM). First, each sample was cleaned as described in the previous section, and coupons were stored in a desiccator until analyzed. Coupons were then observed on a JPK Nano Wizard II AFM (JPK Instruments Inc., Germany) operating at contact mode as described (Scher et al., 2007). The measurements were carried out in air. Data were obtained by scanning the samples using μMasch CSC21/NoAI cantilever (Rc < 10 nm, typical force constant 2 N/m). The scanning probe image processor (SPIP, Image Metrology, Denmark) Software was used for the roughness analysis of the images. We examined three roughness parameters: Ra, Rz and Rpk. Rz, also known as Peak–Peak Height, is defined as the height difference between the highest and lowest pixels in the image, Rpk, also known as the Reduced Summit Height, is the measure of the peak height above the core roughness as calculated from the bearing ratio curve (Abbott–Firestone curve), and Ra is defined as the mean value of the surface height relative to the center and is calculated by:

$$Ra = \frac{1}{LxLy}\int_0^{Ly}\int_0^{Lx}|f(x,y)|\mathrm{d}x\mathrm{d}y$$

where, $f(x,y)$ is the surface relative to the center plane, and *Lx Ly* are the dimensions of the scanned surface (in this case both *Lx* and *Ly* are 10 μm). All three parameters were calculated according to ISO 13565-2 (ISO 13565-2 1996). Each of these parameters was examined in two different experiments for an entire 10 × 10 μm surface area.

2.3. Surface analysis of chemicals using X-ray photoelectron spectroscopy

Coupon was prepared for the X-ray photoelectron spectroscopy (XPS) as was previously described (Nazneen et al., 2012). Surface analysis was performed using a Thermo VG Scientific Sigma probe, operating at a base pressure of $<3 \times 10^{-9}$ Torr and fitted with a monochromatized Al Kα (1486.6 eV) X-ray source. The measurements were performed in the standard and the bulk-sensitive modes at a takeoff angle between the direction of an analyzer and the normal to the specimen plane of 53.0° and 30.5°, respectively. All measurements were taken at the center of the sample to ensure reproducibility and to minimize the effects of scratches or contamination at the edges.

2.4. Strains and plasmids

S. Typhimurium ATCC 14028 (wild type), and its mutant, MAE52 which produces biofilm semi-constitutively (Zogaj et al., 2001) were used in this study. Bacteria were transformed with the pGFP plasmid harboring a kanamycin (KN) resistance gene (Clontech, Palo Alto, Calif.) to obtain green fluorescent protein (GFP)-labeled cells as was previously described (Scher et al., 2005). Bacterial starters were prepared by inoculation of a colony in Luria–Bertani (LB) broth without NaCl supplemented with 30 μg/ml KN and incubation overnight at 37 °C.

2.5. Attachment experiments

Overnight cultures of *S.* Typhimurium were diluted in fresh broth media to a final OD_{600} of 0.3 (approx. 10^7 cells/ml) in a total volume of 50 ml in Erlenmeyer flasks. The metal coupons were inserted to the flasks, and the flasks were incubated with a gentle shaking of 20 rpm for 1 h at room temperature. Next, metal coupons were rinsed several times with sterile saline in order to remove loosely attached cells. The GFP expressing *Salmonella* cells on the coupons were visualized and enumerated using a florescence microscope (Olympus BX51, Olympus Inc.). Attached cells

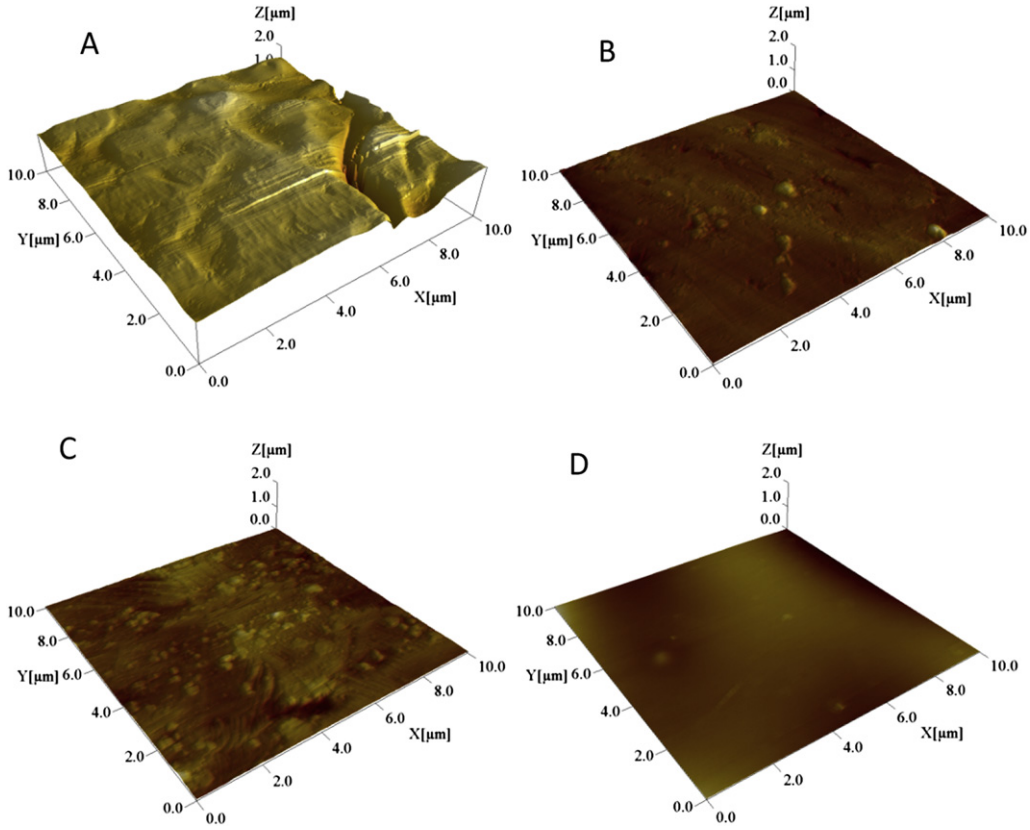

Fig. 1. Three dimensional 10 × 10 μm AFM micrographs of stainless steel surfaces of different surface finish: A) Not treated, B) Mechanical sanding, C) Bright Alum, D) Electropolished.

were counted in at least 60 fields which were chosen randomly (a total surface of 1 mm^2) in each coupon.

2.6. Biofilm growth and determination of biofilm coverage and depth

The metal coupons were colonized and rinsed as described above in the attachment experiments. Next, the washed coupons with the attached bacteria were inserted into clean flasks containing fresh broth and incubated at room temperature with gentle shaking of 20 rpm. Coupons were removed from the broth after 1- or 5-days (culture medium was replaced every day). Measurements of an averaged biofilm thickness and covered (the percent of the surface covered with fluorescent material) were made with Laser Scanning Confocal Microscope (LSCM, Confocal Zeiss LSM 510 META) using ImageJ (http://rsb.info.nih.gov/ij). Biofilm used for observation with a scanning electron microscope (SEM) were grown for 1-day as described above. Samples were fixed using 4% formaldehyde (Frutarom, Israel) and dehydrated using an increasing gradient of 25%, 50%, 75% and 95% of ethanol in ddH_2O for 20 min each. Samples were visualized by using a Zeiss Ultra Plus high-resolution scanning electron microscope (HR-SEM) (Carl Zeiss, Inc).

2.7. Biofilm susceptibility to disinfection with chlorine

1-day old biofilms were produced on the coupons as described above. Next, coupons were treated in solution of 50 ppm or 100 ppm of sodium dichloroisocyanurate (NaDCC) (SIGMA) for 10 min at room temperature with gentle shaking. Each treated coupon was washed several times with saline, and the survivors were recovered using glass beads as described previously with few modifications (Kim et al., 2008). Briefly, each coupon was put in a conical tube containing 10 ml of saline and ca. 30 (3-mm) glass beads. The coupons and the beads were vortexed for 30 s at the highest speed. The supernatant was serially diluted, and plated to enumerate the cells.

2.8. Statistical analysis

Results were statistically processed using the One Way Analysis of Variance (ANOVA) method, followed by the Tukey–Kramer test when needed. p values ≤ 0.05 were regarded as significant.

3. Results and discussion

3.1. Stainless steel surface topography and chemistry analysis

Attachment to surfaces and biofilm formation are affected by many factors. In this study we focused on two surface properties supposed to affect bacterial attachment: topography and chemical composition (Arnold and Bailey, 2000). Surface topography was studied using AFM images of the coupons and chemical composition was measured by XPS. Fig. 1 shows representative AFM micrographs of each of the coupon types. As can be seen in the images, the untreated surface contains deep crevasses and high peaks. Similar cracks were previously proved hard to clean and can potentially harbor bacterial cells (Boyd et al., 2001). The other surfaces tested appear rather smooth with the EP surfaces being almost leveled except for a few imperfections or contaminations; this is followed by the BA and MS surfaces which seem to be slightly more irregular.

Two main parameters widely used to compare surface topology are Ra and Rz. The first is an arithmetical mean deviation of the absolute ordinate values within a sampling length and the second is the sum of the height of the largest profile peak height and the largest profile valley depth, within a sampling length (ISO 4287 1997). It was also suggested that the reduced peak height within the evaluation length parameter (Rpk) (ISO 13565-2 1996) might give a better correlation between surface topology and bacteria attachment (Jullien et al., 2003). Therefore we calculated all three parameters Ra, Rz and Rpk of the four different metal finishes (Table 1). Even though all the surfaces tested appear rather smooth (Ra between 4 and 106 nm), there was a high degree of variance especially in the Rz ratios (ranging between 67 and 1907 nm). Rpk values were very similar to the Ra values (ranging from 8 to 115 nm), and both values correlated with the Rz values. The quantitative analysis confirmed the qualitative analysis, already seen in the images, with NT having the highest roughness values and EP having the smoothest surface. The MS finish showed approximately two times higher the roughness values as compared to BA. Overall, all four surfaces were relatively smooth, because their Ra values were significantly lower than the threshold values (Ra $<$ 800 nm) recommended to reduce fouling and contamination (EHEDG, 2004). Flint et al. (2000) observed that attachment of *Streptococcus* to stainless steel was higher when roughness values were close to bacterial length (0.9 μm) (Flint et al., 2000). Based on this theory, given that the irregularity of EP, MS and BA surfaces is smaller by more than one order of magnitude from bacterial cell length, and irregularity of NT surfaces is smaller than one order, entrapment of bacterial cells, if any, is expected only in the NT surface.

Analysis of surface chemistry was performed in order to evaluate chemical variances in the outermost layer of each metal surface. These differences might explain favorable bacterial attachment and biofilm formation in a given surface. We performed XPS analysis of the four metal finish types (Table 2). The most abundant elements in all surfaces were O (35.56–53.87 wt.%) and C (31.54–49.66 wt.%). A high C signal is typical to surface contamination found on air-exposed metals (Boyd et al., 2001; Johansson and Saastamoinen, 1999). Other detectable elements were N, Cr, Fe, Ni, Si, P, Zn and Ca. The chemical composition of O, C, N and Fe in all metal surfaces is somewhat similar to a recent report regarding NT and EP stainless steel surfaces (Nazneen et al., 2012). Ni was at least two times more abundant in the MS and EP surfaces than in the other surfaces and this was also somewhat similar with Cr, which was more abundant in EP. Fe concentration was also slightly higher in MS and EP. Overall the higher Cr to Fe ratio indicates better passivation procedure and a greater exposure of the bulk elements of stainless steel in the outermost layer (Haidopoulos et al., 2006; Nazneen et al., 2012).

Table 1
Roughness parameters values for the stainless steel AISI 316 surface finishes examined.[a]

Surface finish[b]	Ra (nm)[c]	Rz (nm)[c]	Rpk (nm)[c]
NT	106	1907	115
MS	30	301	41
BA	13	145	21
EP	4	67	8

[a] Results are the mean of two experiments as performed on an entire 10 × 10 μm slide.
[b] NT: Not treated; MS: Mechanical sanding; BA: Bright alum; EP: Electropolished.
[c] Ra is the mean value of the surface height relative to the center. Rz is the Peak–Peak Height also defined as the height difference between the highest and lowest pixel in the image. Rpk (Reduced Summit Height) is a measure of the peak height above the core roughness. These values were calculated according to ISO-13565-2 (ISO 13565-2, 1996).

Table 2
Chemical composition of the surfaces of stainless steel AISI 316.

Surface finish[a]	Elements (wt.%)									
	O (1s)	C (1s)	N (1s)	Cr (2p)	Fe (2p3)	Ni (2p3)	Si (2s)	P (2p)	Zn (2p3)	Ca (2p)
NT	43.20	43.11	5.07	3.54	3.19	0.42	1.46	–	–	–
MS	53.87	31.54	1.74	5.47	4.43	0.87	1.31	0.77	–	–
BA	41.48	43.81	2.94	3.20	3.60	0.36	3.01	1.14	0.45	–
EP	35.56	49.66	3.06	6.81	4.02	0.63	–	–	–	0.26

[a] NT: Not treated; MS: Mechanical sanding; BA: Bright alum; EP: Electropolished.

3.2. The ability of Salmonella to attach the stainless steel is affected by the surface finish

The attachment of *Salmonella* to the metal coupons following 1 h of incubation in a solution of approx. 7 log CFU/ml was measured. Analysis of the attached bacteria seen in the fluorescence-microscope images demonstrated that significantly fewer bacteria attached to both BA and EP surfaces ($p < 0.05$). As can be seen in Fig. 2, number of bacteria on MS or NT surfaces was four times higher compared with the BA and EP surfaces. We did not observe significant differences in numbers of attached bacteria between BA and EP surfaces, or MS and NT surfaces. Similar to our results with type 316 stainless steel, an investigation of bacterial attachment to 304 type stainless steel revealed bacterial attachment was the lowest in EP type surface followed by sanded surface then sand-blasted and NT surfaces being the most colonized surface (Arnold and Bailey, 2000). Taking together the attachment results with surface roughness measurements discussed above, it is clear why an almost featureless surface such as the surface of EP and BA would hinder bacterial settlement. MS, on the other hand, showed high levels of initial bacterial attachment like the untreated surface. This is in spite of the observation that roughness parameters for MS surface were higher than the parameters of EP and BA, but still lower than what would be expected to entrap bacterial cells, and were much lower compared with the roughness parameters of untreated surfaces. Thus, we could not find a direct correlation between surface roughness and initial bacterial attachment, in contrast to the previously implied connection between the two (Jullien et al., 2003; Percival et al., 1998). Meaning that in our system other parameters

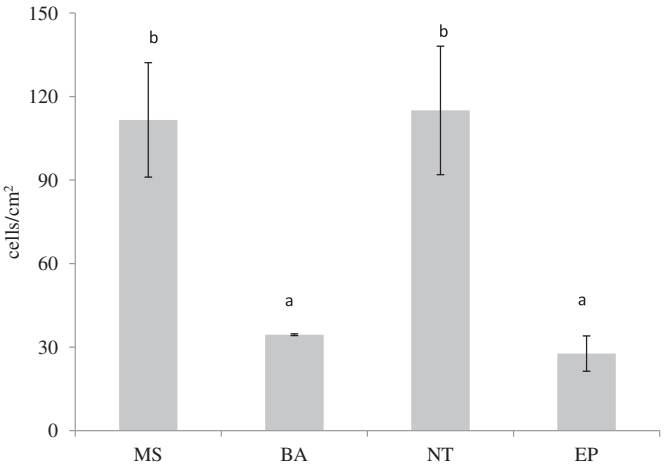

Fig. 2. Initial attachment of *Salmonella enterica* serovar Typhimurium WT cells to steel surfaces of different surface finish. Cells were enumerated in 60 different fields of images obtained using an epifluorescence microscope. Experiments were conducted three times and bars represent the standard deviations. Columns with a different letter indicate statistical differences between bars ($p < 0.05$).

such as chemical composition, hydrophobicity and surface charge also affect the initial attachment. To conclude, we believe that there is some connection between surface roughness and initial bacterial attachment but probably this cannot ensure on its own control of initial bacterial attachment.

Similarly, surface composition did not correlate directly with the ability of *Salmonella* to adhere to the stainless steel surface. BA and NT surfaces had very similar abundance of the main elements, but very different levels of bacterial attachment. It was suggested that metals are less susceptible to bacterial attachment and biofilm development, when they contain molybdenum in addition to chromium and nickel (Percival et al., 1998). However in our experiments the concentration of molybdenum on the surface was undetectable in all surfaces. It should be noted that AISI-316 stainless steel usually contains between 2.00 and 3.00% molybdenum; however, we could not identify molybdenum on the surface using XPS. This was previously described (Haidopoulos et al., 2006) and could be resulted from surface contamination of atmospheric exposed steel surfaces.

3.3. Kinetics of surface coverage and biofilm development

Results discussed above reveal that surface finish affects the initial adherence of *Salmonella* to stainless steel. Since biofilm formation poses great threat to the food industry (Chmielewski and Frank, 2003), we further investigated whether the differences in initial bacterial attachment levels continue to be significant even after several days of biofilm development. For this aim we used a *S.* Typhimurium strain that produces biofilm semi-constitutively. Similar mutants were identified in food and in food plants such as in the fish industry (Vestby et al., 2009). Fig. 3 shows representative SEM micrographs of each surface in the initial biofilm formation stage (after 1-day of incubation). SEM images reveal differences in bacterial colonization. We observed that the crevices in the NT coupons harbored considerable amount of bacteria, but we could also identify many individual cells and micro-colonies on the entire surface. In other locations the bacteria formed 3-D clusters with typical properties of biofilms with hills and valleys structures that covered large areas. Regarding the MS surface, we observed abundant scratches, which were found to harbor bacteria in higher density. This result demonstrates that although the roughness parameters seem too low (Table 1), occasional cracks and crevasses found could be potential pockets for initial bacterial colonization. On the BA surface bacteria formed biofilms on specific pouches, but individual cells were also visible throughout the metal surface. Surfaces of EP harbored very few visible cell clumps, and bacteria were visible throughout the entire surface. Similar observations with other bacteria were described in the past regarding sanded, electropolished and a non-treated control surfaces (Arnold and Bailey, 2000).

We measured biofilm development using two parameters, surface coverage and biofilm depth. Measurements were executed following 1- and 5-days of biofilm development using a confocal microscopy. Table 3 shows the results of percent of covered area (florescent area) on the coupon surface. EP was the least covered surface and NT was the most covered surface after 1-day. EP and BA that had the lowest numbers of attached bacteria after 1 h (Fig. 2), continued to have the lowest bacterial loads after 1 day. However, as a result of the high variability in measurements, no significant differences were observed between BA, MS and NT. After incubation for 5-days the covered area of all coupons continued to increase. This increase can only be explained by cell proliferation, because media were replaced by fresh sterile media after the initial attachment and subsequently every day. At day five the most

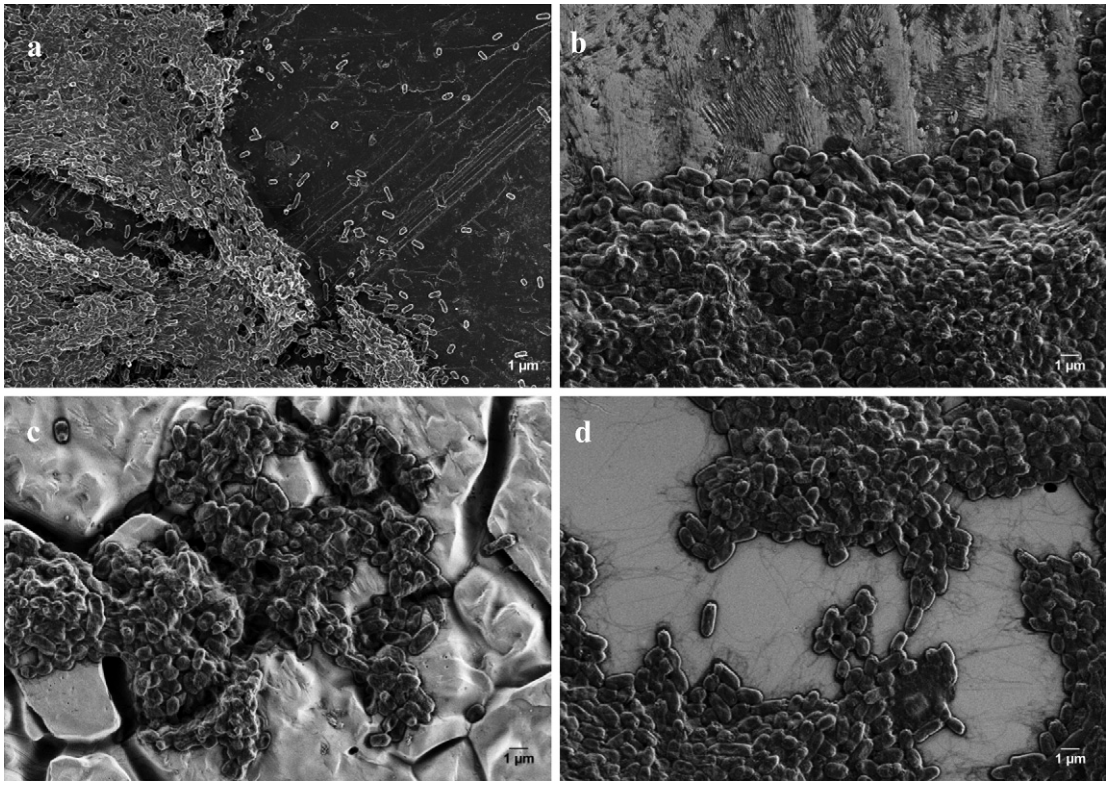

Fig. 3. Representative micrographs taken from a HR-SEM of stainless steel surfaces type 316 following incubation with *Salmonella* Typhimurium for 1-day. Mechanical Sanding (MS) surface (a). Bright-Alum (BA) surface (b). The Not Treated (NT) surface (c). Electro-Polished (EP) surface (d).

Table 3
Percentage of bacterial surface coverage on each of the four coupon types following 1- and 5-days of incubation.

Time (days)	Surface coverage (%)[a,b]			
	NT	MS	BA	EP
1	$49 \pm 8^{x,A}$	$40 \pm 9^{x,A}$	$35 \pm 8^{x,A}$	$23 \pm 5^{y,A}$
5	$88 \pm 12^{y,B}$	$43 \pm 9^{x,A}$	$69 \pm 22^{x,y,B}$	$53 \pm 3^{x,B}$

[a] Each coupon was visualized using a confocal microscope in six different places and the experiment was conducted twice in duplicates. Means within a column with different uppercase letter (A,B) differ significantly ($p < 0.05$). Means within a row with different letter (x,y) differ significantly ($p < 0.05$).
[b] NT: Not treated; MS: Mechanical sanding; BA: Bright alum; EP: Electropolished.

covered surface was still in the NT coupon with almost the entire surface covered (88%). Remarkably following the first day and up to the fifth day, the MS showed only three percent biofilm increase.

An increase in the biofilm volume indicates that the number of bacteria attached to the steel increase even if the coverage area remains low. In addition, thicker biofilms have more exopolysaccharides (EPS) and are more resistant to antimicrobial agents. Such biofilms are also more likely to promote detachment of microcolonies which may result in cross-contamination (Flemming and Wingender, 2010; Mah and O'Toole, 2001). Measurements of biofilm depth revealed that following 1-day of incubation the biofilm depth was quite similar in the four surfaces examined with an average thickness of 15 μm–18 μm as shown in Table 4. Following 5-days of incubation the biofilm thickness on MS and EP surfaces increased by approximately two folds, and by three folds on BA surface, but the not-treated metal surface had the most profound biofilm depth. It is interesting to note that biofilm formed on the control metal (NT) following 5-days was more than four times thicker than the biofilm formed following 1-day in addition to the increase in the biofilm area on the surface by approximately 77%. The finding that the higher surface roughness induces a thicker biofilm is not in line with a recent study that showed the opposite correlation on titania surfaces (Singh et al., 2011). A review of recent literature provides only one example of a study performed on biofilm formation of pathogens on different steel surfaces. Rodriguez et al. (2008) reported that for the 304 type surface similar amount of biofilm was formed with *L. monocytogenes* either on MS or on EP surfaces. However after 5-days of incubation the biofilm formed by *L. monocytogenes* was much less developed, with only less than ten micrometers in diameter and 0.45 μm in height, values which are below the *Salmonella*'s biofilm dimensions even following the first day.

To conclude this part, following 1-day, EP surface was the least covered surface with increasing values for BA, MS and NT. Biofilm depth, however, was quite similar in all surfaces following 1-day but varied following 5-days with EP and MS having similar values and NT being associated with the highest biofilm formation. The most intriguing result is that following 5-days, surface coverage barely changed with MS but grew massively in the other surfaces with the NT being almost entirely covered (Table 4). In the past it was found that metal ions such as Cr, Ni and Fe are accumulating in relatively high concentrations in potable-water biofilm (Percival et al., 1998). EP and MS surfaces contained the highest levels of Cr, Ni and Fe (Table 2); therefore, we suggest that there might be an association of surface chemistry of both EP and MS surfaces and the finding that bulk elements such as Cr, Fe and Ni were more abundant in these surfaces, with the bacterial ability to develop biofilms. However, this must be further investigated.

3.4. Susceptibility of biofilms formed on different surfaces to chlorine

In the food industry metal surfaces are routinely being treated to remove the bacterial load using different detergents and antibacterial agents such as chlorine, ethanol and soaps. Chlorination may be applied alone or after usage of other agents during the cleaning process. We investigated whether the susceptibility of a one-day old biofilm of *Salmonella* to chlorine depends on surface finish of the stainless steel. Previously, it was found that a 15-min chlorine treatment (100 ppm) completely eradicated a 10-day old biofilm, while 50 ppm did not eradicate the biofilm even after 25 min (Joseph et al., 2001). We also established that 100 ppm chlorine completely killed *Salmonella* in the biofilms following a 10-min treatment on all metal surfaces tested (data not shown). However, we were interested in the possible differences of biofilm sensitivity between different metal surfaces. For this aim we treated 1-day biofilms with 50 ppm of chlorine for 10 min. Surviving cells were recovered from the coupons and plated. The initial biofilm load in each of the surfaces was fairly similar when enumerating biofilm cells (8.2–8.5 log CFU/coupon) (Table 5) and also when measuring the covered (fluorescent) area of 1-day old biofilms (Table 3). After disinfection we observed significant differences among the bacterial counts on the different surfaces. Disinfection was mostly effective on the EP surface with only 0.005% of cells surviving the treatment (3.8 log CFU/coupon); this was followed by the NT control, BA and MS with 0.658% having the largest amount of surviving cells. Remarkably, this means that biofilm formed on the EP surface was between 50 and 130 times more sensitive to chlorine than the other metal finishes tested. To the best of our knowledge, only one report investigated the effectiveness of chlorine on different surface finishes. Airey and Verran (2007) investigated chlorine treatment against *S. aureus* cells on a 304 stainless steel. This report showed that the amount of remaining cells was quite similar on both finishes, EP and BA. However, since the two studies differ so markedly in bacteria, methods and stainless steel type used, it is not possible to compare the findings.

Electro-polishing not only affected the ability of the bacteria to adhere to the surface and develop biofilms, but also increased their sensitivity to chlorine. This means that surface characteristics

Table 4
Biofilm depth on each of the four coupon types following 1- and 5-days of incubation.

Time (days)	Biofilm depth (μm)[a,b]			
	NT	MS	BA	EP
1	$15 \pm 1^{x,A}$	$16 \pm 2^{x,A}$	$16 \pm 3^{x,A}$	$18 \pm 4^{x,A}$
5	$62 \pm 21^{y,B}$	$30 \pm 10^{x,B}$	$47 \pm 19^{x,y,B}$	$30 \pm 12^{x,B}$

[a] Each coupon was visualized using a confocal microscope in four different places (measurements were done only where biofilm was visible) and the experiment was conducted three times in duplicates. Means within a column with different uppercase letter (A,B) differ significantly ($p < 0.05$). Means within a row with different letter (x,y) differ significantly ($p < 0.05$).
[b] NT: Not treated; MS: Mechanical sanding; BA: Bright alum; EP: Electropolished.

Table 5
Number of *Salmonella* cells associated with stainless steel coupons prior and following a treatment with 50 ppm chlorine (log CFU/coupon ± SD).

Coupon type[a]	Before treatment[b,c]	After treatment[b,c]	% of surviving[d]
NT	$8.5 \pm 0.3^{A,x}$	$5.7 \pm 0.7^{A,y}$	0.231%
MS	$8.3 \pm 0.4^{A,x}$	$6.2 \pm 0.4^{A,y}$	0.658%
BA	$8.4 \pm 0.3^{A,x}$	$6.2 \pm 0.1^{A,y}$	0.561%
EP	$8.2 \pm 0.2^{A,x}$	$3.8 \pm 0.3^{B,y}$	0.005%

[a] NT: Not treated; MS: Mechanical sanding; BA: Bright alum; EP: Electropolished.
[b] Experiment was conducted three times in duplicates. Means within a column with different uppercase letter (A,B) differ significantly ($p < 0.05$). Means within a row with different letter (x,y) differ significantly ($p < 0.05$).
[c] Experiments were done on a 1-day biofilm.
[d] % of surviving cells were performed on the average count values.

which control chemical reactivity of surfaces not only affect binding of bacteria, but also affect susceptibility of bacteria to disinfection. Since EP is the smoothest surface (Table 1, Figs. 1 and 3), results support the possibility that surviving bacteria hide in cracks (Shi and Zhu, 2009). Differences in the biofilm topology or properties of the biofilm matrix resulting from differences in colonization and growth on the 4 surfaces types may also affect the sensitivity to chlorination. On the other hand the differences were not related to the biofilm depth, which was very similar in all surfaces (Table 4). Much more work is needed in order to determine which parameters (structure, topography, chemical composition, electric properties, etc.) have a role regarding the influence of surface finish on effectiveness of an antimicrobial agent on a given bacterium.

4. Conclusions

The objective of this study was to evaluate the effect of stainless steel surface finish on attachment, biofilm formation and susceptibility to chlorine treatment of surface adhered *Salmonella* cells. Identification of characteristics of the metal surface that contribute to or disrupt the formation of biofilms, as well as revealing of parameters affecting the biofilm susceptibility to disinfection is crucial in finding ways to enhance food safety and to reduce cross contamination in processing plants with minimal use of chemicals. Past studies were performed mainly on Gram-positive bacteria, using metal type 304. This is the first study aimed at investigating the kinetics of biofilm development by the common pathogen *S.* Typhimurium and the effect of disinfecting treatment on stainless steel of different metal finish. Evaluation of initial attachment (1 h) revealed that while the NT and MS surfaces were the most colonized, the EP and BA surfaces were the least colonized. The development of young (1-day) and old (5-days) biofilm was also investigated. MS and EP had the least surface coverage and biofilm depth following both 1 day and 5 days; on the other hand the NT control was the most covered surface with the deepest biofilm formed. Results demonstrated that topography of the surface may influence the initial attachment of *S.* Typhimurium, but it does not always show a direct correlation. On the other hand, during biofilm development the composition of the surface may have a significant role, in addition to the topography. Thus, the surface properties that led to different attachment levels may differ from the characteristics that enhance biofilm development. Finally, we investigated whether surface finish improves or worsens effectiveness of chlorine treatment against biofilm cells; we found that although all the surfaces harbored similar amounts of biofilm, the disinfecting treatment resulted in much less surviving bacteria with the EP surface compared to the other three surface finishes.

Overall our results suggest that EP surface was the least colonized surface 1 h after initial attachment, and also it was the most effective substrate for chlorine disinfection against *Salmonella* biofilm cells. Regarding biofilm formation for a long period of time, the MS surface seems to be the least susceptible surface with the least amount of biofilm formed. These results should be taken into account in food processing plants when determining which surface finish should be used. For instance, for surfaces which are routinely being cleaned, based on our results with *S.* Typhimurium, it is best to use an electro-polished surface. In contrast, for surfaces, which are not accessible to regular cleaning, it is logical to consider mechanically-sanded surface. Careful selection of the material used for surfaces of the production lines would improve safety and quality of the products, particularly when bacteria develop resistance to antimicrobials. It should be noted, however, that each surface becomes rough and scratched under conditions of continuous reuse, and accumulates organic material such as proteins and lipids; thus, further research is required in order to determine the characteristics of used surfaces and to investigate the role of additional parameters such as surface charge and hydrophobicity.

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