

## Improved Recovery of Viable *Listeria monocytogenes* From Stainless Steel Surfaces for Subsequent Detection

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### Abstract

The contamination of food contact surfaces in the food manufacturing environment provides an important avenue for dissemination of pathogenic bacteria such as *Listeria monocytogenes* to food products. Bacteria adhered as biofilms are particularly problematic and may not be effectively recovered by standard swabbing techniques, leading to under-estimation of the contamination status of surfaces. Using stainless steel coupons as a model surface, we examined the efficacy of biofilm-dispersing agents in improving the recovery of *L. monocytogenes* for subsequent detection by culture techniques. A combination of pectinase and Tween 20 produced an approximately four-fold improvement in the recovery of *L. monocytogenes* originally deposited under biofilm forming conditions. We propose that the reliability of current surface swabbing techniques in the detection of persistent microbial surface contaminants can be significantly enhanced through the incorporation of biofilm-dispersing agents in the sampling method.

**Key words:** *Listeria monocytogenes*, biofilm, stainless steel surface, recovery, detection.

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### Introduction

*Listeria monocytogenes* is the causative agent of listeriosis, a potentially fatal foodborne illness (Mead et al., 1999 ; Swaminathan and Gerner-Smidt, 2007). The ability of this organism to contaminate food contact surfaces and survive in the manufacturing environment increases the risk of food product contamination, with possible serious public health consequences. Routinely monitoring the food manufacturing environment for the presence of pathogenic bacteria such as *L. monocytogenes* is a key element in preventing the contamination of food products. The occurrence of persistent environmental contamination may be related to the capacity of some strains to form biofilms (Jay et al., 2005), which involves tight adherence of the cells to surfaces. The adhesion of cells to surfaces is believed to involve hydrophobic and ionic interactions of the cells with the surface (Di Bonaventura et al., 2007), as well as the formation of an extracellular polymeric substance (EPS) matrix composed of lipids, proteins and polysaccharides in which the cells are enmeshed (Annous et al., 2009).

The standard procedure prescribed by the Health Canada

Compendium of Analytical Methods for sampling food contact and other surfaces for subsequent microbiological analysis involves swabbing with a sponge pre-moistened with a neutralizing buffer (Microbiological Methods Committee, 2010). However, the recovery efficiency of this approach may be poor especially if the bacterial cells are adhered as a biofilm (Jay et al., 2005; Kang et al., 2007), hampering verification of the contamination status of surfaces. It is presently hypothesized that the recovery of surface-adhered bacteria such as *L. monocytogenes* by swabbing might be improved through the incorporation of a biofilm-dispersal treatment which preserves cell viability for downstream microbiological analysis using standard culture techniques.

A variety of agents have been reported to have biofilm-dispersing capability, including polysaccharide-degrading enzymes such as pectinase (Lequette et al., 2010) and Dispersin B (Kaplan et al., 2004), D-amino acids (Kolodkin-Gal et al., 2010) and DNase (Hall-Stoodley et al., 2008). To examine the applicability of biofilm-dispersing agents in food microbiology testing, we studied the effect of pectinase and a mild detergent (Tween 20) on the quantitative recovery of viable *L. monocytogenes* cells from biofilms deposited on model stainless steel surfaces.

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## Material and Methods

**Preparation of *L. monocytogenes* biofilms on stainless steel surfaces.** The method of Leriche and Carpentier (2000) was adapted for the preparation of *L. monocytogenes* biofilms on circular stainless steel chips (5 mm in diameter, 2 mm thickness, type 304, no. 4 finish) and stainless steel square plates (4 cm x 4 cm, 2 mm thickness, type 304, no. 4 finish). Prior to use the stainless steel surfaces were washed in 95% ethanol for 10 min then rinsed 5 times with sterile distilled water and autoclaved at 121°C for 10 min. Biofilms were prepared using *L. monocytogenes* (biofilm forming) strain KB (Kane Biotech, Winnipeg, Manitoba) grown in Brain Heart Infusion (BHI) broth at 30°C for 24 h. A 100 µL portion of enrichment broth culture was transferred to a 1.5 mL microfuge tube and centrifuged at 10 000 g for 10 min. The pellet was re-suspended in 1.0 mL of sterile 0.01 mol L<sup>-1</sup> phosphate-buffered (pH 7.2)/0.85% (w/v) NaCl (PBS), followed by centrifugation and re-suspension in sterile PBS as above. A stainless chip was placed smooth side up in a 2 mL-capacity microfuge tube and covered with 100 µL of washed cell suspension, then incubated at room temperature for 3 h in the dark. The chips were washed 10 times by gentle vortexing in 500 µL of PBS, and the washed chips were then overlaid with 100 µL of BHI diluted 1:10 in sterile distilled water, followed by incubation at room temperature for 2 days in the dark. The chips were then washed by holding with forceps over a receptacle and squirting a gentle stream of sterile PBS on each side for 20 s. These biofilm-coated chips were used immediately in the recovery experiments described below. For biofilms formed on stainless steel squares, the preceding protocol was used with the exception that 1.0 mL of washed cell suspension was used to overcoat the squares placed in a sterile petri dish, and all washes were carried out under a gentle stream of sterile PBS over a receptacle.

**Recovery of *L. monocytogenes* from stainless steel chips.** Biofilm coated stainless steel surfaces were subjected to a variety of treatments for recovery of *L. monocytogenes* cells, and the efficacy of the treatments was evaluated by performing aerobic plate counts of the eluates. Acidic phosphate buffer (APB; 0.05 mol L<sup>-1</sup> phosphate/0.85% (w/v), adjusted to pH 5.0 with HCl) was used as diluent in the preparation of all treatment solutions. The pectinase stock solution used in these experiments is commercially available as Pectinex Ultra SP-L (≥3800 units/mL)(Sigma Aldrich, St. Louis, MO). For each trial, one biofilm-coated chip was placed in the bottom of a 2 mL-capacity microfuge tube and covered with 200 µL of treatment solution (APB with or without the treatment agents: 380 units/mL of pectinase or 0.01% [v:v] Tween 20), then incubated at room temperature for 30 minutes in the dark. After gently vortexing, the supernatant fluid was removed and serially diluted in sterile PBS followed by spread plating 100 µL of each dilution on BHI agar. After

incubation at 30°C for 48 h the number of colonies on each plate was recorded. Each treatment was repeated three times and the enumeration data subjected to statistical analysis using SAS software (SAS Institute Inc., Cary, NC). **Recovery of *L. monocytogenes* from stainless steel squares by swabbing.** Each biofilm-coated stainless steel square was placed in a sterile petri plate and covered with 1.0 mL of treatment solution, then incubated at room temperature for 30 minutes in the dark. A sterile SpongeSicle swab (3M Canada, London, Ontario) pre-moistened in 10 mL of peptone water was used to swab the surface of the square by rubbing each side of the sponge head for 10 seconds. The swab was then placed in a stomacher bag and stomached in 20 mL of sterile PBS (30 seconds at speed 3) using an Interscience BagMixer (Saint Nom, France). The homogenate was serially diluted and plated on BHI agar as described above. Each treatment was repeated three times and the enumeration data subjected to statistical analysis using SAS software as above.

## Results

The efficacy of biofilm-dispersing agents in enhancing the recovery of *L. monocytogenes* from stainless steel surfaces was studied using a model system in which a known biofilm forming strain was deposited on uniform stainless steel chips. Since the basis for the adherence of biofilm-deposited bacterial cells generally involves entrapment within an EPS matrix, the treatment options focused primarily on the application of solutions containing pectinase and the mild detergent Tween 20 to disrupt the EPS. In the interest of devising a treatment regimen which is compatible with a typical microbiological sampling operation in a food manufacturing setting, treatment times were limited to 30 minutes under ambient conditions (which in our laboratory averaged 22 °C during the period of this study). Of paramount importance to the subsequent detection of the released bacteria by standard culture techniques is the preservation of cell viability during the treatment. Suspending *L. monocytogenes* strain KB in solutions of pectinase (380 units/mL) and Tween 20 (0.01%) in APB for 30 min had no measurable impact on the viability of the bacteria as determined by viable counts on plating media (Not shown).

Biofilm-coated chips were treated by covering with solutions of biofilm-dispersing agents and incubating for 30 minutes, followed by measuring bacterial recovery in the eluate by performing viable counts on plating media. The use of pectinase alone produced an approximately two-fold higher recovery of *L. monocytogenes* cells from the chips compared to treatment with APB devoid of agent (Table 1). On the other hand, Tween 20 used alone did not significantly improve the recovery of *L. monocytogenes* cells.

Table 1. Effect of pectinase and Tween 20 on the recovery of *L. monocytogenes* cells from stainless steel chips<sup>a</sup>.

Treatment	Cells recovered (cfu/mL x 10 <sup>6</sup> )
None	1.8 ± 0.2
Tween 20	2.2 ± 0.2
Pectinase	4.2 ± 0.5
Tween 20 and pectinase	6.6 ± 0.7

<sup>a</sup>Treatments included the use of pectinase (380 units/mL) and Tween 20 (0.01 %, v:v) either individually or in combination, or APB alone (no agent). Each determination was done in triplicate (results expressed as mean cell density ± standard deviation). Results of the pectinase treatments (with and without Tween 20) were significantly different from the control ( $P \leq 0.05$ ).

However, when pectinase was combined with Tween 20 a significant improvement was observed in the recovery of *L. monocytogenes* over treatment with pectinase alone, with an almost 4-fold improvement in recovery over treatment with buffer devoid of agent.

The preceding results suggest that an application of pectinase in combination with Tween 20 significantly enhanced the recovery of biofilm-adsorbed *L. monocytogenes* from small-diameter chips. To demonstrate the applicability of this approach in the recovery of *L. monocytogenes* from large surfaces by swabbing, we prepared biofilm-coated stainless steel plates with sufficient surface area to permit swabbing using a typical sponge device marketed for environmental sampling in microbiological analysis. Treatment solution consisting of either pectinase diluted in APB containing 0.01 % Tween 20, or APB devoid of biofilm-dispersing agents, was applied by spreading over the surface of the plate and allowing to sit for 30 minutes. The surface was then swabbed with a sampling sponge, and the recovered bacteria eluted by stomaching in buffer, followed by performing aerobic plate counts of the resulting suspension. Pre-treatment of the surface using a combination of pectinase and Tween 20 resulted in an approximately 4-fold increased recovery compared to pre-treatment with buffer alone (Table 2), a result which mirrors the recovery efficiencies observed with the disc model.

Table 2. Effect of combined pectinase and Tween 20 on the recovery of *L. monocytogenes* cells by swabbing<sup>a</sup>.

Treatment	Cells recovered (cfu/mL x 10 <sup>6</sup> )
None	2.1 ± 0.2
Tween 20 and pectinase	8.1 ± 0.8

<sup>a</sup>Treatments consisted of the application of pectinase (380 units/mL) combined with Tween 20 (0.01 %, v:v) or APB alone (no agent). This was followed by swabbing with a sampling sponge and performing viable counts of recovered cells. Each determination was done in triplicate (results expressed as mean cell density ± standard deviation). Results of the pectinase treatments (with and without

Tween 20) were significantly different from the control ( $P \leq 0.05$ ).

We also examined the efficacy of other agents reported to exhibit biofilm-dispersing properties. These included D-amino acids (D-tyrosine, D-tryptophan, D-alanine, D-leucine and D-methionine), either individually at 1 mM in APB or in combination, and DNase I at 2 units. None of these agents had any impact on the viability of cells suspended in solutions with the aforementioned concentrations, but neither did their application to biofilm-coated chips produce significant increases in the recovery of *L. monocytogenes* compared to the use of APB alone (not shown).

## Discussion

While previous work focused primarily on the use of polysaccharidases as a means of eliminating biofilms from surfaces in food processing environments (Lequette et al., 2010), the results of the present studies suggest that pectinase used in combination with a mild detergent is highly effective for the recovery of *L. monocytogenes* in viable form for subsequent microbiological analysis using standard culture techniques. Pectinase is a food grade mixture of enzymes with predominantly polygalacturonase activity which may act by breaking down the polysaccharide components of the EPS matrix. Tween 20 is a mild detergent which may contribute to destabilization of the EPS matrix by disrupting hydrophobic interactions between constituent lipid components, and also, perhaps, between cells and the adsorbent surface. The use of these agents in a food manufacturing setting in environmental sampling procedures is further supported by their low cost, ease of application and their innocuous nature (i.e., lack of toxicity).

The reason for the recovery of significant quantities of cells from stainless steel surfaces subjected to the control treatments (buffer devoid of biofilm-dispersing agents) is not known. Perhaps the biofilms formed under the present conditions were comprised of both loosely and tightly adhered cell aggregates, with the former shedding readily in the buffer, and the latter requiring active degradation of the EPS for release. On the other hand, continual sloughing of cells may be a natural feature of biofilms.

One possible approach for food microbiology testing purposes is to apply these agents on surfaces using a simple spray bottle, waiting 30 min, and then swabbing the treated area with a sampling sponge in the usual manner. While the 30 minute timeframe may not be suitable for some food manufacturing settings, the advantage of increased likelihood of recovery (and subsequent detection) of surface-bound pathogens such as *Listeria monocytogenes* warrants consideration. Further work will be required to investigate whether other biofilm-dispersing agents can provide enhanced recovery of surface-adhered bacteria for

food microbiology testing purposes. In this work stainless steel coupons were used as a model, though other types of surfaces made from different materials are also prevalent in food manufacturing environments, and more work will be required to determine the efficacy of the present treatment regimen with a broader variety of surfaces.

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