BIOFILM FORMATION BY *LISTERIA MONOCYTOGENES* IN FRESH BEEF DECONTAMINATION WASHINGS

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Background

Listeria monocytogenes is a foodborne pathogen of major concern since it is ubiquitous, able to proliferate under refrigeration temperatures, and has been isolated from food processing environments. The highly publicized, fatal outbreaks of foodborne disease as a result of contamination with *L. monocyotgenes*, especially with postprocessing contaminated meat products (CDC, 1999), has increased consumer and industry awareness and drawn much attention to the need for improved control measures during meat production and processing. Meat processing plants in the U.S. employ many meat decontamination strategies including the application of various organic acids and/or hot or cold water solutions (Bacon et al., 2000). The runoff and aerosol dispersion from these wash applications may settle and collect on food contact surfaces of equipment, providing an environment favorable for colonization and proliferation of bacterial contamination that may have been present on the carcass, leading to possible attachment and biofilm formation. Wong (1998) indicated that *L. monocytogenes* is a bacterium that has an exceptional ability to form biofilms on various kinds of surfaces used in the production of food processing equipment. The attachment and subsequent biofilm formation of bacteria on food contact equipment surfaces in food processing environments is a significant potential source of contamination. Upon de-attachment, such biofilms may serve as sources of microorganisms resulting in food spoilage or transmission of disease.

Objectives

The objectives of this study were to: (i) determine the effect of inoculation level (10^2, 10^5, 10^7 CFU/ml) on the level of attachment and biofilm formation of *L. monocytogenes* strain N-7144/rif+ to stainless steel surfaces under a typical processing plant abuse temperature (15°C), and (ii) investigate the level of attachment of inoculated (10^5 CFU/ml) acid-adapted or nonadapted *L. monocytogenes* strain N-7144/rif+ to stainless steel coupons at four different processing plant abuse temperatures (5, 15, 25, 35°C).

Methods

Meat decontamination washings were prepared by spraying individual portions (approximately 2 kg) of beef top rounds with water (10 or 85°C) in a simulated spray-washing apparatus (CHAD CO., Olathe, KS). The water (10 or 85°C) washings were then mixed at a ratio of 1:1 to obtain composite water washings (Samelis et al., 2001). Fifty milliliter aliquots of the composite water washings were distributed into sterilized centrifugation tubes (50 ml, 28.5 x 104 mm (O.D. x L); Nalgene) containing individual stainless steel coupons in an upright position. For the first investigation, composite water washings containing the stainless steel coupons (5cm x 2cm; 0.08 mm thickness) were inoculated (0.5 ml) with an overnight culture (tryptic soy broth with 0.6% yeast extract, TSBYE) of *L. monocytogenes* strain N-7144/rif+ to obtain inocula of 10^2, 10^5, 10^7 CFU/ml and the tubes were incubated, statically, at 15ºC, for up to 7 days. For the second investigation, composite water washings containing the stainless steel coupons were inoculated (10^5 CFU/ml) with either an acid-adapted [previously grown in glucose-free tryptic soy broth plus 0.6% yeast extract (TSBYE-G) with 1% added glucose (TSBYE+G) at 30°C for 24 h] or nonadapted (previously grown in TSBYE-G) inocula of *L. monocytogenes* strain N-7144/rif+ and the tubes were incubated, statically, at 5, 15, 25 or 35°C for up to 7 days. Determination of microbial populations present and pH measurements of samples was done at 0, 2, and 7 days post-inoculation. To determine the level of attachment, individual stainless steel coupons containing attached cells were removed (2 and 7 days) from the associated tubes with the inoculated washings and were thoroughly rinsed with sterile distilled water to remove unattached cells. The coupons were then placed into 40 ml of sterile 20 mM PBS (pH 7.2) in centrifugation tubes (50 ml, 28.5 x 104 mm (O.D. x L); Nalgene) containing ten glass beads to aid in de-attachment of cells from the surface. Cells were de-attached from the surfaces of the coupons into suspension by vortexing the tubes containing the coupons for 2 min. Cells in suspension were obtained (0, 2, and 7 days) by removing a volume of the washings in which the coupons were suspended. For microbiological analysis, 1 ml of each sample was serially diluted using 9 ml of sterile 0.1% buffered peptone water (BPW) (Difco) and the appropriate dilutions were plated onto duplicate agar plates. Tryptic soy agar with 0.6% yeast extract (TSAYE) was used to determine total bacterial population counts, while TSAYE supplemented with 100 µg/ml of rifampicin (Sigma Chemical Co., St. Louis, MO) (TSAYE+Rif) was used for the selective enumeration of the inoculated *L. monocytogenes*. Colonies were manually counted following incubation at 30ºC for 48 h. Two replicate experiments were conducted, with two samples evaluated per replicate (n=4). Microbial counts were transformed to logarithms before computing means and standard deviations and counts were reported as log CFU/cm^2 for attached cells and log CFU/ml for cells in suspension. The detection limit was 1.3 log CFU/cm^2 and 1.0 log CFU/ml for detection of attached cells and cells in suspension, respectively.

Results and Discussion

In one phase of this study, stationary-phase cells of a *L. monocytogenes* culture were inoculated at three different levels (10^2, 10^5, 10^7 CFU/ml) in fresh beef water decontamination washings and stored for 7 days at 15°C. The reason for studying the different levels of inoculation was to evaluate the effect which different loads of naturally contaminated *L. monocytogenes* would have on the rate of biofilm formation and the competitive response the level of inoculation would offer against naturally occurring microflora. The temperature of 15°C chosen for evaluating the effect of the inoculation levels was to simulate a common processing plant abuse temperature as well as to evaluate the influence of the competitive flora on *L. monocytogenes* at a non-optimal growth temperature as would be expected in food processing facilities. Under the conditions of the study, *L. monocytogenes* attached to the stainless steel with varied efficacy for the different inoculation levels. The results were consistent in revealing the lower (1.0 to 2.2 log CFU/cm^2) attachment of the pathogen (TSAYE+Rif) observed at the inoculation level of 10^2 CFU/ml as compared with that at the two higher inoculation levels (Table 1). The difference in attachment among incoulum levels was highest at day-2, while attachment increased slightly (3.3-3.5 log CFU/cm^2) from day-2 to day-7 at the inoculation level of 10^2 CFU/ml and the opposite behavior was displayed by the two higher inoculation levels (Table 1). The decrease (from 5.1 to 4.7 and from 7.0 to 4.8 log CFU/cm^2) in pathogen cells in the attached state from day-0 to day-7 for the inoculation levels of 10^5 and 10^7 CFU/ml, respectively, is possibly explained by the increased attachment (5.6-6.3 log CFU/cm^2) of the total microbial cells (TSAYE) and the effect which nutrient competition might have had at the later stage of storage, with the increased number of bacteria present at that stage (Table 1). The increase in attached pathogen (TSAYE+Rif) populations at day-7 for the inoculation level of 10^2 CFU/ml may be due to the fact that the number of total bacteria (TSAYE) present were low initially and thus had more available nutrients at the later stage of storage to still increase in numbers. As expected growth of the pathogen (TSAYE+Rif) in suspension was much lower (1.8 to 2.4 log CFU/ml) for the inoculation

level of 10^2 CFU/ml as compared with that of the higher inoculation levels, which would account for the level of attachment at the inoculation level of 10^2 CFU/ml being lower than the attachment at the higher inoculation levels (Table 1). Pathogen populations suspended in the washings inoculated with 10^2 CFU/ml increased from 2.2 log CFU/ml at day-0 to 5.3 log CFU/ml by day-2 and remained unchanged through day-7, while in the washings inoculated with 10^5 and 10^7 CFU/ml, pathogen levels reached 7.2-7.3 logs by day-2 and remained unchanged throughout storage (data not shown).

In the second phase of the study, stationary phase cells of acid-adapted or nonadapted *L. monocytogenes* culture were inoculated (10^5 CFU/ml) in fresh beef water decontamination washings and stored for 7 days at four different temperatures (5, 15, 25, 35ºC). The temperatures of growth and attachment evaluated are all representative of potential processing plant and storage abuse temperatures in food processing facilities. Attachment of the inoculated *L. monocytogenes* was highest at 15 and 25ºC (4.6-4.8 log CFU/cm2) by day-2, while the attachment at 5 and 35°C was approximately 1.0-1.5 logs lower (Table 2). The higher attachment observed at 15 and 25ºC by day-2, as compared with that at 5ºC, was expected since we know that *L. monocytogenes* is able to grow at refrigeration temperatures, although its growth rate is slower under these conditions. The indication that attachment at day-7 at 35ºC incubation was lower (1.1-1.2 logs) than that at the other three temperatures is an interesting finding, considering that the level of the pathogen was between 6.2 to 6.5 log CFU/ml in suspension by day-2 (Table 2). This would lead us to believe that 35ºC is not an optimal temperature for biofilm formation by *L. monocytogenes*, even though the rate of growth in suspension (data not shown) was not suppressed at this temperature. The pathogen counts (TSAYE+Rif) decreased from day-2 to day-7 at 15, 25 and 35ºC, while those at 5ºC increased slightly by day-7, which is possibly a result of nutrient competition and subsequent competitive exclusion of the pathogen by the natural flora. By day-7 the attachment of the pathogen observed at 5ºC was not different from that at 15 and 25ºC, as the decrease in attachment at the higher temperatures was met by the increase in attachment seen at 5ºC (Table 2). The inoculated (5 logs) pathogen in suspension of washings stored at 5°C decreased to 3.8 logs by day-2 and was unchanged throughout storage, while in the washings stored at 15 and 25°C the pathogen decreased gradually from 4.6-4.8 at day-2 to 3.9-4.0 at day-7 and in washings stored at 35°C counts decreased to 3.3 and 2.8 logs by day-2 and day-7, respectively (data not shown).

Conclusions

The data indicate a consistent trend of what appears to be a "maximum limit" of attachment, a phenomenon revealing that the inoculation levels of 10^5 CFU/ml and 10^7 CFU/ml did not differ in the populations of bacteria attached to stainless steel at the incubation temperature of 15ºC in meat decontamination washings. Temperature ranges for optimal attachment and biofilm formation of *L. monocytogenes* to the stainless steel was observed to be at 15 and 25ºC. The influence of the natural meat microflora was more pronounced at higher temperatures (> 25ºC) of incubation and was responsible in part for the suppressed growth and attachment of the pathogen resulting in a decrease of *L. monocytogenes* presence on the stainless steel surface. These findings indicate that temperatures of 15 or 25°C in meat processing plants may be the "hot spots" or niches where *L. monocytogenes* presence is expected and where it will colonize and form biofilms given favorable combinations of contamination loads and environmental temperatures. Additional studies are in progress to develop sanitation strategies for prevention of formation or inactivation of *L. monocytogenes* biofilms formed in residual meat decontamination washings in meat plants.

Pertinent literature

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Table 1. Changes (mean log CFU/cm^2 ± SD; n=4) in attachment to stainless steel coupons of the total flora (TSAYE; Tryptic Soy Agar supplemented with 0.6% yeast extract) and the populations of inoculated (10^2, 10^5, 10^7 CFU/ml) *Listeria monocytogenes* strain N- 7144/rif+ (TSAYE+Rif; TSAYE supplemented with 100mg/l of rifampicin) in fresh beef decontamination washings stored at 15°C for 0, 2 or 7 d.

TSAYE			TSAYE+Rif		
day-0 (log CFU/ml)	day-2	day-7	day-0 (log CFU/ml)	day-2	day-7
2.6 (0.1)	2.6 (0.1)	2.6 (0.1)	2.2 (0.1)	3.3 (0.1)	3.5 (0.1)
5.5 (0.2)	5.5 (0.2)	5.5 (0.2)	5.1 (0.3)	5.2 (0.4)	4.7 (0.2)
7.5 (0.5)	7.5 (0.5)	7.5 (0.5)	7.0 (0.3)	5.5 (0.3)	4.8 (0.1)

Table 2. Changes (mean log CFU/cm^2 ± SD; n=4) in attachment to stainless steel coupons of the total flora (TSAYE; Tryptic Soy Agar supplemented with 0.6% yeast extract) and the populations of inoculated (10^5 CFU/ml) acid-adapted *Listeria monocytogenes* N- 7144/rif+ (TSAYE+Rif; TSAYE supplemented with 100mg/l of rifampicin) in fresh beef decontamination washings stored at 5, 15, 25 or 35°C for 0, 2 or 7 d.

Temperature of storage (ºC)	TSAYE			TSAYE+Rif		
	day-0 (log CFU/ml)	day-2	day-7	day-0 (log CFU/ml)	day-2	day-7
5	5.4 (0.2)	4.8 (0.1)	6.6 (0.2)	5.1 (0.3)	3.8 (0.3)	3.9 (0.2)
15	5.4 (0.2)	5.6 (0.2)	6.5 (0.2)	5.1 (0.3)	4.8 (0.1)	3.9 (0.3)
25	5.4 (0.2)	5.6 (0.1)	6.7 (0.1)	5.1 (0.3)	4.6 (0.3)	4.0 (0.1)
35	5.4 (0.2)	5.5 (0.4)	6.4 (0.3)	5.1 (0.3)	3.3 (0.2)	2.8 (0.1)