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Growth Media and Surface Conditioning Influence the Adherence of *Pseudomonas fragi, Salmonella typhimurium,* and *Listeria monocytogenes* Cells to Stainless Steel

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ABSTRACT

Microorganisms have been shown to adhere to food-contact surfaces and may provide a route for the contamination of processed food. To better understand this phenomenon, the effects of growth media and surface conditioning on the adherence of *Pseudomonas fragi, Salmonella typhimurium* and *Listeria monocytogenes* cells to stainless steel were studied. The microorganisms were grown in tryptic soy broth (TSB), 1% reconstituted skim milk (RSM) and RSM with 1% sucrose (RSM + S). Stainless-steel surfaces were conditioned by immersion in growth media for 1 h and then were rinsed in phosphate-buffered saline (PBS) prior to the adherence assay. After growing in each medium, cells were harvested, resuspended in PBS, and then allowed to contact the stainless steel for 30 min. Adherence was quantified by acridine orange-staining the cells and viewing under epifluorescence microscopy. Growth media had little influence on adherence to stainless steel that had not been preconditioned. *P. fragi* and *L. monocytogenes* cells adhered in the highest numbers when grown in RSM plus sucrose. *S. typhimurium* cells showed the highest level of adherence when grown in TSB. Analysis of variance yielded *P* values of less than 0.01, indicating that both growth media and surface conditioning were significant in the level of adherence observed.

Key words: Adherence, media effects, surface conditioning

Bacterial attachment in food-processing environments is a potential source for contamination of foods that may lead to spoilage and the transmission of food-borne pathogens. Microorganisms been shown to attach to chicken tissue *(8),* beef, pork, and lamb tissue *(1).* Adherence to food-contact surfaces such as stainless steel and rubber has been demonstrated by a number of researchers *(5, 12).*

Once cells have attached to food-contact surfaces, attempts to remove them by traditional cleaning and sanitizing procedures may be ineffective. Stone and Zottola *(11)* used a model clean-in-place (CIP) system to demonstrate that *P. fragi* cells may not be removed during the CIP cycle. Other researchers have shown that adherent *L. monocytogenes* cells exhibited increased resistance to benzalkonium chloride, anionic acid sanitizer, and heat *(2).*

In the food-processing environment, stainless-steel surfaces come in contact with fluids containing various levels of food components. One of the first events to occur is the adsorption of molecules to the surface. This effect is often referred to as conditioning. The amount of conditioning that occurs by milk protein has been modeled as a function of contact-surface tension, temperature, and time, and depending on the conditions, may provide a better environment for microbial adherence *(6).*

The public-health ramifications of contamination by pathogens such as *S. typhimurium* and *L. monocytogenes* are a major concern of all food processors. *Pseudomonas* species are often associated with the spoilage of perishable foods such as milk and meats and, therefore, have a major impact on the quality of these foods. The objectives of this study were to determine the effect of growth conditions and surface conditioning on the adherence of *S. typhimurium, L. monocytogenes* and *P. fragi* cells to stainless steel.

MATERIALS AND METHODS

Bacterial cultures

S. typhimurium, L. monocytogenes strain V7, and *P. fragi* ATCC 4973 were obtained from the culture collection of the University of Minnesota, Department of Food Science and Nutrition. All cultures were maintained in tryptic soy broth (TSB) (Difco Laboratories, Detroit, MI) at 4°C. Prior to use, the cultures were grown in TSB at 22°C and subcultured twice. Bacterial cultures were grown in the test medium at 22°C for 18 to 24 h.

Test surface and media

The test surface was 304 stainless-steel chips, finish no. 4 (6 by 6 mm). The stainless-steel chips were prepared by rinsing them in acetone for a minimum of 30 min, rinsing in distilled water, and then soaking in 1 N NaOH for 1 h. After a final rinse in distilled water, the chips were allowed to air dry. The chips were sterilized by autoclaving at 121°C for 15 min in glass jars.

The test media were TSB, 1% reconstituted skim milk (RSM) (Land O'Lakes, Arden Hills, MN), and 1% reconstituted skim milk

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with 1% sucrose (RSM + S). The conditioning media were TSB, RSM, RSM + S, and phosphate-buffered saline (PBS). The TSB, RSM, RSM + S, and PBS were sterilized by autoclaving at 121°C for 15 min.

Surface conditioning

To condition the surface, sterile stainless-steel chips were placed in plastic petri plates containing the conditioning medium. The chips remained in the medium for 1 h. The medium was removed and replaced with PBS. The PBS remained for up to 5 min and was then replaced with the adherence medium.

Adherence testing

The cells were harvested from the test cultures grown as described above by centrifugation and resuspended in PBS. The cells were then added to the petri plates containing the stainless-steel chips. Adherence was allowed to occur over 30 min.

Quantification of adherence

Pairs of chips were aseptically removed at selected time intervals. The chips were rinsed by placing them in a section of silicone tubing (5-mm i.d.) and then pumping sterile 0.1% peptone water through the tubing for 10 s with a peristaltic pump. The peptone water was pumped at a rate of 166 ml/min. The chips were then placed in 10 ml of sterile distilled water. The water was removed using a vacuum apparatus, and the chips were placed in a 0.025% sterile acridine orange solution (Sigma Chemical Co., St. Louis, MO) in 0.1 N citrate buffer, pH 6.6, to stain the attached cells. Following staining, the chips were rinsed in distilled water and allowed to air dry. Adherent microorganisms were quantified by viewing the chips using epifluorescence microscopy. Ten fields of a known area per chip were viewed and the fluorescing cells were enumerated. The viewing area was determined with the use of a stage micrometer. A computerized image-analysis system (Global Lab Image, Data Translation, Maraboro, MA) was used to enumerate the cells. The average number of cells per field was converted to the number of cells per cm^2.

Populations in the test media were determined by plating, in duplicate, on tryptic soy agar (TSB with 1.5% agar added) (Difco). The plates for *S. typhimurium* and *L. monocytogenes* were incubated at 37°C for 24 h and for *P. fragi* at 23°C for 48 h.

Analysis of variance was performed on the data by using the software MYSTAT *(13)*.

RESULTS

The concentrations of the microorganisms used in the adherence assay after resuspending in PBS are shown in Table 1. Each of the three microorganisms grew to the highest population in TSB. When growth in RSM and RSM + S were compared, it was seen that the addition of sucrose did not enhance growth.

TABLE 1. *Concentrations of test microorganisms grown in various test media after centrifuging and resuspending in PBS*

	Concentration (CFU/ml) in medium		
Test microorganism	TSB	RSM	RSM + S
S. typhimurium	1.38×10^9	3.70×10^8	4.60×10^8
L. monocytogenes	1.30×10^8	4.9×10^7	5.2×10^7
P. fragi	1.30×10^8	4.15×10^7	4.10×10^7

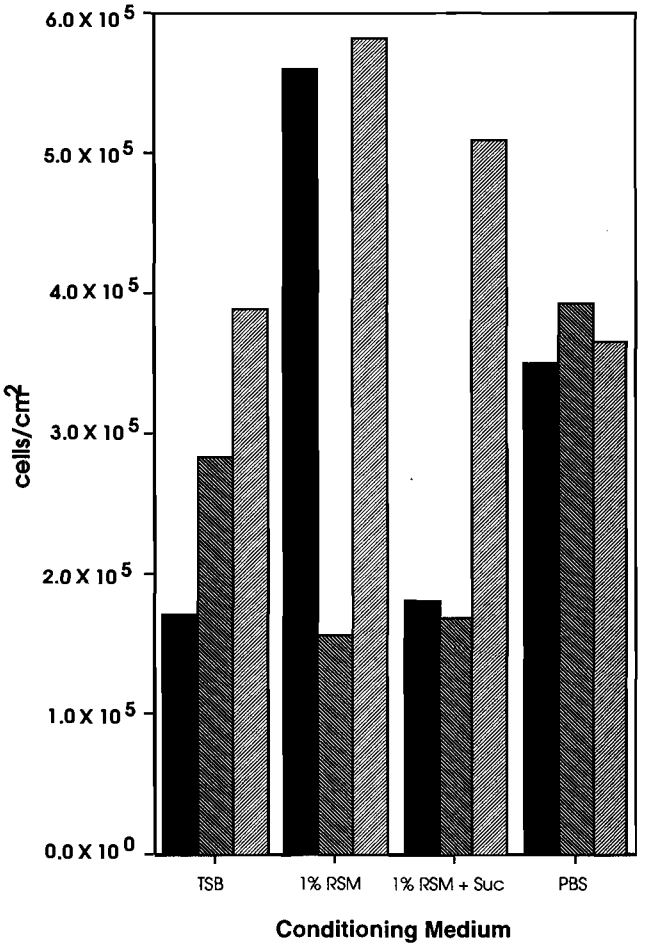

FIGURE 1. *Number of adherent* P. fragi *cells observed after 30 min of contact time with conditioned stainless steel. Growth medium:* ■, *TSB;* ▧, *RSM;* ▨, *RSM*+S.

P. fragi cells adhered in substantial numbers under all conditions tested (Fig. 1). Within each conditioning medium, the greatest *P. fragi* cell adherence levels were observed when the culture had been grown in RSM + S. When the surface was conditioned in PBS, the growth media did not appear to affect adherence. In addition, when RSM + S was used as a growth medium, adherence appeared to be enhanced.

S. typhimurium cells also appeared to adhere in high numbers under all conditions. However, *S. typhimurium* cells showed the highest levels of adherence when grown in TSB (Fig. 2).

L. monocytogenes cells did not adhere in substantial numbers except under certain conditions. As with *S. typhimurium,* the highest observed levels of adherence were seen when *L. monocytogenes* cells were grown in TSB. Both growth in and surface conditioning by RSM and RSM + S appeared to significantly restrict the adherence of *L. monocytogenes* cells to stainless steel (Fig. 3).

Analysis of variance *(13)* was used to determine if either growth media or surface-conditioning media influ-

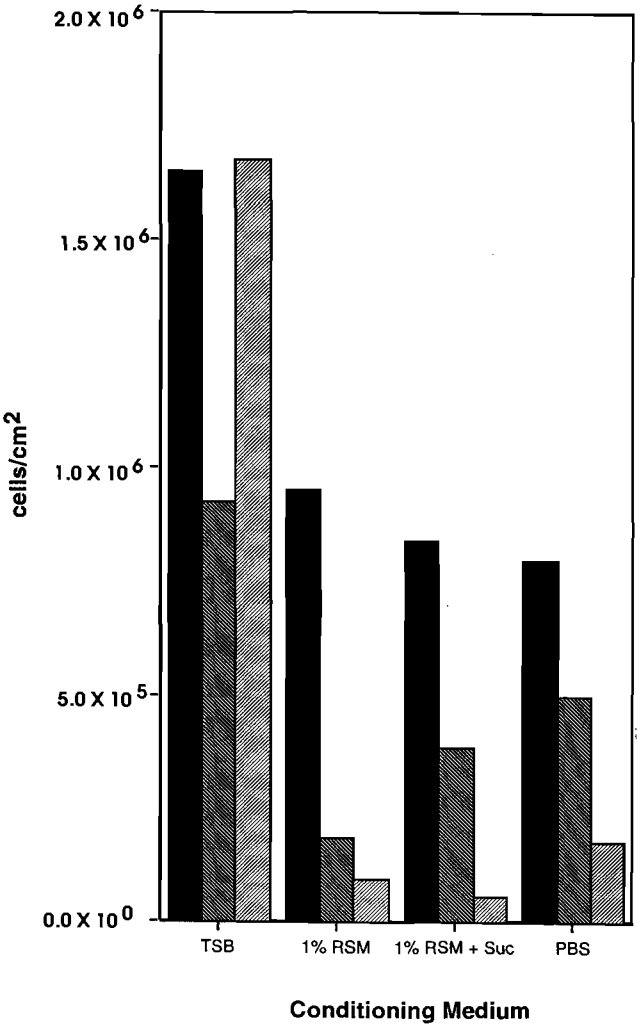

FIGURE 2. *Number of adherent* S. typhimurium *cells observed after 30 min of contact time with conditioned stainless steel. Growth medium:* ■, *TSB;* ▧, *RSM;* ▨, *RSM + S.*

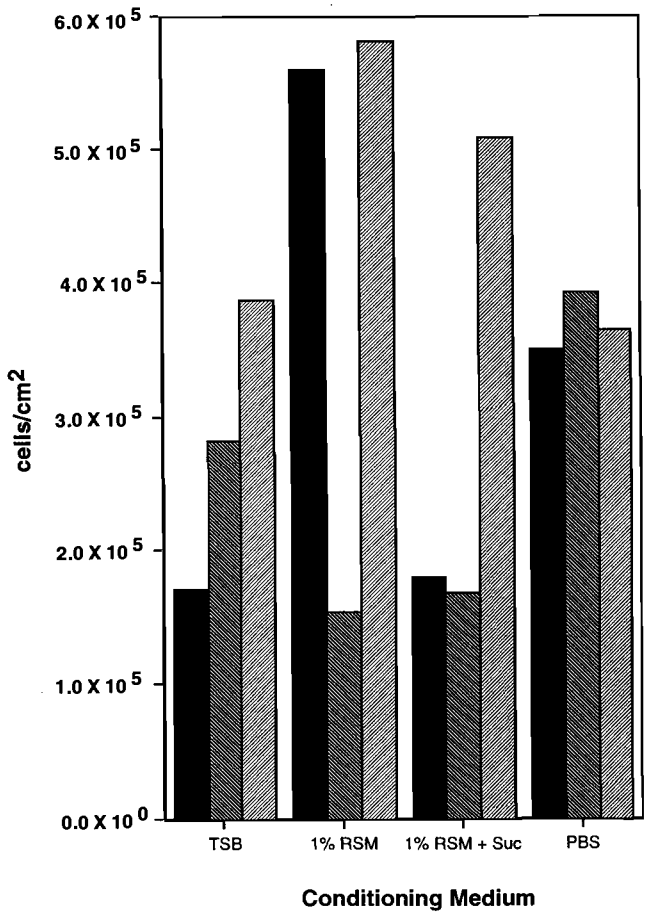

FIGURE 3. *Number of adherent* L. monocytogenes *cells observed after 30 min of contact time with conditioned stainless steel. Growth medium:* ■, *TSB;* ▧, *RSM;* ▨, *RSM + S.*

TABLE 2. *Results of analysis of variance performed on* S. typhimurium, L. monocytogenes, *and* P. fragi *cells grown in either TSB, RSM, or RSM + S and surfaces conditioned in either PBS, TSB, RSM, or RSM + S*

Species Factor	Sum of squares	*DF*	Mean squares	*F* ratio	P
		S. typhimurium			
Media	1.68×10^{15}	2	8.41×10^{14}	98.96	0.00
Conditioning	4.23×10^{15}	3	1.41×10^{15}	165.81	0.00
Media, conditioning	8.90×19^{14}	6	1.48×10^{14}	17.45	0.00
		L. monocytogenes			
Media	3.86×10^{14}	2	1.93×10^{14}	104.35	0.00
Conditioning	1.96×10^{14}	3	6.54×10^{13}	35.34	0.00
Media, conditioning	3.84×10^{14}	6	6.40×10^{12}	34.55	0.00
		P. fragi			
Media	1.62×10^{14}	2	8.10×10^{13}	22.72	0.00
Conditioning	9.46×10^{13}	3	3.15×10^{13}	8.84	0.00
Media, conditioning	2.20×10^{14}	6	3.67×10^{13}	10.30	0.00

enced adherence. The results of the ANOVA for each microorganism are shown in Table 2.

DISCUSSION

Surface conditioning by TSB, RSM, or RSM + S represents the potential of soiling of stainless steel under food-processing conditions; conditioning in PBS represents a clean surface. Results of this study show that the effect of soiling on adherence varied with each microorganism and each growth medium. In addition to the soiling effect, the growth conditions also appeared to influence adherence. Other researchers have observed similar results. Helke et al. *(3)* found that exposing both stainless steel and Buna-N to the individual components of milk inhibited the adherence of *S. typhimurium* and *L. monocytogenes* cells. They also observed that the attachment menstruum has a significant effect on attachment. They reported pretreatment with skim milk and β-lactoglobulin decreased attachment.

Similar variability in adherence was observed by Speers and Gilmour *(10)*. They studied a variety of microorganisms originally isolated from milk-soiled surfaces and found that, in general, when milk components were included in a suspending medium, adherence was lower than when Ring-

er's solution was used as a suspending medium. McGuire *(6)* suggested a scenario that might explain the observation that lower levels of adherence are common when surface is conditioned with milk. McGuire's model offers the possibility that adsorbed proteins may establish an equilibrium with the proteins in the bulk fluid, resulting in a passive surface that is unable to further adsorb particles such as microorganisms *(6)*.

Marshall et al. *(7)* hypothesized that bacterial adherence to surfaces followed a two-step mechanism. The first step was reversible adherence: cells associated with a surface could easily be removed. The second step was irreversible adherence accompanied by extracellular polymers that helped anchor the cell to the surface. However, other researchers have found that in the initial step of adherence to a surface, polymers such as polysaccharides may actually inhibit adherence. Wrangstadh et al. *(15)* observed that a marine *Pseudomonas* sp. showed a higher level of cell adherence after starvation. The starvation caused a decrease in the production of extracellular polysaccharide as observed by transmission electron microscopy.

In our study, sucrose was added to diluted skim milk for two reasons. First, it is not uncommon for a dairy to make sweetened fluid products such as flavored milk and ice cream mix. Secondly, the addition of sucrose provided additional carbohydrate for utilization by the bacterial cells for the production of polysaccharides. *P. fragi* cells always adhered in high numbers when grown in 1% RSM + S, while *S. typhimurium* cells generally showed a lower adherence with RSM + S as the growth medium. Polysaccharides do appear to be involved in the adherence of *P. fragi* cells to stainless steel. Herald and Zottola *(4)* showed that compounds that bind or disrupt carbohydrates tend to decrease the adherence of *P. fragi* to stainless steel.

It is not surprising to see the low adherence of *L. monocytogenes* cells relative to the other microorganisms tested. Wirtanen and Mattila-Sandholm *(14)* observed that it took 5 days for *L. monocytogenes* to reach 1×10^5 cells per cm^2 while *P. fragi* was over 1×10^6 cells per cm^2 after only 2 days. Other researchers have noted that gram-positive species often adhere at lower levels to inert surfaces such as glass and stainless steel *(10)* as well as to meat tissue *(1)*.

Although there were instances in this study that suggested an unclean surface may actually allow fewer cells to adhere, cleaning is still an essential part of any sanitation program. It has been shown that *L. monocytogenes* cells are much more likely to adhere when growing in the presence of *P. fragi (9)*. Therefore, when considering cleaning and sanitizing programs in a food-processing environment, it must be assumed that a mixed flora is present and in that mixed flora may be microorganisms that adhere well.

Clearly, microbial adherence to food-contact surfaces by *P. fragi, S. typhimurium,* and *L. monocytogenes* cells is significantly influenced by both the growth medium and the conditioning of the surface, as was verified by the statistical analysis done in this study. With each culture used, P values of less than 0.01 were obtained (Table 2). These data indicated that both growth media and surface conditioning were significant factors affecting the level of adherence observed. However, there is variability between microorganisms of different species and in the specific conditions that exist in the food-processing environment. Consequently, conditions other than those reported here may also influence microbial adherence in the environment of a food-processing facility.

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