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Biofilm development by *Listeria innocua* in turbulent flow regimes

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1 **BIOFILM DEVELOPMENT BY *LISTERIA***
2 ***INNOCUA* IN TURBULENT FLOW REGIMES**

3

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20 **Key words:** *Listeria innocua*, biofilm, Reynolds number

21 **ABSTRACT**

22 Chemostat-cultured cells of *L. innocua* were continuously recirculated through a
23 stainless steel tubes at Reynolds numbers ranging from 9,500 to 16, 500 for 7 days.
24 Samples of the tubes were removed by a special method and were examined using
25 SEM. Biofilm formation had occurred after only 1 day at Reynolds numbers of 9,500
26 and 11,500 and the extent of coverage increased with time. At the higher Reynolds
27 numbers (13,000 and 16,500) only individual cells were evident after 1 day, but these
28 developed to form microcolonies. After 7 days biofilms had become established at all
29 four Reynolds numbers.

INTRODUCTION

Food may become microbiologically contaminated simply by contact with abiotic surfaces that are themselves colonised by pathogenic micro-organisms (Midelet & Carpenter, 2002; Lundén, Autio & Korkeala, 2002; Midelet & Carpentier, 2004). The transfer of micro-organisms will depend on the state of the micro-organisms at the surface. Individual cells may simply adsorb to a surface or proliferate at the surface in the form of a biofilm. A biofilm has been defined as a functional consortium of micro-organisms attached to a surface and is embedded in the extracellular polymeric substances (EPS) produced by micro-organisms (Costerton et al., 1978). Biofilms present a greater threat to food safety because they are capable of self-regeneration if portions of the biofilm become detached or ‘sloughed off’ (Carpentier & Cerf, 1993), and because they are resistant to chemical disinfection (Krysinski, Brown & Marchisello, 1992). The ability to form biofilms appears to be shared by many food borne bacteria including *Vibrio* (Prouty & Gunn, 2003), *Listeria* (Hammer & Bassler, 2003) and *Salmonella* (Ryu & Beuchat, 2004).

Bacterial attachment studies are typically conducted under conditions where there is no gross flow of liquid relative to the surface (Norwood & Gilmour, 1999; Beresford, Andrew & Shama, 2001). Whilst such studies have undoubtedly contributed to an understanding of the phenomenon of attachment, there are many circumstances in both natural and artificial environments where solid surfaces are either continuously, or periodically, in contact with flowing liquids that contain micro-organisms. Fluid flows are characterised by a dimensionless parameter known as the Reynolds number.

The Reynolds number (Re) is defined as $\frac{\rho v D}{\mu}$ where, ρ is the fluid density, v its

velocity, D a characteristic dimension and μ the fluid viscosity. In practical terms this parameter represents the ratio of inertial to viscous flow; values below about 2,000 are termed laminar whilst those above 4,000 are turbulent.

The trend in industrialized countries is for food processing to become increasingly centralized (Hjaltested, Gudmundsdottir, Jonsdottir, Kristinsson, Steingrimsson, & Kristajansson, 2002; Wing & Gregory, 2002). As a result of this, processing facilities increase in scale and operations such as cleaning become fully automated. Such changes are inevitably accompanied by increases to the flow rates – and concomitantly Reynolds numbers – of ingredients and products. Despite these trends, surprisingly little work has been done on the formation of biofilms at high Reynolds numbers. Previous studies have focussed on the pseudomonads because certain species such as *P. putida* and *P. fluorescens* are good biofilm formers (Pujo & Bott, 1991; Melo & Viera, 1994; Lewandowski & Stoodley, 1995; Stoodley, Lewandowski, Boyle & Lappin-Scott, 1998).

An additional feature of many previous studies on biofilm formation has been the use of translucent abiotic surfaces such as glass or certain synthetic polymers because this aids in the visualisation of the biofilms. In fact these materials are rarely employed in the modern food industries: glass is fragile, and when it breaks fragments can potentially contaminate foods. Whilst polymers are unsuitable in any applications where their surfaces might become scratched because this can encourage biofilm formation (van Haecke, Remon, Moors, Raes, Derudder & Vanpeteghem, 1990). Stainless steel on the other hand is widely used in the food industry owing to its excellent corrosion resistance and because it is able to withstand the cleaning and

80 sanitising regimes routinely employed in the food industry (Zottola & Sasahara,
81 1994).
82
83 Tubes represent a particularly convenient means of contacting microbial suspensions
84 with solid materials, but accessing the lumen of tubes is difficult particularly if the
85 tubes are made of hard materials such as steel. In the work presented here we
86 describe a method for monitoring biofilm formation in stainless steel tubes through
87 which were continuously recirculated chemostat-grown culture of *L. innocua* at
88 Reynolds numbers ranging from 9,500 to 16,500.

89 MATERIAL AND METHODS

90 *Bacterium – Maintenance and Cultivation*

91 *Listeria innocua* (ATCC 33090) was purchased from the National Collection of Type
92 Cultures, Colindale, Middx. Cultures were maintained on Brain Heart Infusion agar
93 (Oxoid, Basingstoke, U.K.) slopes at 4 °C. *L. innocua* was grown both in shake flasks
94 and in a chemostat on a Tryptone –Yeast medium having the following composition:
95 Glucose 0.50 g, NaCl 15.00 g, NaH₂PO₄ 0.50 g, Na₂HPO₄ 0.50 g, MgSO₄ · 7 H₂O
96 0.15 g, Yeast extract 2.50 g, Tryptone 2.50 g. (All quantities per litre of distilled
97 water). The medium was sterilised by autoclaving at 121 °C for 15 minutes.

98 *Chemostat*

99 *Listeria innocua* was grown in a fully instrumented bioreactor operated as a
100 chemostat. The bioreactor (FT Applikon, Gloucester, U.K.) was operated at a working
101 volume of 1 litre. Temperature control (30± 0.5 °C) was maintained by placing the
102 bioreactor in a temperature controlled water bath. pH was maintained and controlled
103 at 7.0 by the automated addition of either 0.2 M NaOH or 0.2 M HCl. Air was
104 supplied to the sparger at a flow rate of 1.3 litres min⁻¹ and the impeller was operated
105 at 200 rpm. The dilution rate was kept constant at 0.015 hr⁻¹ and steady state was
106 deemed to have been reached after at least five volume throughputs. Daily checks
107 were made for contamination by examination of culture samples using a microscope
108 and by plating on Palcam Agar (Oxoid) and Tryptone Soya Agar (Oxoid), and
109 incubating at 30 °C, and Malt Extract Agar (Oxoid), and incubating at 25 °C, and
110 examining the plates daily for 3 days. In all cases samples were taken directly from
111 the bioreactor.

112 *Cell Counts*

Samples from the chemostat were serially diluted in $\frac{1}{4}$ strength Ringers solution and plated onto Tryptone Soy Agar (Oxoid). The plates were incubated overnight at 30° C before counting.

Biofilm Apparatus

A schematic of the apparatus used to study biofilm formation is given in Figure 1. Culture from the chemostat was fed directly into a 2 litre stirred mixing vessel located in a water bath and maintained at 30 °C by means of a chilling unit (CZ1, Grant Instruments, Cambridge, U.K.) equipped with a temperature controller ('Fi-monitor', Fisons Ltd., Loughborough, U.K.). Cell suspension was circulated through test sections and via a coiled tube heat exchanger by means of a centrifugal pump (Model JP5, Grundfos Pumps Ltd., Leighton Buzzard, U.K.) installed with a by-pass line. The volume of liquid in suspension was approximately 7 litres. The stainless steel tubes (AISI 304, length, 1 m, O.D., 0.012 m and I.D., 0.01 m; East Midlands Alloys, Loughborough, U.K.) were mounted in a specially-designed manifold.

Overall flow to the manifold was controlled by means of the pump by-pass valve but the flow to each tube was set by means of a ball valve. The flowrate to each tube was measured by means of orifice plates equipped with differential pressure transducers.

The liquid volume in the mixing vessel was maintained constant by means of a peristaltic pump (Model 302S, Watson-Marlow Ltd, Falmouth, UK).

Sanitisation Protocol

All equipment downstream of the chemostat was sanitised by the continuous recirculation for 24 hours of a biocide solution (RBS pF, Borghgraef S. A., Belgium). Following this the biocide was drained out and the apparatus was flushed through

137 with 100 litres of tap water sterilised by passage through a 0.2 μm in-line filter
138 (Sartoclean CA, Sartorius, Epsom, Surrey, U.K.).

139 *Treatment of test sections*

140 Removable test sections 0.05 m long were located in each of the 1m stainless steel
141 pipes 0.1 m from the ends by means of push fit couplings (Flow-Tech, Loughborough,
142 U.K.). This was within the region of fully developed flow at each of the Re used.

143 Before replacing the test sections, the system downstream of the chemostat was
144 drained and the liquid collected in pre-sterilised vessels. After the new sections had
145 been inserted into place the bacterial suspension was re-introduced into the mixing
146 vessel and circulation was resumed.

147 Prior to connection, the test sections were grooved to enable small coupons (1×10^{-2}
148 by 5×10^{-3} m) to be cut from them once they were removed from the stainless steel
149 tubes without unduly disturbing the biofilm colonising the bore of the section. Four
150 equidistant longitudinal grooves (length, 0.01 m) were milled into the outside of the
151 test sections (figure 2a). The depth of the grooves (8×10^{-4} m) was such as to permit
152 ready detachment of the coupon whilst at the same time allowing the test section to
153 retain strength and rigidity whilst in location in the biofilm apparatus. Once removed
154 from the biofilm apparatus, the test sections were first washed in sterile phosphate
155 buffer solution (PBS) to removed unattached cells. They were then fixed in 2%
156 glutaraldehyde (buffered with PBS) for 2 hours and finally washed three times in PSB
157 for 15 minutes.

158 In order to provide coupons for examination by SEM, the ungrooved portions of the
159 test sections were cut away using a pipe cutter (Figure 2b) and a fine saw was used to
160 make two longitudinal cuts (figure 2c) that resulted in the detachment of a coupon
161 having a curved surface (figure 2d).

Coupons were stored in PBS at 4 °C before being analyzed by SEM. Coupons were never stored for more than 4 days.

Preparation for SEM

SEM was chosen as it can be used for the examination of surfaces that are not flat, however it does not allow *in vivo* studies and some artifacts can be generated (Surman et al., 1996). Coupons were prepared for Scanning Electron Microscopy (SEM) by first washing in distilled water for 5 minutes. They were then dehydrated in alcohol of increasing strength, starting with 70% followed by 90% and finally absolute alcohol, exposure in all cases being for 10 minutes. Then the samples underwent a critical point drying (Baltec CPD 030 Critical Point Dryer, EM Systems Support Ltd, Cheshire, UK) and then they were sputter-coated with gold for 90 seconds at 20mA (Polaron SC7640, Quorum Technologies Ltd., Newhaven, UK).

All observations were carried out with SEM (Hitachi S3000H - Hitachi Scientific Instruments, London, UK).

RESULTS

Operation of the chemostat under the conditions specified under Materials and Methods led to a steady state concentration of cells in the overflow line of 7.4×10^9 CFU/ml which was equivalent to an absorbance at 600 nm of 1.03. The velocities in each of the four tubes were 0.95; 1.15; 1.30; 1.65 m/s which correspond to Reynolds numbers of 9,500, 11,500, 13,000 and 16,500 respectively (based on the properties of pure water at 30° C). The system devised here for sampling the stainless steel tubes proved successful in practice and stainless steel coupons were detached from the test sections in a way that minimised disruption of attached biofilms. SEM images of the

development of the biofilm at Reynolds numbers ranging from 9,500 to 16,500 after 1, 4 and 7 days are shown in Figures 3 to 5.

L. innocua had colonised a significant proportion of the surface at a Reynolds number of 9,500 after only 1 day (Figure 3a). Increasing the Reynolds number to 11,500 resulted in less attachment, but appreciable surface colonization is still evident (Figure 3b). However, at a Reynolds number of 13,000 only small aggregates of cells are visible (Figure 3c). Figure 3d reveals a surface largely free of cells at the highest Reynolds number of 16,500. After 4 days, the extent of surface attachment at the lower Reynolds numbers, 9,500 and 11,500 (Figures 4a & b) appears very similar. Figure 4c reveals cell aggregates similar to those seen previously after 1 day. The SEM at the highest Reynolds number, 16,500 (Figure 4d) shows cell clusters where only a previously small numbers of single cells were visible (Figure 3d). By day 7, surface attachment at a Reynolds number of 9,500 appears to have become more widespread. Surface coverage for the higher Reynolds numbers (Figs 5b-d) seems not significantly different to those obtained after 4 days.

Some of the SEM images reveal the presence of fissures at the surface of the stainless steel. In particular, Figure 4c shows two such fissures at high magnification. It is noticeable that these surface features do not appear to be the focus for cell attachment. In fact the attached growth visible in the figure is quite distinct from the fissures.

The stainless steel tubes and associated pipes, fittings and pumps could only be sanitized rather than sterilized. Therefore the possibility of contamination could not be excluded. However, samples plated out on a variety of agars did not reveal

obvious contaminants. Examination of the attached cells reveals the presence of low numbers of morphologically distinct cells. Elongated cells are indicated by an arrow in Figure 4c and squat cells by an arrow in Figure 5a.

DISCUSSION

The techniques developed here enabled samples to be removed from stainless steel tubes without causing obvious damage to the attached biofilms. Using these techniques we were able to demonstrate that *L. innocua* is capable of establishing biofilms in turbulent flow conditions at Reynolds numbers up to 16,500.

The question of whether listerial species can form biofilms was until recently one that had not been resolved. Kalmokoff, Austin, Wan, Sanders, Banerjee & Farber (2001) claimed that *L. monocytogenes* does not form true biofilms but merely adheres to surfaces, and most of the visual evidence of surface colonisation published before 2001 would appear to support this view. However, Marsh, Luo & Wang (2003) provided convincing evidence to the contrary by publishing SEMs of biofilms produced by *L. monocytogenes*. The studies reported here are the first to demonstrate biofilm formation by a listerial species under flow conditions and at Reynolds numbers that are industrially relevant.

There have been only few previous studies relevant to the food industry performed at such high Reynolds numbers. Pujo & Bott (1991) operated with Reynolds numbers up to 16,800 and Lewandowsky & Stoodley (1995) examined turbulent flows with Reynolds numbers as high as 20,500. In both cases these studies were performed with

pseudomonads. Lewandowsky & Stoodley (1995) measured the effect of biofilm formation on pressure drop in pipes and claimed that the pressure drop can double over a period of 25 days. Attempts to measure pressure drop caused by *L. innocua* biofilms proved unsuccessful (Perni, 2005).

Biofilms are often associated with exopolysaccharide (EPS) which is thought to play a role in surface attachment (Stoodley et al., 1999). In general, the SEM images presented here do not reveal unambiguous EPS formation. Fine strands that might be constituted of EPS are visible in the biofilms at 7 days at the lowest Reynolds number (Fig 5a). Similarly, SEMs of biofilms of *L. monocytogenes* presented by Chavant, Folio & Hebraud (2003) did not show the presence of EPS matrix.

Preliminary experiments (not reported here) conducted in the absence of NaCl in the growth medium led to rapid contamination of the mixing vessel and of the biofilms that formed on the stainless steel tubes. Supplementing the medium with NaCl at 1.5 % was successful in eliminating contamination in the mixing vessel. Figures 3 to 5 reveal the presence of small numbers of cells with distinctive morphologies. As previously stated, it is possible that these are contaminants. However, there is a alternative explanation for the presence of these cells: Zaika & Fanelli (2003) applied stresses to growing cultures of *L. monocytogenes* by manipulating the growth temperature and the concentration of NaCl added to cultures. These workers were able to achieve elongations of between 4 and 10 times the length of unstressed cells, and also a shortening of cells. The stresses imposed on *L. innocua* cells attached to stainless steel surfaces in this work are not so readily identified. However, the prevailing conditions were those of constant high hydrodynamic shears and it is not

inconceivable that this might have manifested itself in changes to the cell morphology of a certain sub population of cells.

The results obtained here cast doubt on the ‘rule of thumb’ widely quoted in industry that velocities of 1 m/s are sufficient to prevent biofilm formation (Pujo & Bott, 1991). It was earlier mentioned that biofilm formation carries with it the chance of contamination. Many of the techniques currently being advocated for the decontamination of the surfaces of foods or processing plant such as UV (Gardner & Shama, 2001) or cold plasma treatment (Vleugels, Shama, Deng, Greenacre, Brocklehurst and Kong, 2005) would benefit from an ability to conduct large scale trials under realistic conditions inside food processing facilities. However, such studies could only be undertaken if it could be guaranteed that public health would not thereby be compromised. Moreover, there is a considerable amount of interest in the elimination of *L. monocytogenes* from food processing environments (Carpentier & Chassaing, 2004) but studies of the sort advocated above with this bacterium would not be possible because of the containment requirements that are demanded in most industrialised countries. Indeed, we were not able to repeat the experiments reported here with *L. monocytogenes* because we were unable to provide the necessary containment necessary for the industrial pumps and valves we used.

L. innocua has been used as a surrogate for *L. monocytogenes* sometimes with little or no stated justification (Wouters, Duttreux, Smelt & Lelieveld, 1999) or on the basis that both organisms were similarly resistant to tetracycline, ozone and the bacteriocins produced by *Carnobacterium* spp., (Vaz-Velho, Fonseca, Silva & Gibbs, 2001). In addition, both organisms have been shown to have a similar susceptibility to

287 antibiotics and heavy metals (Margolles, Mayo & Reyes-Gavilan, 2001) and to have
288 similar responses to heat treatment, gamma irradiation, lactic acid and sodium nitrite
289 treatment (Kamat & Nair, 1996). The lone voice of dissent would appear to be that of
290 Meylheuc, Giovannacci, Briandet & Bellon-Fontaine, (2002) who concluded on the
291 basis of microelectrophoresis and physicochemical surface characterization tests
292 based on microbial adhesion to solvents that the two organisms were dissimilar to the
293 extent that *L. innocua* should not be used as a substitute for *L. monocytogenes*.
294
295 Notwithstanding, both species have been shown to occupy identical niches in food
296 processing plants (Gudbjornsdottir et al., 2004) and on a variety of foods (Duffy et al.,
297 2000; Cornu, Kalmokoff & Flandrois, 2002). Therefore, the finding that *L. innocua*
298 forms biofilms on a material widely-used in the food industry under conditions
299 generally regarded as preventing film formation evidently must at the very least
300 increase the possibility that *L. monocytogenes* also possesses similar biofilm-forming
301 abilities under conditions of high Reynolds numbers flow.

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Figure 1 Schematic of the biofilm formation apparatus

Figure 2 Stages in the removal of test sections of stainless steel tube for examination by SEM

- (a) Test piece showing exterior lateral grooves
- (b) Truncation across grooves
- (c) Vertical incision
- (d) Removal of test piece

a

b

Figure 3 L. innocua biofilms after 1 day

(a Re = 9,500; b Re = 11,500; c Re = 13,000; d Re = 16,500)

a

b

c

d

Figure 4 L. innocua biofilms after 4 days (Arrow depicts an elongated cell)

(a Re = 9,500; b Re = 11,500; c Re = 13,000; d Re = 16,500)

a

b

c

d

Figure 5 L. innocua biofilm after 7 days (Arrow depicts squat cell)

(a Re = 9,500; b Re = 11,500; c Re = 13,000; d Re = 16,500)