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Initial adhesion of *Listeria monocytogenes* to fine polished stainless steel under flow conditions is determined by prior growth conditions

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ABSTRACT

Listeria monocytogenes is a food-borne pathogen known to persist in food production environments, where it is able to attach and form biofilms, potentially contaminating food products ready for consumption. In this study the first step in the establishment of *L. monocytogenes* in a food-processing environment was examined, namely the initial adhesion to stainless steel under specific dynamic flow conditions. It was found that the intrinsic ability of *L. monocytogenes* to adhere to solid surfaces under flow conditions is dependent on nutrient availability. The addition of L-leucine to the growth medium altered the fatty acid composition of the *L. monocytogenes* cells and increased adhesion. The growth conditions resulting in the highest adhesion (growth medium with added glucose) had cells with the highest electron donating and lowest electron accepting properties, whereas growth conditions resulting in lowest adhesion (growth medium with added mannose) had cells with the lowest electron donating properties and highest electron accepting properties. The highest and lowest adhesion conditions correlated with differences in expression of cell surface protein of *L. monocytogenes* and among these the autolysin amidase (Ami). This study implies that food composition influences the adhesion of *L. monocytogenes* to solid surfaces during dynamic flow conditions.

1. Introduction

One of the pathogens of particular concern in food processing environments is *Listeria monocytogenes,* that causes listeriosis in humans (Painter and Slutsker, 2007). Although cases of listeriosis are relatively rare; the mortality rate is high and at risk are immunocompromised, elderly and the foetus of pregnant women (Bhunia, 2008; Todd and Notermans, 2011). *L. monocytogenes* has an ability to persist in various food producing environments (Ortiz et al., 2010; Carpentier and Cerf, 2011). Strains have been found to persist from months to several years in pig slaughter houses and processing facilities (Ortiz et al., 2010), in fermented meat sausage production sites (Ferreira et al., 2011), in cheese production environments (Fox et al., 2011) and in fish slaughter houses (Wulff et al., 2006). It has recently been suggested, that it may not be strains of *L. monocytogenes* with unique properties that lead to persistence, but harborage sites in food industry premises and equipment, where cleaning is difficult and nutrients are available, that makes *L. monocytogenes* able to grow and persist (Carpentier and Cerf, 2011). The first step in *L. monocytogenes* establishment at a harborage site is its ability to attach to solid surfaces. Adhesion is a multifactorial process, and the degree of adhesion may be dependent on many factors such as solid surface properties and environmental conditions (Ploux et al., 2010). Some studies show that persistent strains of *L. monocytogenes* adhere to surfaces and form biofilms more easily than strains not associated with persistence, implying that adherence and biofilm formation on surfaces are important for persistence of *L. monocytogenes* in the food processing environment (Lundén et al., 2000; Norwood and Gilmour, 1999). If a biofilm is formed, it may protect microorganisms against cleaning and disinfection (Chavant et al., 2004) and allow pathogens like *L. monocytogenes* to establish in the food processing environment. Even a limited number of *L. monocytogenes* cells may eventually form biofilm potentially contaminating food products (Møretrø and Langsrud, 2004; Shi and Zhu, 2009; Simões et al., 2010).

Although little is known about the physiological conditions that predispose *L. monocytogenes* for persistence, nutrient availability may influence the intrinsic ability of *L. monocytogenes* to adhere (Palmer et al., 2007). Cell surface characteristics differ in response to variations in growth condition and have also been found to

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influence adhesion and biofilm formation (Briandet et al., 1999a,b; Chavant et al., 2002; Di Bonaventura et al., 2008; Gordesli and Abu-Lail, 2012; Jensen et al., 2007; Tresse et al., 2006; Zhou et al., 2012). It has been shown that different growth temperatures, growth media, and storage temperatures, influence the physiochemical properties of *L. monocytogenes* and thereby adhesion and biofilm formation (Briandet et al., 1999a,b; Chavant et al., 2002; Di Bonaventura et al., 2008; Stepanović et al., 2004). While carbon sources (glucose, cellobiose, fructose, mannose and trehalose) do not influence attachment to stainless steel under static conditions, an increase in ammonium chloride and decrease in iron concentration in the growth medium result in a decreased attachment (Kim and Frank, 1994). In contrast, a similar study found biofilm development to be influenced by the presence of mannose and trehalose and various levels of phosphate and amino acids (Kim and Frank, 1995). Additionally, Briandet et al. (1999b) showed that addition of glucose to trypticase soy broth supplemented with 6 g yeast extract (TSYE) alters the physicochemical properties of *L. monocytogenes*. Neither of these studies evaluated how specific nutrients, such as carbohydrates and amino acids, influence the very initial adhesion process in combination with how the specific growth conditions influence cell physiochemical properties; and if high or low adhesion ability, due to specific nutrient availability, could be due to other factors such as alteration of surface protein expression and fatty acid composition.

Studies evaluating the influence of growth conditions and nutrient availability on adhesion have primarily been done under static conditions and have been evaluated after extended periods of time (Briandet et al., 1999a,b; Chavant et al., 2002; Di Bonaventura et al., 2008; Kim and Frank, 1994; Stepanović et al., 2004). However, in food production systems *L. monocytogenes* are often exposed to sheer stress conditions, and adhesion under such conditions is critical for biofilm establishment (Doijad et al., 2011; Gudbjörnsdóttir et al., 2004; Perni et al., 2006; Silva et al., 2003). *L. monocytogenes* has been isolated form high shear environments, such as vats and pipes in milk processing environments (Doijad et al., 2011; Perni et al., 2007; Silva et al., 2003) and cooking facilities, flow lines, and RTE-food production drains in meat processing environments (Gudbjörnsdóttir et al., 2004). Application of flow perfusion systems allows determination of real-time initial adhesion at single cell level, making it possible to monitor the very initial adhesion step (Skovager et al., 2012). The aim of the present study was to examine the influence of single nutrient components (mannose, glucose and L-leucine) on the ability of *L. monocytogenes* to adhere to fine polished stainless steel under flow; and the contribution of cellular macromolecules to the process.

2. Materials and methods

2.1. Strain and growth conditions

The GFP labelled, fluorescent *Listeria monocytogenes* strain EGDe/pNF8 (strain EGDe was obtained from Werner Goebel (Biozentrum)) (Fortinea et al., 2000; Larsen et al., 2006) was used in the present study. The strain was maintained on Tryptone Soya Agar (TSA) (Oxoid) supplemented with 5 μg/mL erythromycin (erm) at 5 °C, inoculated into 100 ml Tryptone Soya Broth (TSB) (Oxoid) with a total of 1% (w/v) glucose and 5 μg/mL erm, and grown at 37 °C with agitation (225 rpm) for 24 h. Subsequently, each strain was re-inoculated (25 μL per 10 ml) into fresh TSB (Difco, without dextrose) but containing 5 μg/mL erm and different nutrients namely either 2.5 g/L glucose or 2.5 g/L mannose (designated glu-medium and man-medium, respectively) or supplemented additionally with 100 mM L-Leucine (designated glu + leu-medium and man + leu-medium, respectively). Cells were grown for 22 h (stationary phase) at 37 °C with agitation (225 rpm). The cell culture was washed twice in 0.15 M NaCl (Merck) (4000 ×*g*, 5 min).

2.2. Preparation for flow perfusion experiments

2.2.1. Surface characteristics and preparation of pine polished stainless steel coupons

Fine polished stainless steel, SS 304 (FPSS) (Outo Kumpu, Sheffield, UK) was prepared as described in Skovager et al. (2012). In short, FPSS was cut into coupons (7.5 cm × 3.5 cm) using a guillotine. The surface characteristics of the fine polished stainless steel can be found in Skovager et al. (2012). The steel coupons were soaked and rinsed in acetone overnight, after which they were rinsed in 96% alcohol for 5 min. Finally, the steel coupons were rinsed with distilled water and air dried, standing on a table overnight. CoverWell Perfusion chambers (622503, PC3L-0.5, CoverWell, Grace Bio-Labs, Inc) were glued (Super attak, Loctite, Henkel Norden AB) onto the surfaces. Silicone tubes (Watson Marlow Alitea) were glued on the inlet (ID: 1.6 mm; Wall: 1.6 mm) and the outlet (ID: 4.8 mm; Wall: 1.6 mm) of the perfusion chambers (Skovager et al., 2012).

2.3. Flow perfusion experiments

The optical density (600 nm) of the prepared cell suspension was adjusted to 0.100 ± 0.005, corresponding to a cell density of approx. $8 \cdot 10^7$ CFU/ml, determined by pour plating on TSA medium (Oxoid) + 5 μg/mL erm.

The same setup and procedure as described by Skovager et al. (2012) was used. However, another epi-fluorescence microscope (Zeiss Axioplan 2, Carl Zeiss; mercury lamp: HBO100w) and digital camera (Leica DFC340 FX) controlled by the LAS Software V3.6.0 (Leica) were used. Fluorescent images were captured after 1 min, 3 min, 5 min, 10 min and 15 min of perfusion. For each image, cells were excited for 4–6 s (excitation filter BP450-490; beam splitter FT 510 and emission filter BP 515–5650) visualizing GFP labelled cells. For each of three biological replicates and for each growth condition, triplicate surfaces were used. Wall shear stress was set to 0.10 Pa (shear rate ~ 100 s^{-1}) corresponding to a flow rate of 0.75 ml/min. Image analysis and calculation of initial adhesion rate (IAR) were made as described by Skovager et al. (2012).

2.4. Microbial adhesion to solvents (MATS) analysis

The MATS-analysis was carried out as described previously (Bellon-Fontaine et al., 1996), with minor modifications as described by Skovager et al. (2012). In short, *L. monocytogenes* was grown as previously described. Stationary phase cells were harvested (4000 ×*g*, 5 min) and the supernatant discarded. The cells were washed twice in 0.15 M NaCl (4000 ×*g*, 5 min), and re-suspended in 0.15 M NaCl to $OD_{400} \approx 0.8$ (A_0 value) (UV-1800 Shimadzu spectrophotometer). Two mL of cell suspension was added to a test tube with 0.4 mL of one of the following solvents, chloroform (Merck), hexadecane (Sigma-Aldricht), ethyl acetate (Merck), or decane (Fluka). The cell suspensions were vortexed with the solvent for 1 min. The emulsions were left to stand for 15 min to allow phase separation, and the OD_{400} (A value) of the aqueous phase was measured. Affinity of the cells for each solvent was calculated by use of Eq. (1):

$$\% \text{ affinity} = 100 \ (1 - A/A_0) \quad (1)$$

A_0 and A are the OD_{400} values of the cell suspension in the aqueous phase before and after mixing, respectively. Analysis were carried out in triplicate.

2.5. Determination of cell size and sedimentation velocity

Stationary phase cells were harvested and washed twice in 0.15 M NaCl (4000 ×*g*, 5 min), placed on an objective glass slide under the