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Microbial Adhesion to Processing Lines for Fish Fillets and Cooked Shrimp: Influence of Stainless Steel Surface Finish and Presence of Gram-Negative Bacteria on the Attachment of *Listeria monocytogenes***

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Summary

Microflora adhering to surfaces of processing lines in a shrimp factory and a fish processing plant was identified *in situ* and adhesion of mixed culture of *Listeria monocytogenes* and Gram-negative bacteria on stainless steel surfaces (untreated, polished and glass beaded) was studied *ex situ*. The predominant genus attached to the surfaces was *Pseudomonas* spp. (66 %) in the shrimp factory and *Enterobacteriaceae* (27 %) in the fish factory. Shrimp juice was used as an enrichment broth during the study of adhered bacteria. Three different Gram-negative strains and a mixture of *Pseudomonas* spp. were selected to study their attachment together with *L. monocytogenes* to stainless steel surfaces. Highest numbers of the attached bacteria were obtained after the contamination with a mixed culture of *L. monocytogenes* and *Serratia liquefaciens*. A lower number of bacteria adhered to stainless steel surfaces when mixed cultures of *L. monocytogenes* and *Pseudomonas fluorescens* or *Aeromonas* spp. were tested. No significant differences ($p<0.05$) were observed in the bacteria attached to differently treated steel surfaces with different roughness (Ra=0.1–0.8 μm). Bacterial adhesion increased with longer contact time. Colonisation of *L. monocytogenes* on stainless steel surfaces was significantly enhanced only in the presence of mixed *Pseudomonas* spp. These results indicate that smooth surfaces do not necessarily provide hygiene benefits over rougher surfaces.

Key words: seafood processing, hygienic design, biofilm, adherence, *Listeria*, stainless steel

Introduction

Bacteria are an indigenous part of raw materials for food production but they can also be found on the food processing equipment where they can subsequently contaminate the products (*1*). Attachment of pathogens and other bacteria to food equipment surfaces can lead to product contamination, spoilages and surface deterioration. Research in the food industry has revealed that in natural habitats most bacteria are able to colonize surfaces (*2*). Attached bacteria are reported to have enhanced resistance to disinfectants compared to free-liv-

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ing cells (*3*). Some Gram-negative bacteria (*e.g. Pseudomonas* spp.) may have certain resistance mechanisms against commonly used disinfectants (*e.g.* quaternary ammonium compounds) (*4–6*). *Listeria monocytogenes* is a foodborne pathogen of major public health concern. It is very difficult to control this organism in the food processing environment. This pathogen can adhere to food contact surfaces in the processing environment (*7–12*). This is important, as the presence of other bacteria, *e.g. Pseudomonas* spp., has been reported to enhance the number of *L. monocytogenes* on the surface and their resistance to disinfectants (*3*). Although *L. monocytogenes* has been found in a variety of fishery products (*13–15*), such sources have not been associated with large outbreaks of listeriosis (*16*). However, these products have been considered potential sources of human listeriosis (*17–19*). The greatest challenge in controlling *L. monocytogenes* in the food industry is therefore to prevent the establishment of the bacterium in specific niches, where routine cleaning and disinfection are ineffective. The aim of this study was to evaluate the microflora attached to the surfaces in shrimp and fish processing lines and to determine the effect of stainless steel surface finish and the presence of Gram-negative bacteria on the adhesion of *Listeria monocytogenes* to these surfaces. This will provide useful information on bacterial attachment as well as contribute to the appropriate choice of stainless steel surface finish, hence promoting the production of safe food products.

Materials and Methods

Preparation of steel coupons

Stainless steel coupons (SS type 304, no. 2B, untreated, polished, pickled and polished/pickled) of 10×4 cm^2 were placed near food contact surfaces in different locations in a shrimp factory (16 samples) and a fish factory (20 samples). They were kept in the factories for three months. Both plants used similar cleaning procedures involving water rinsing, followed by caustic wash in 0.5 % NaOH and water rinsing before disinfecting with quaternary compounds. In the shrimp factory an acid wash was conducted weekly.

Three types of stainless steel coupons (1×7 cm^2) were used in the *ex situ* study. They were of type 304 no. 2B, untreated, polished and glass-beaded. They were soaked in 1 M NaOH overnight to etch the surface clean and after that for 1 h in acetone to remove grease (*9*).

Surface roughness

Surface roughness or topography was analysed using a surface tracing apparatus (Mitutoyo Surftest-201, Japan) equipped with a stylus with the radius of 5 μm. Three measurements were taken from each steel sample and the average surface roughness (Ra) and the sum of the height of the largest profile valley depth within a sampling length (Rz) were estimated. Ra and Rz are criteria for surface roughness as defined by ISO standard 4287, 1997 (*20*).

Enumeration of adherent cells and characterization of selected isolates

The stainless steel coupons (*in situ*) were removed aseptically and transported to the laboratory and samples for microbiological analysis were taken from the entire surface of each coupon with a cotton swab coated with hydrophobic cotton. The swab heads were broken off into a plastic bottle. The bottle containing the cotton swab blended with 10 mL of Butterfield´s buffer was shaken for 15 s. Serial dilutions were prepared for each sample and plated onto Iron Agar (*21*). The plates were incubated at 15 °C for 7 days. The bacteria were removed by ultrasound from 10 stainless steel coupons taken from the fish processing plant and the results were compared to those obtained with the swabbing method. Then the coupon was submerged in 20 mL of sterile water containing 0.5 % of Tween 80 in a glass tube and ultrasonicated in a bath ultrasonicator (Bransonic 3510E-DTH, 100 W, 42 kHz; Branson Ultrasonics Co., Danbury) for 1 min. The content of the tube was then serially diluted, plated and incubated as described before. From the plates twenty-five colonies were randomly selected, isolated and identified by different tests to the genus level (Gram stain, form and size, catalase, oxidase, motility, oxidation/fermentation of glucose, API 20E for *Enterobacteriaceae* and API 20NE for non-enteric Gram negative rods (BioMerieux, France)). A special check for *Listeria* was made according to the USDA method (*22*) where the stainless steel coupons were placed in a pre-enrichment broth.

After each contact time of the *ex situ* study the stainless steel coupons were removed aseptically from the test suspension in the glass tubes and rinsed gently with 3×25 mL of sterile water to remove unattached cells. Microbiological samples were taken from each surface with the swabbing technique described previously. Serial dilutions were prepared and samples plated onto TSA-YE for total count (incubated at 22 °C for 72 h) and onto modified Oxford agar (MOX from Difco) for *Listeria* count (incubated at 35 °C for 48 h).

Microorganisms

Mixed cultures of *Listeria monocytogenes* and Gram-negative bacteria were used to study the bacterial adhesion. The Gram-negative bacteria selected were *Serratia liquefaciens*, *Aeromonas* spp., *Pseudomonas fluorescens* and a mixed culture of different *Pseudomonas* strains. These bacteria were randomly selected and isolated from samples taken from the stainless steel coupons that were placed in the processing environment for the study of microflora (as described before) and the *L. monocytogenes* strain was isolated from a shrimp factory in a previous study (*23*) according to the USDA method (*22*). All bacterial strains were cultured and maintained on tryptic soy agar or in tryptic soy broth supplemented with 0.6 % of yeast extract (Difco) (TSA-YE or TSB-YE, respectively). Stock cultures were maintained in TSB and 20 % glycerol at –70 °C.

Microbial adhesion

Each coupon was placed vertically in a glass tube and autoclaved at 121 °C for 15 min. Shrimp juice was

used to simulate the processing conditions. It was prepared by mixing one part of minced cooked shrimp with 2 parts of deionised water, heated and boiled for 2 min and filtered. It was then supplemented with w(NaCl)= 0.019 to reach a salt concentration of cooked shrimp. The final NaCl-concentration in the shrimp juice was 2.3 % in the water phase measured according to Volhard method ($AgNO_3$, NH_4SCN and $FeNH_4(SO_4)_2$) (*24*). The pH was adjusted to pH=(7.7±0.1) using Radiometer PHM 80. The shrimp juice was autoclaved at 121 °C for 15 min. Thirty mL of sterile shrimp juice was pipetted into each sterile glass tube. One mL of relevant bacterial suspension ($1.5–3.1 \cdot 10^6$ cfu/mL) was transferred into the shrimp juice. Final concentration in the shrimp juice was $5 \cdot 10^4–1 \cdot 10^5$ cfu/mL. Prior to use, the cultures were grown in tryptic soy broth with 0.6 % of yeast extract (TSB-YE, Difco) at 22 or 35 °C for 24 h and subcultured twice. Contact time was 24, 72 and 120 h. Incubation was at 19–21 °C with shaking at 70 rpm. Tests were performed in triplicate.

Statistical analysis

Data analysis was carried out using Number Cruncher Statistical Software (NCSS, 329, North 1000 East, Kaysville, Utah 84037). Analysis of variance (ANOVA) expressed at the 95 % level was used to determine significant differences between the means for surface type, bacterial cultures and contact times. When the ANOVA showed a difference, Duncan's Multiple-Comparison Test was carried out to assess further significant differences between the means.

Results

Composition of microflora adhered to stainless steel surface in seafood processing lines (in situ)

Microbial adhesion was found in many locations and after cleaning the microbial population was $10^1–10^4$ cfu/cm^2 (after swabbing) on samples taken from the shrimp factory and $10^4–10^5$ cfu/cm^2 (after swabbing) and $10^4–10^6$ cfu/cm^2 (removed by ultrasound) on samples taken from the fish factory. The difference between removing bacteria from the coupons by swabbing method or ultrasound revealed 0.5–1 log difference with more recovery by ultrasound treatment. The presence of *Listeria* was not detected on any of the coupons. A total of 356 and 140 isolates were obtained and identified from shrimp and fish factory, respectively, both showing a high proportion of Gram-negative rods (89 and 74 %, respectively) (Table 1). The composition of the microflora in the samples removed by ultrasound showed higher proportion of Gram-negative rods or 82 % compared to 74 % (data not shown). This difference may be explained by the fact that more bacteria were lost during identification methods after swabbing, or 35 strains compared to 2 strains after the ultrasound treatment.

Table 1. Distribution pattern of isolates from the stainless steel coupons in the seafood factories

Type of organisms	Number of isolates	
	Shrimp factory	Fish factory
Gram-positive	4 (1 %)	36 (26 %)
Gram-negative	317 (89 %)	104 (74 %)
Yeasts	35 (10 %)	
Total	356	140

The predominant genus attached to the surfaces was *Pseudomonas* (66 %) in the shrimp factory and *Enterobacteriaceae* (27 %) in the fish factory. The main species of *Pseudomonas* were *P. fluorescens* and *P. putida* and of *Enterobacteriaceae* was *Serratia liquefaciens*. The other Gram-negative microflora included *Aeromonas*, *Moraxella* and *Acinetobacter* spp. The composition of Gram-negative microflora is shown in Fig. 1 (shrimp processing plant) and Fig. 2 (fish processing plant). The Gram-positive microflora from both plants included *Micrococcus* spp. and coryneforms. Overall distribution patterns are shown in Table 2 (shrimp processing plant) and Table 3 (fish processing plant).

Bacterial adhesion (ex situ)

All three types of stainless steel surfaces tested were shown to be very smooth with Ra from 0.16–0.8 μm. Glass beaded steel was the roughest with Ra from 0.7–0.8 μm and Rz value from 5.4–6.5 μm. The roughness of the untreated and polished steel were similar, with Ra value of

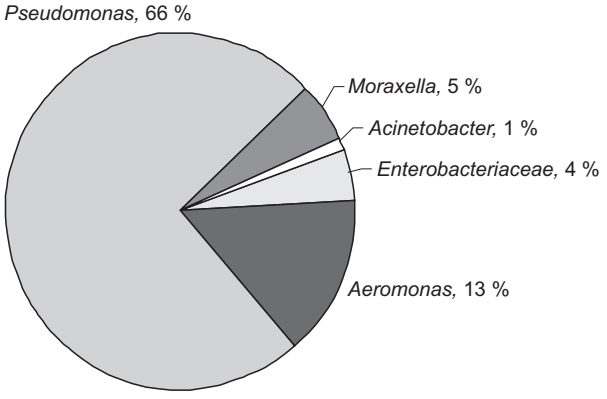

Fig. 1. Distribution pattern of Gram-negative isolates in shrimp processing plant

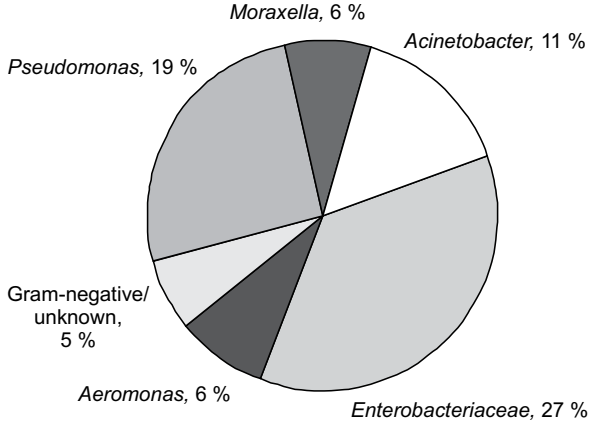

Fig. 2. Distribution pattern of Gram-negative isolates in fish processing plant

Table 2. Composition of microflora on stainless steel coupons in shrimp processing plant

Type of microflora	Number of isolates					
	Platform in cooking area	Cooking equipment	Peeling machine	Cleaning machine (a blower)	Total	Fraction %
Enterobacteriaceae	10	2	3		15	4
Aeromonas spp.	1	40	5		46	13
Gram-negative/unknown				1	1	<1
Pseudomonas spp.	63	25	90	56	234	66
Moraxella spp.				5	5	1
Moraxella-like spp.		1		12	13	4
Acinetobacter spp.	2			1	3	1
Micrococcus spp.				2	2	<1
Coryneforms		1		1	2	<1
Yeasts	14	19	1	1	35	10
Total	90	88	99	79	356	

Table 3. Composition of microflora on stainless steel coupons in fish processing plant

Type of microflora	Number of isolates										
	I*	II	III	IV	V	VI	VII	VIII	IX	Total	Fraction/%
Enterobacteriaceae	–	2	4	9	–		2		21	38	27
Aeromonas spp.	–	5		3	–					8	6
Gram-negative/unknown	–		5		–	1		1		7	5
Pseudomonas spp.	–	7	9	7	–		1	3		27	19
Moraxella spp.		2	1			2	2	1		8	6
Acinetobacter spp.	–	6		2	–	1		7		16	11
Micrococcus spp.	–	1		2	–	9	2	6		20	14
Coryneforms							15			15	11
Planococcus spp.								1		1	1
Total	0	23	19	23	0	13	22	19	21	140	

* I-portioning machine-1, II-flowline, III-weighing machine-1, IV-conveyer belt, V-degrading machine, VI-IQF, VII-weighing machine-2, VIII-stairbelt, IX-portioning machine-2

0.16–0.22 μm and Rz value from 1.3–1.9 μm. The highest number of bacteria attaching to the surfaces was obtained after contamination with a mixed culture of *Serratia liquefaciens* and *L. monocytogenes* (Fig. 3). A lower number of bacteria adhered to the steel surfaces when a mixed culture of *Pseudomonas fluorescens* and *L. monocytogenes* (Fig. 4) or *Aeromonas* spp. and *L. monocytogenes* were tested (Fig. 5). But the presence of the mixed culture of *Pseudomonas* spp. significally enhanced the colonisation of *L. monocytogenes* ($p<0.05$) (Fig. 6) compared to the other bacterial group tested. The number of adherent bacteria increased with longer contact time

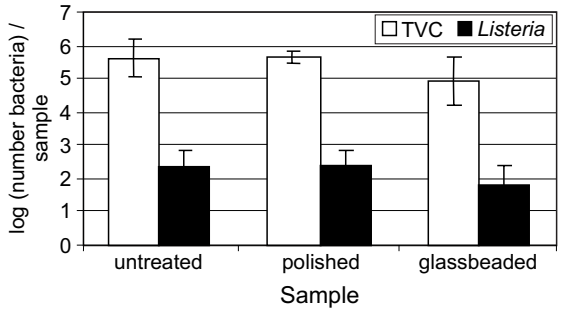

Fig. 3. Adherence of *L. monocytogenes* to stainless steel surfaces in co-culture with *Serratia liquefaciens* after 120-h contact time at 22 °C. Means of triplicate (bars represent the standard error of the mean value)

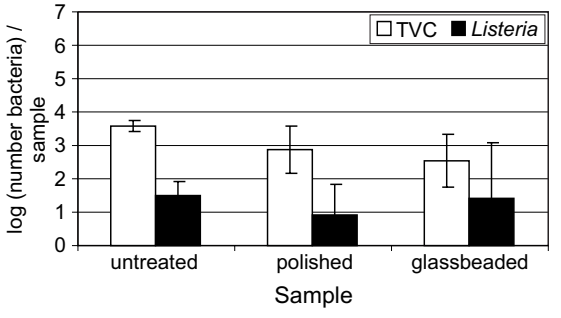

Fig. 4. Adherence of *L. monocytogenes* to stainless steel surfaces in co-culture with *Aeromonas* spp. after 120-h contact time at 22 °C. Means of triplicate (bars represent the standard error of the mean value)

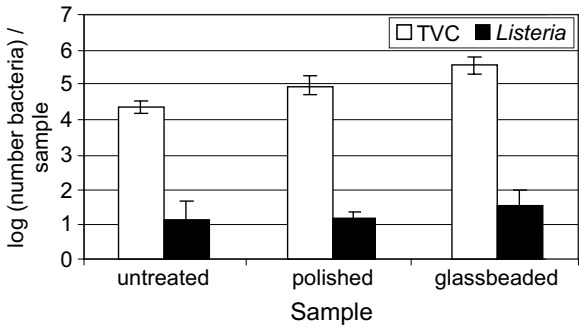

Fig. 5. Adherence of *L. monocytogenes* to stainless steel surfaces in co-culture with *Pseudomonas fluorescens* after 120-h contact time at 22 °C. Means of triplicate (bars represent the standard error of the mean value)

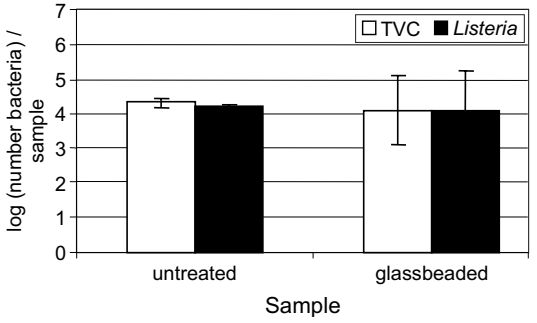

Fig. 6. Adherence of *L. monocytogenes* to stainless steel surfaces in co-culture with a mixed culture of *Pseudomonas* spp. after 120-h contact time at 22 °C. Means of triplicate (bars represent the standard error of the mean value)

($p<0.05$) and no difference was observed between different types of steel surfaces after 96- to 120-hour incubation ($p>0.05$).

Discussion

Adhered bacteria were found in many locations of both processing lines with densities of 10^1–10^4 cfu/cm^2 in the shrimp factory and 10^4–10^5 cfu/cm^2 in the fish processing plant. It has been stated that large biofilm structures common in other industries (multilayer of bacteria, typically above 10^8 cfu/cm^2) rarely occur in food processing environments (*25*). These results support this statement and indicate that regular cleaning and disinfection limit the progression of the attached cells to extensive biofilms on food contact surfaces in both factories. In this study a swabbing method was used for sampling and therefore it is necessary to have in mind that this method provides only limited information on actual surface hygiene and it will not remove all bacteria from the surface. The fact that slightly higher counts of bacteria were recovered after ultrasound treatment and fewer bacteria were lost during identification process indicates that this treatment for removing bacteria from the surfaces is more efficient. Higher counts of bacteria detected on the stainless steel samples in the fish factory indicate that the cleaning procedure in this place was not effective. If the cleaning procedures used are ineffective and the conditions are favourable, biofilms incorporating *L. monocytogenes* may be formed on different surfaces (*11*). The distribution pattern of the isolates revealed a high proportion of Gram-negative rods. *Pseudomonas* spp., *Aeromonas* spp., *Enterobacteriaceae* and yeast dominated in the shrimp processing plant while *Pseudomonas* spp. and *Enterobacteriaceae* were mainly found in the fish processing plant. *Listeria* was not detected in any of the samples. Of the *Pseudomonas* spp., *P. putida* and *P. fluorescens* were the main species isolated from the surfaces in the factories. Most importantly, it should be pointed out that these bacterial groups are considered as main spoilers of fresh/chilled fish (*26*). The main species of *Enterobacteriaceae* was *Serratia liquefaciens*, which is ubiquitous in nature and used as an indicator bacteria to evaluate cleaning effectiveness. High incidence of *Pseudomonas* spp. indicates that the disinfectant used was not effective, but these bacteria are known to have some resistance mechanisms against quaternary ammonium compounds widely used in seafood industry (*5,6*). Yeasts were identified in two places in the shrimp processing plant, especially on surfaces which had been polished and pickled. After studying the microbial ecology of processing equipment in herring production, Bagge-Ravn *et al.* (*27*) pointed out that yeasts should get special attention when developing cleaning and disinfecting procedures. The shrimp industry might need to do the same. These organisms may be dispersed into the finished product, leading to product spoilage.

All three types of stainless steel surfaces were very smooth with Ra from 0.16–0.8 μm and the results show that the adhesion of bacteria was not affected by the surface topography. Hilbert and her colleagues came to a similar conclusion but their study showed that the attachment and removal of microorganisms from stainless steel surfaces did not depend on surface roughness when Ra value was between 0.01 and 0.9 μm (*28*). It should be pointed out that for all the steel types, even the glass beaded steel, Ra values are within the limits given by EHEDG, which is Ra≤0.8 μm (*29*). The highest numbers of the attached bacteria were obtained after contamination with the mixed culture of *Serratia liquefaciens* and *L. monocytogenes*. Lower numbers of bacteria adhered to the steel surfaces when the mixed culture of *Pseudomonas fluorescens* and *L. monocytogenes* as well as the mixed culture with *Aeromonas* spp. and *L. monocytogenes* were tested. These results show that *L. monocytogenes* cells in the co-cultures were recoverable in all cases. Colonisation of *L. monocytogenes* on stainless steel was only significantly enhanced in the presence of mixed *Pseudomonas* spp. compared to other bacterial groups tested. It should be pointed out that in the processing environment it is unlikely that *L. monocytogenes* should be present as a single species culture, at least when the results from the study *in situ* are taken into account. The results also show that contact time influences the adhesion, independently of the types of steel. These results show that smooth surfaces do not always provide hygiene benefits over rougher surfaces. Other hygienic design criteria like welding, joints and corners may be of greater importance and equipment should be designed to prevent the accumulation of soil and allow easy cleaning, so that biofilm will not develop.

Conclusions

In the food processing environment microorganisms can attach themselves to stainless steel with different surface finishes and increase in number relatively rapidly, although this development is generally limited by cleaning and disinfection procedure. Microorganisms attached to surfaces may be an significant source of contamination and it is therefore important to improve the design of processing equipment with hygienic requirements in mind. Control of biofilm formation or adherence of bacteria relies on the implementation of effective cleaning and disinfecting procedures and on the design of processing equipment. The choice of surface treatment of stainless steel is an important factor to have in mind when food processing equipment for open process is designed, but smooth surfaces do not always provide hygiene benefits over rougher surfaces.

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Mikrobna adhezija u postrojenjima za proizvodnju ribljih fileta i kuhanih rakova

Utjecaj površinske obrade nehrđajućeg čelika i prisutnosti Gram-negativnih bakterija na prijanjanje bakterije *Listeria monocytogenes*

Sažetak

Mikroflora koja prijanja na površine postrojenja u tvornici rakova i tvornici za preradu ribe utvrđena je *in situ,* a adhezija miješane kulture *Listeria monocytogenes* s Gram-negativnim bakterijama *ex situ* na površinama nehrđajućeg čelika (neobrađenog, poliranog i visoko poliranog). Dominantni prijanjajući sojevi bili su *Pseudomonas* spp. (66 %) u tvornici rakova, a *Enterobacteriaceae* (27 %) u tvornici za preradu ribe. Sok rakova koristio se za obogaćivanje podloge tijekom ispitivanja prijanjajućih bakterija. Za ispitivanje prijanjanja na površinu čelika odabrana su 3 različita soja Gram-negativnih bakterija i smjesa *Pseudomonas* spp. zajedno s *Listeria monocytogenes.* Najveći broj prijanjajućih bakterija dobiven je kontaminacijom s miješanom kulturom *Listeria monocytogenes* i *Serratia liquefaciens*. Primjenom miješane kulture *Listeria monocytogenes* i *Pseudomonas fluorescens* ili *Aeromonas* spp. smanjen je broj bakterija koje su prijanjale na površinu čelika. Nisu opažene razlike ($p > 0,05$) u prijanjanju bakterija na različito obrađenim površinama nehrđajućeg čelika s različitom hrapavošću ($Ra=0,1–0,8$ μm). Adhezija bakterija povećavala se dužim vremenom dodira. Kolonizacija *Listeria monocytogenes* na površini nehrđajućeg čelika bila je bitno pojačana samo u prisutnosti *Pseudomonas* spp. Ovi rezultati pokazuju da za postizanje higijenskih uvjeta nije važno radi li se o glatkim ili grubim čeličnim površinama.