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Persistent *Listeria monocytogenes* Strains Show Enhanced Adherence to Food Contact Surface after Short Contact Times

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ABSTRACT

Adherence of 3 persistent and 14 nonpersistent *Listeria monocytogenes* strains to stainless steel surfaces after short and long contact times was investigated. *L. monocytogenes* strains were obtained from poultry plants and an ice cream plant throughout several years. Adherence tests were performed in tryptic soy broth at 25°C for 1, 2, and 72 h. Test surfaces were rinsed after the contact time, and attached cells were stained with acridine orange and enumerated with an epifluorescence microscope. The persistent poultry plant strains showed adherence 2- to 11-fold higher than the nonpersistent strains following 1- and 2-h contact times. The adherence of the persistent ice cream plant strain after 1- and 2-h contact times was higher than most of the nonpersistent strains. Seven of 12 nonpersistent ice cream strains showed an adherence of less than half that of the persistent strain. After 72 h, the differences in adherence were not as marked, since half the nonpersistent strains had reached adherence levels comparable with the persistent strains. In fact, three nonpersistent strains showed even higher adherence than the persistent strains. Thus, results of this study reveal that persistent *L. monocytogenes* strains show enhanced adherence at short contact times, promoting their survival in food processing facilities and possibly having an effect on initiation of persistent plant contamination.

The adherence of pathogenic bacteria to food contact surfaces and biofilm formation are sources of concern in food processing plants, because adhered cells are more difficult to remove mechanically from surfaces and more resistant to disinfectants than planktonic cells (4, 5, 16). The ability to adhere is important in the survival of bacteria in food processing plants, where cleaning and disinfection are a daily routine. *Listeria monocytogenes* adheres and forms biofilm under conditions prevailing at food processing plants (7, 8, 18) and has been observed to adhere after short contact times (9), challenging cleaning procedures. *L. monocytogenes* has been shown to adhere to stainless steel, rubber, glass, and polypropylene surfaces and other surface materials used in food processing plants (2, 6, 9, 16).

Immediate eradication of *L. monocytogenes* from production lines, especially post heat treatment production lines, is of great importance not only because of product safety but also for facilitation of cleaning. It is easier to remove and eradicate cells that are not yet adhered or are loosely adhered to surfaces. It is also easier to eradicate young biofilms than old biofilms, because young cells are more sensitive to sanitizers (13, 19). The eradication of *L. monocytogenes* has, however, often proved difficult, requiring special measures to succeed (1, 10).

Earlier studies show that *L. monocytogenes* has caused prolonged contamination in food processing plants (1, 10, 11, 20). These contamination studies reveal that some *L. monocytogenes* strains may dominate and persist in a plant or production line for years (10, 20). Miettinen et al. (10)

showed that a dominating strain persisted in an ice cream plant for 7 years. *L. monocytogenes* was eradicated from the packaging machine, which was considered the main contamination source, by targeted cleaning and disinfection. Autio et al. (1) conducted a contamination study in a fish processing plant, identifying one dominating strain during at least 1 year. Targeted cleaning eradicated the organism.

The fact that some *L. monocytogenes* strains dominate and persist in food processing plants, whereas other strains do not, supports the theory of persistent strains having qualities promoting survival in food processing facilities. Ronner and Wong (16) studied the adherence of seven *L. monocytogenes* strains to stainless steel and Buna-n rubber and observed that some of the strains adhered with higher cell counts than others. Norwood and Gilmour (12) observed differences in adherence of persistent and nonpersistent *L. monocytogenes* strains to food contact surfaces. It seems that in production plants, where cleaning and disinfection procedures form part of the daily routine, adherence at short contact times is important in the survival of *L. monocytogenes*. The bacteria have to be able to adhere to surfaces, preferably in high numbers, before cleaning and disinfection procedures take place. The aim of this study was to evaluate the adherence of persistent and nonpersistent strains to stainless steel surfaces after short and long contact times in relation to persistent plant contamination.

MATERIALS AND METHODS

***L. monocytogenes* strains.** *L. monocytogenes* strains were isolated from earlier surveys performed at an ice cream plant (10) and poultry plants (Table 1). Samples were collected throughout several years from the environment, raw material, equipment, and

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TABLE 1. Characterization, source, and persistence of *L. monocytogenes* strains used in the study

Pulsed-field gel electrophoresis type	Serotype	Source	Persistent or nonpersistent
I	1/2b	Ice cream plant	Persistent
II	1/2b	Ice cream plant	Nonpersistent
III	1/2b	Ice cream plant	Nonpersistent
IV	1/2b	Ice cream plant	Nonpersistent
V	1/2b	Ice cream plant	Nonpersistent
VI	1/2b	Ice cream plant	Nonpersistent
VII	1/2b	Ice cream plant	Nonpersistent
VIII	1/2b	Ice cream plant	Nonpersistent
IX	1/2b	Ice cream plant	Nonpersistent
X	1/2b	Ice cream plant	Nonpersistent
XI	1/2b	Ice cream plant	Nonpersistent
XII	1/2 ^a	Ice cream plant	Nonpersistent
XIII	4b	Ice cream plant	Nonpersistent
XIV	1/2c	Poultry plant A	Persistent
XV	1/2c	Poultry plant B	Persistent
XVI	1/2a	Poultry plant A	Nonpersistent
XVII	1/2a	Poultry plant B	Nonpersistent

^a H antigens could not be determined because of poor growth in the brain heart infusion motility agar (10).

products. Characterization of strains was conducted by serotyping and by pulsed-field gel electrophoresis (PFGE) typing (10). The strains from the ice cream and the poultry plants were characterized with three restriction enzymes (*ApaI*, *AscI*, and *SmaI*) and two enzymes (*ApaI* and *AscI*), respectively. Seventeen strains with different PFGE types were chosen for the study according to persistence or nonpersistence. A strain was considered to be persistent if it was a dominating strain in the plant and was repeatedly found during a period of several months or years. Persistent strains were isolated not only from the environment or equipment but also from products. A strain recovered sporadically was considered to be nonpersistent. Cultures were maintained at -70°C .

Test surface. Stainless steel surfaces (type 316, Outokumpu, Finland) were cut into 20 by 70 by 1-mm coupons and immersed in 1 N NaOH for 24 h to etch the surfaces clean. Coupons were then rinsed in distilled water and immersed in acetone for 1 h to remove possible grease. Finally, the surfaces were rinsed in distilled water and air dried. Coupons were placed vertically in glass jars and autoclaved.

Test suspension. Each strain was cultured onto blood agar plates and incubated at 37°C for 24 h. To attain a test suspension with cells in the same phase, the following procedure was performed. One colony was transferred into 10 ml of tryptic soy broth (TSB) and incubated at 25°C for 20 h, and then 0.1 ml was inoculated into 10 ml of TSB and incubated at 25°C for 20 h. Then 1 ml was inoculated into 100 ml of TSB and grown at 25°C to log phase. When the concentration of the suspension was 9×10^7 CFU/ml, as previously determined by plating, 1.5 ml was inoculated into 150 ml of TSB to attain a test suspension with a concentration of 9×10^5 CFU/ml.

Adherence of cells. The test suspension was transferred into an autoclaved glass jar with vertically placed test coupons. Contact times were 1, 2, and 72 h. Incubation was at 25°C with shak-

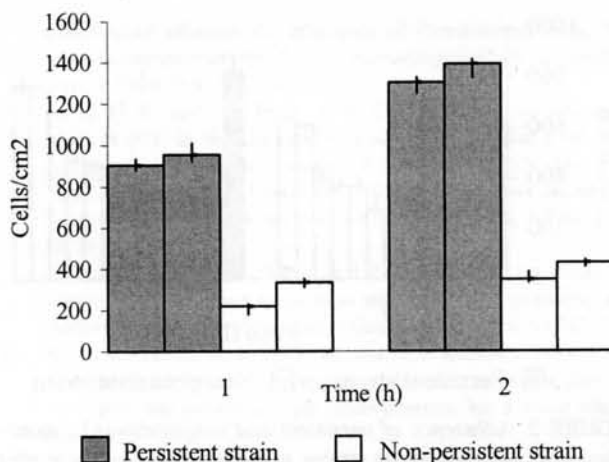


FIGURE 1. Adherence of persistent and non-persistent *L. monocytogenes* poultry plant strains to stainless steel surface after 1- and 2-h contact times. Tests were made in triplicate.

ing at 70 rpm (Promax 2020, Heidolph, Germany). Tests were performed in triplicate, including preparation of test suspensions.

Enumeration of adhered cells. After contact time, coupons were removed from test suspension and vortexed at speed one (Cyclo-Mixer, Clay Adams, Parsippany, N.J.) for 5 s in a test tube containing 50 ml of sterile water to remove loosely adhered cells and leave only firmly adhered cells. Following vortexing, coupons were immersed in distilled water. Coupons were then stained with filter-sterilized (Minisart 0.2 μm , Sartorius, Göttingen, Germany) 0.05% acridine orange (Certistain, Merck, Darmstadt, Germany) for 2 min, rinsed in sterile water, and air dried. Enumeration of adhered cells was conducted with an epifluorescence microscope (Optiphot-2, Nikon, Japan) at a magnification of 400. Minimums of 30 and 10 fields were observed on every short-contact coupon and long-contact coupon, respectively.

RESULTS

Persistent *L. monocytogenes* strains obtained from poultry plants showed the highest adherence levels of all strains after 1- and 2-h contact times (Fig. 1). Adhering cell counts of both persistent strains at 1- and 2-h contact times varied between 9.1×10^2 cells/cm² and 1.4×10^3 cells/cm², which were 2.7- to 4.6-fold higher than adhering cell counts of both nonpersistent poultry plant strains.

The adherence level of the persistent *L. monocytogenes* strain obtained from the ice cream plant varied between 6.3 and 8.3×10^2 cells/cm² at 1- and 2-h contact times (Fig. 2). Twelve strains recovered from the ice cream plant were categorized as nonpersistent strains, with adherence levels ranging from 8.8×10 cells/cm² to 7.7×10^2 cells/cm² at 1- and 2-h contact times. All nonpersistent strains except two showed lower adherence than the persistent strain at 1- and 2-h contact times. Two nonpersistent strains reached the same adherence level as the persistent strain at 1-h contact time. Seven nonpersistent strains showed an adherence of less than half that of the persistent strain at 1- and 2-h contact times. The poorest adherence (8.8×10 cells/cm²) was shown by a nonmotile strain.

Observed adherence of each strain at 72 h (Fig. 3) was higher than at short contact times. Adherence levels of the persistent strains were between 2.1 and 2.4×10^5 cells/

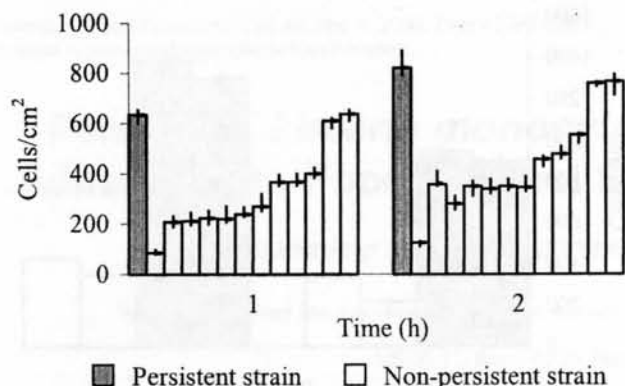


FIGURE 2. Adherence of persistent and nonpersistent *L. monocytogenes* ice cream plant strains to stainless steel surface after 1- and 2-h contact times. Tests were made in triplicate.

cm². Seven nonpersistent ice cream plant strains reached adherence levels of persistent strains, whereas seven strains, including both nonpersistent poultry plant strains, showed lower adherence levels. Three nonpersistent strains showed higher adhering cell counts than any of the persistent strains. Some nonpersistent strains, including the nonmotile strain, that were adhering poorly at short contact times were observed to adhere with higher adhering cell counts at 72 h than most of the other strains.

Adherence of both *L. monocytogenes* strains of serotype ½c was higher than the adherence of other serotypes at short contact times. Strains of serotype ½b were observed to adhere with large variation. The persistent strain of serotype ½b showed marked adherence, whereas some strains of serotype ½b adhered poorly.

DISCUSSION

The persistent poultry plant strains were observed to adhere at short contact times with higher cell counts than nonpersistent strains, and the persistent ice cream plant strain also adhered with higher numbers than most of the strains categorized as nonpersistent, which suggests that adherence to stainless steel surfaces after short contact times may have an effect on persistent plant contamination.

Adherence to surfaces is of great relevance in the success of cleaning and disinfection (5). Efficient adherence at an early stage gives the cells an opportunity to adhere more firmly before the cleaning and disinfection procedures take place. Strains that adhere with lower cell counts are easier to destroy (13).

Two *L. monocytogenes* strains were found to have persisted in poultry plants for at least 2 years, whereas other strains were recovered sporadically. At the ice cream plant, one strain persisted for 7 years. It is probable that these prolonged contaminations were due to factors affecting the survival of strains in the food processing environment rather than continuous contamination by incoming raw materials. This theory is supported by the fact that persistent strains were not found in the raw materials during the sampling period (1, 3, 10, 17). Eradication of the persistent *L. monocytogenes* strains from the food processing plants re-

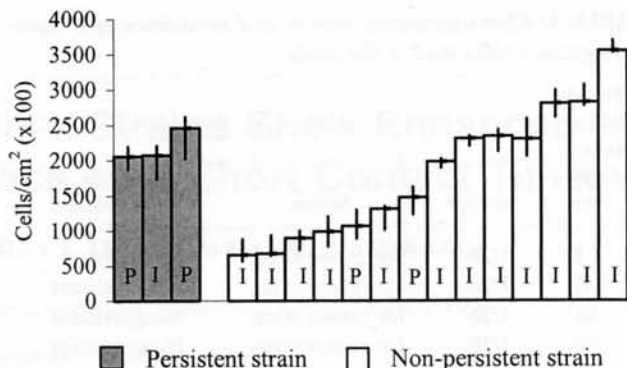


FIGURE 3. Adherence of persistent and nonpersistent *L. monocytogenes* poultry plant strains (P) and ice cream plant strains (I) to stainless steel surface after 72 h. Tests were made in triplicate.

quired improved and targeted cleaning and disinfection (1, 10).

Strains of serotype ½c were observed to adhere with the highest cell numbers. This observation was also made by Norwood and Gilmour (12). Strains of serotype ½c seem to have qualities that provide them with good adhering abilities. Serotype ½c differs in flagellar antigen from other serotypes in this study. Flagella have been shown to have an effect on initiation of adherence of *Pseudomonas aeruginosa* and *Escherichia coli* to surfaces (14, 15). The nonmotile strain of serotype ½ expressed the poorest adherence of all strains at short contact times. It adhered, however, with high cell numbers at long contact times, which suggests that flagellar-induced motility is important mainly at the initiation of adherence. Strains of serotype ½b showed variation in adhering cell counts. The persistent strain showed high adhering cell counts, and most of the nonpersistent strains showed lower adhering cell counts. It is obvious that qualities affecting adherence that are not distinguished by serotyping exist.

L. monocytogenes strains obtained from the ice cream plant expressed variation in adherence at short contact times. The persistent strain adhered with higher cell counts than most of the nonpersistent strains. Nevertheless, two nonpersistent *L. monocytogenes* strains adhered with high cell counts. It is possible that each cleaning and disinfection procedure is not equally effective, influencing the survival of *L. monocytogenes*. The resistance of strains to disinfectants is important in their survival. The resistance of strains to disinfectants is influenced, however, by adherence to surfaces (5, 16). Time of entry of the strain and amount and distribution in the plant may affect its survival. Cells that are acted on with effective measures of cleaning and disinfection soon after their entry are likely to be destroyed despite enhanced adherence. In contrast, cells that are allowed to adhere for some hours may survive the stress caused by cleaning and disinfection, especially if cleaning is insufficient. Locations that are difficult to clean provide cells with protection and time to establish.

Adherence at 72 h was 100- to 1,000-fold higher than at short contact times. A high adherence level at long contact time is important in the resistance against stress factors

in food processing plants. However, reasons leading to persistent plant contamination cannot be explained by adherence of long contact time because half the nonpersistent strains reached adherence levels similar to those of the persistent strains at 72 h. Furthermore, some nonpersistent strains that were adhering poorly at short contact times were observed to adhere better at long contact time than most of the other strains. These observations indicate that there are factors intrinsic to *L. monocytogenes* strains that affect adherence to surfaces differently at short versus long contact times. The flagella seem to affect adherence mainly at short contact times.

To conclude, the enhanced adherence shown by persistent *L. monocytogenes* strains at short contact times facilitates their survival on food processing surfaces. This may have an effect on the initiation of persistent plant contamination.

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