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The effect of mild preservation treatments on the invasiveness of different *Listeria monocytogenes* strains on Greenshell™ mussels

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ABSTRACT

High pressure processing (HPP) and thermal treatments are food preservation technologies used by the food industry to inactivate *Listeria monocytogenes*. However, the safe shelf-life of treated products could be compromised by the presence and/or recovery of surviving bacteria. The aim of the present study was to evaluate the effect of these technologies on the invasion capacity of *L. monocytogenes*. Eleven *L. monocytogenes* strains (serovars 1/2a,1/2b,1/2c and 4b) isolated from food processing plants and food products were grown in culture media, associated with Greenshell™ mussel surfaces and subjected (or not) to HPP (350 MPa, 2 min) or mild heating (MH, 55 °C, 10 min). The invasiveness of the strains was compared immediately after processing and after 14 days of storage at 8 °C using human intestinal epithelial Caco-2 cells. All strains on mussels had decreased invasion capacity compared with those coming directly from culture media. Invasiveness of L. *monocytogenes* also decreased immediately after pressurization but returned to the rate in untreated samples after 14 days of refrigerated storage. In contrast, MH did not affect invasion capacity, and treated samples showed the same rates as non-MH treated ones. Lineage I strains were more invasive than lineage II strains after both mild processing treatments. Based on these results, the risk of *L. monocytogenes* infection by the consumption of treated mussels will not be increased, as the invasiveness of possible survivors is not increased.

1. Introduction

Listeria monocytogenes is the causative agent of listeriosis, a foodborne disease that can be life threatening to at-risk populations, such as immunocompromised people, pregnant women, children or the elderly. The pathogen is of great concern for the food industry, as consumption of contaminated food is the main mode of transmission to humans (Todd & Notermans, 2011). In 2010, *L. monocytogenes* was estimated to have infected 23,150 people worldwide, accounting for 5463 deaths (23.6%) (de Noordhout et al., 2014).

L. monocytogenes can be detected in a wide variety of food products (Farber & Peterkin, 1991) although the increase in the consumption of ready-to-eat (RTE) products in recent years makes this pathogen a serious threat, as it can survive a wide range of environmental stress conditions, such as low temperature, acidic pH and high osmolarity (Sue, Fink, Wiedmann, & Boor, 2004). Raw materials are one source of potential *L. monocytogenes* contamination for seafood products (Eklund et al., 1995). However, because of the ability of *L. monocytogenes* to form biofilms and the long-term persistence of some strains, the processing plant environment can also play an important role (Rørvik, Aase, Alvestad, & Caugant, 2000; Rørvik, Caugant, & Yndestad, 1995; Warriner & Namvar, 2009). A study investigating the prevalence and biofilm-forming ability of *L. monocytogenes* in New Zealand mussel-processing plants confirmed the presence of the pathogen in raw and processed products, and the importance of cross-contamination from external and internal environments (Cruz & Fletcher, 2011). In 1992, an outbreak of listeriosis from Greenshell™ mussels (hot-smoked, vacuum-packed) was reported in New Zealand, involving four cases and two deaths (Brett, Short, & McLauchlin, 1998).

The virulence of *L. monocytogenes* varies among different strains (Liu, Ainsworth, Austin, & Lawrence, 2003; Roche et al., 2003).

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Additionally, bacteria have been shown to modify their metabolism in response to stress and to alter their virulence (Wałecka, Molenda, Karpíšková, & Bania, 2011). *L. monocytogenes* had increased *in vitro* virulence in Caco-2 cells when exposed to acid (Conte et al., 2000; Olesen, Vogensen, & Jespersen, 2009) and salt stress (Garner, James, Callahan, Wiedmann, & Boor, 2006; Olesen et al., 2009). In relation to temperature, Garner et al. (2006) and Loepfe, Raimann, Stephan, and Tasara (2010) observed a significant reduction in the invasiveness of cold-stressed (7 °C) *L. monocytogenes,* whereas Conte et al. (1994) did not observe any difference in *in vitro* invasion capacity of *L. monocytogenes* grown at 37 °C, 25 °C and 4 °C. Although all these studies investigated food-related stresses, they were performed in laboratory media, which may not reflect the behavior of the pathogen in food matrices. In this regard, studies performed on fermented sausages and cured cooked ham (Larsen, Koch, & Ingmer, 2010), RTE turkey meat (Lin, Wang, Tsai, & Chou, 2010), rillettes extract (Midelet-Bourdin et al., 2006) and milk (Pricope-Ciolacu, Nicolau, Wagner, & Rychli, 2013) showed that environmental factors (physicochemical properties of the food product/culture media, storage temperature, etc.) highly affect the *in vitro* virulence of *L. monocytogenes*.

Greenshell mussels (*Perna canaliculus*), which are found only in New Zealand, are one of the world's most successful farmed mussel species, and their processing usually includes a heating step (blanching) which contributes to food safety (Bremer & Osborne, 1997). However, overheating of the mussel flesh causes a decrease in the product's properties, affecting yield, texture, color and shelf life (Boyd & Wilson, 1978). Moreover, because of the high processing costs of blanching (Fletcher, Youssef, & Gupta, 2008), alternative processing technologies such as High Pressure Processing (HPP), which inactivates microorganisms while better preserving organoleptic properties of the food products, have been considered (Campus, 2010).

From a food safety perspective both heat and HPP treatments have to be thoroughly assessed. Small changes in the core temperature of the product during a thermal treatment have a significant effect upon the rate at which contaminating *L. monocytogenes* cells are killed (Bremer & Osborne, 1995). In relation to HPP treatment, it is known that bacteria can be protected during the process when associated with seafood compared with laboratory media (Bremer, Osborne, Kemp, van Veghel, & Fletcher, 1998; Smiddy et al., 2005). *L. monocytogenes* cells that were not inactivated by either heat or HPP treatments could grow during refrigerated storage and be a threat for consumers. Thus, sub-lethally injured cells should be carefully studied in relation to their virulence and potential for causing illness (Van Boeijen, Moezelaar, Abee, & Zwietering, 2008). The effect of HPP treatment (350 MPa) on *L. monocytogenes* in Greenshell mussels has been studied (Fletcher et al., 2008), but to date, no study has been published investigating the effect of HPP on the virulence of sub-lethally injured *L. monocytogenes* in these mussels. The aim of this study was to assess the effect of the food matrix (culture media *vs*. mussel meat) and the processing treatment (HPP and heat) on the virulence potential of *L. monocytogenes* strains, belonging to different serotypes and PFGE types, based on their ability to invade human epithelial Caco-2 cells.

2. Materials and methods

2.1. Characterization of the strains by pulsed-field gel electrophoresis (PFGE)

PFGE of *L. monocytogenes* isolates used in this study (Fig. 1) was performed using a modified US Center for Disease Control and Prevention (CDC) Pulsenet protocol (Graves & Swaminathan, 2001). Selected isolates belong to serotypes 1/2b and 4b (lineage I) and 1/2a, 1/2c (lineage II), the most frequently reported in human listeriosis cases (Paul et al. 2014). In brief, isolates were grown in Brain Heart Infusion (BHI) (Difco, Becton, Dickinson and Co., Sparks, MD, USA) agar plates for 24 h at 37 °C. A fresh bacterial suspension was prepared in Tris-EDTA buffer (Sigma-Aldrich) with absorbance (610 nm) adjusted to 0.48–0.52 (MicroScan Turbidity Meter, Siemens, West Sacramento, CA, USA). Bacterial cultures were embedded in 1.2% Seakem Gold agarose (Lonza, Rockland, ME, USA). The plugs formed were lysed for 2 h at 54 °C and washed with Tris-EDTA (Sigma-Aldrich) buffer. One-third of the plug size was then separately digested with 25 units of *Asc*I (New England Biolabs, Beverly, MA, USA) for 4 h at 37 °C or with 50 units of *Apa*I (Roche, Mannheim, Germany) for 4 h at 30 °C. Restriction fragments were separated by electrophoresis through 1% Seakem Gold agarose (Lonza) in 0.5% Tris-Borate-EDTA (Sigma-Aldrich) at 6 V/cm on a CHEF DR III system (Bio-Rad, Hercules, CA, USA). A linear ramping factor with pulse times from 4.0 to 40.0 s at 14 °C and 120 °C was applied for 18 h. *Xba*I (Roche)-digested plugs of *Salmonella enterica* serovar Braenderup (CDCH9812) were applied as molecular markers. After electrophoresis, PFGE gels were stained with ethidium bromide (Bio-Rad, NZ) and photographed using Kodak Electrophoresis Documentation and Analysis Systems 290 (EDAS) (Kodak, Rochester, NY, USA) software. Gel images were analyzed using Infoquest™ fingerprinting software (Bio-Rad) and visually checked by operator prior to assign a pattern designation. Similarity between fingerprints was determined by the Dice coefficient and using a band position tolerance of 1%. Dendrograms were generated by the unweighted pair group method with arithmetic mean (UPGMA). Combined pulsotypes (based on *Apa*I and *Asc*I pulsotypes) were identified with a unique number. Pulsotypes with three or fewer fragment differences were regarded as being closely related and in the same pulsogroup (Tenover et al., 1995). Results were compared with the New Zealand *L. monocytogenes* database (pers. comm., Brent Gilpin, ESR, Christchurch), and with results from Cruz and Fletcher (2011).

2.2. Preparation of inocula

Strains were stored at −80 °C on glass bead vials. Resuscitation of the cultures was done by adding one bead to 10 mL of BHI and incubation for 24 h at 37 °C with shaking (200 rpm, MaxQ 2000 orbital shaker, ThermoFisher, USA). *L. monocytogenes* strains were kept on blood agar (Columbia Sheep Blood Agar, Fort Richard, New Zealand) at 4 °C for use in all experiments. To prepare the inoculum, a loop of culture from an isolated colony was inoculated into 10 mL of BHI and incubated as described above. Exponential growth phase cells were obtained by adding 500 μL of an overnight culture in 10 mL of fresh BHI and incubation for *ca.* 3 h at 37 °C with shaking (200 rpm, MaxQ 2000 orbital shaker, ThermoFisher), to reach an optical density at 600 nm of 1–1.2. The estimated concentration of bacterial suspension was about 1×10^8–1×10^9 CFU/mL. Broth cultures were centrifuged for 5 min at 2150 × *g* and 20 °C, resuspended in 10 mL of salt peptone water (SPW, 0.1% Difco Peptone with 1% added NaCl) and used for mussel inoculation.

2.3. Mussel preparation and inoculation

Live Greenshell mussels (*Perna canaliculus*) were supplied by a local grower, brushed under tap water, hand shucked, trimmed to 10 ± 0.5 g each (if needed), weighed and stored at −80 °C in sealed foil pouches (CasPak). On the day of inoculation, mussel pouches were thawed under cold tap water for 20 min. Mussels were then individually distributed in new foil pouches (100 × 150 mm) and inoculated with two mL of a *L. monocytogenes* suspension. Pouches