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Detachment of *Listeria innocua* and *Pantoea agglomerans* from cylinders of agar and potato tissue under conditions of Couette flow

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1 Detachment of *Listeria innocua* and *Pantoea agglomerans* from
2 Cylinders of Agar and Potato Tissue under Conditions of
3 Couette Flow.

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7 **Abstract**

8 Cylinders of raw potato or agar were contacted with suspensions of *Listeria innocua*
9 and *Pantoea agglomerans* and then used as replacement rotors in a rheometer in order
10 to investigate detachment under the influence of known shear forces. These shear
11 forces were functions solely of the rotational speed of the rotor and the fluid
12 (glycerol) in which the cylinders were caused to rotate. With this system surface shear
13 forces ranging from 1.3 to 125 Pa could be generated corresponding to rotational
14 speeds of 12.5 to 775 rpm. Under these conditions detachment phenomena were quite
15 rapid with in most cases complete detachment being achieved over timescales of the
16 order of 30 s. In general, lower shear forces were required to detach *L. innocua* from
17 both agar and potato. For agar cylinders an applied shear force of only 1.3 Pa was
18 sufficient to achieve 98 % detachment of *L. innocua* after 20 s. By contrast, relatively
19 high shear forces were required to detach *P. agglomerans* particularly from potato;
20 under an applied shear force of 2.8 Pa only 9.5 % detachment was achieved after 30 s.
21 The results obtained at the highest shear forces studied here (125 Pa) with potato
22 cylinders were suggestive of mass transfer into glycerol of one or more constituents
23 present in potatoes that caused detached cells to aggregate causing an apparent

decrease in percentage detachment. The data obtained could be used as a basis for the rational design of washing processes for fresh ready to eat food products.

Keywords: Surface Shear Stress; Plant Tissue; Couette Flow; Bacterial Detachment; Washing Processes

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1. Introduction

The consumption of fresh ready-to-eat (RTE) salad vegetables and fruits has greatly increased over the last two decades (Li et al., 2001). These minimally processed foods are subject to contamination by a wide variety of microbial pathogens at every stage of their cultivation, harvesting and subsequent processing for retail (Mayer-Miebach et al., 2003). It is therefore not surprising that the growth in popularity of these commodities has been accompanied by an increase in incidences of foodborne diseases directly attributable to their consumption (De Roever, 1998).

The traditional method of decontaminating fresh salad products has been to wash them in chlorinated water. However, evidence has steadily been accumulating to show that the efficacy of this type of treatment is limited and may, for certain types of product e.g. sprouted shoots, be wholly inadequate (Gandhi and Matthews, 2003). Allied to this are concerns over the effects on human health of chlorine residuals (Kalmaz and Kalmaz, 1981) that have already resulted in its ban in certain countries.

49 Taken together these factors would appear to spell the demise of this form of
50 decontamination treatment in the foreseeable future.
51

52 It is unlikely that any single treatment will replace washing with chlorine throughout
53 the entire RTE food sector. A number of alternatives have been proposed and these
54 include the use of chlorine dioxide gas (Han et al., 2000), heat shock coupled with
55 calcium lactate (Rico et al., 200&), hydrogen peroxide (Ukuku, 2004) and, UV and
56 ozone treatment (Bialka et al., 2008). However, all such treatments are more costly
57 than chlorine washing and most have yet to clear the final hurdle of consumer
58 acceptance.
59

60 Recent work (Wei et al., 2005) has shown that significant decreases in microbial
61 viability can be achieved simply by the use of acidified water at 50° C. There are
62 therefore, distinct advantages in ensuring that the maximum level of decontamination
63 is achieved by water washing before resorting to any of the newer decontamination
64 techniques cited above. The objective of washing is to remove dirt, pesticide residues
65 and micro-organisms (Baur et al., 2005) and this is typically achieved using flumes,
66 and a wide variety of this type of equipment is commercially available (e.g.
67 Rodriguez, 1999). In virtually all cases these designs have been arrived at largely by
68 heuristics. However, it remains possible that if a systematic approach were taken to
69 the design of washing processes, novel configurations might emerge. This approach
70 would firstly require knowledge of the shear forces required to physically detach the
71 microflora associated with a particular type of produce. It would also rely on an
72 appreciation of the effects on the produce of exerting such forces (Hassan and Frank,
73 2003) in order to minimise losses through physical damage to the plant tissue.

74

75 As a first step towards this process, we present a novel and precise method of
76 estimating the effects of shear forces on the detachment of bacteria from solids. We
77 did this by generating a known shear force at the surface of the solid – itself in the
78 form of a cylinder – by causing it to rotate inside a slightly larger hollow cylinder
79 filled with a viscous liquid (glycerol). The velocity distribution generated in the
80 annular gap between the two cylinders is referred to as ‘Couette flow’. This was
81 achieved in practice by using a commercial rheometer in which the rotational element
82 or ‘rotor’ was replaced by one made from materials of interest. These were agar,
83 chosen here to represent a model food compound (Midelet and Carpentier, 2004), and
84 raw potato. These experiments were conducted using *Listeria innocua* and *Pantoea*
85 *agglomerans*. The former is widely regarded to be a surrogate for the pathogen *L.*
86 *monocytogenes* the causative agent of listeriosis (Perni et al., 2006) and frequently
87 associated with fresh produce (Beuchat, 1995). *P. agglomerans* is a biofilm-forming
88 plant pathogen that is also known to colonise fresh produce (Brocklehurst et al.,
89 1987).

90

91 **2. Materials and methods**

92

93 *2.1 Detachment studies*

94

95 These studies were conducted using a rotational rheometer (Viscotester VT 550,
96 Haake GmbH, Karlsruhe, Germany) comprising a stainless steel cylindrical rotational
97 element or ‘rotor’ and a static element or ‘cup’. We substituted the rotor supplied with
98 the rheometer by ones made either of raw potato or agar. In either case, the cylinders

had to be generated consistently to precise dimensions (dia. 20 mm; length 60 mm) and in such a way that when positioned in the cup, they were concentric with one another and in alignment with the axis of the instrument.

2.2 Production of Potato and Agar Cores

A specially fabricated stainless steel prong comprising three hollow tines at one end and a cylindrical shouldered shaft at the other was employed first. The shaft had been machined in such a way as to enable it to be coupled directly to the motor drive of the rheometer. The prong was pushed by hand into the flat surface of a potato prepared by slicing through a potato with a knife (Figure 1a).

A coring device, consisting of a stainless steel tube (o.d. 21 mm, length 200 mm) sharpened at one end and containing inside it a sliding internal guide, was then located directly over the prong so that the guide fitted onto the shouldered cylindrical shaft (Figure 1b). The corer was forced into the potato until it had completely penetrated it, at which point it was withdrawn yielding a cylinder which was cut to the requisite length (60 mm) with a scalpel to yield the mounted core shown in Fig. 1c

Prior to use, the surface of the potato was decontaminated by spraying with a 70 % (v/v) aqueous ethanol solution and then dried in a laminar flow cabinet for 30 minutes, in addition, all components were flame-sterilised.

In order to produce agar cylinders, the prong was placed vertically into approximately 300 ml of molten TSA (Tryptone Soy Agar, Oxoid) supplemented with 3 % (w/v) agar no. 3 (Oxoid) in a sterilised glass beaker so that the tines were completely

submerged. Once the agar had set, the coring procedure described above was put into operation.

Once produced, the cores were lightly sprayed with 70 % (v/v) aqueous alcohol and then dried for 30 minutes in a laminar flow cabinet prior to immersion in bacterial suspension.

2.3 *Micro-organisms and cultivation*

L. innocua (ATCC 33090) and *P. agglomerans* (isolated from lettuce and kindly donated by Professor T. F. Brocklehurst of the Institute of Food Research, Norwich, UK) were both maintained on TSA agar slopes at 4°C. Sterile TSB (100 ml), contained in 150 ml Duran bottles with specially modified tops enabling the cores to be suspended vertically whilst in contact with its contents, were inoculated with a loopful of bacteria previously grown for 24 hours on TSA plates incubated at 30 °C in the case of *L. innocua* and 20 °C for *P. agglomerans*. The broths were incubated statically at the same temperatures quoted above for 24 hours.

A core equilibrated at 30 °C in the case of *L. innocua* and 20 °C for *P. agglomerans* for 30 min prepared as described above was immersed in cell suspension for 2 hours at the relevant growth temperature. After removal, the core was washed twice by immersing it in succession into each of two 150 ml Duran bottles containing 100 ml of sterile PBS before attaching to the rheometer or prior to conducting counts. The mean initial counts (in CFU/cm²) for *L. innocua* were 9.3 x 10⁶ on potato and 3.0 x 10⁶ on agar. The counts for *P. agglomerans* were 1.47 x 10⁷ and 1.1 x 10⁷ respectively.

149

150 For detachment studies the rheometer cup was filled with 100 ml of sterile glycerol
151 and the core gently lowered into it. Samples of glycerol (100 µl) were taken from
152 approximately the same position in the gap between the core and wall of the cup and
153 plated onto TSA plates after appropriate dilution in PBS. Plates were incubated for 24
154 hours at 30 °C in case of *L. innocua* and 20 °C for *P. agglomerans* before counting.

155

156 We present our results in terms of ‘percent detachment’ and in order to do this
157 obtained estimates of the cell numbers adhering to the cores immediately after
158 washing, as described above, by placing a core in a plastic bag with 50 ml of sterile
159 PBS and processing in a Stomacher (Model 400, Seward Ltd., Thetford, UK) for 2
160 minutes on ‘normal’ setting and plating out on TSA plates.

161

162 The percentage detachment at any time *t* was calculated thus:-

163

164
$$\text{percent detachment} = \left\{ \frac{\frac{\text{cell concentration in glycerol}(t)^1 \times \text{total vol. of glycerol}}{\text{surface area of core}}}{\text{surface conc. of cells at } t = 0^2} \right\} \times 100$$

165

166 1 obtained directly from counts and expressed as CFU/ml

167 2 expressed as CFU/cm²

168

169

170 All experiments were repeated using three independently grown cultures.

171

172 2.4 Calculation of surface shear

173

174 The relationship between shear stress and shear rate is given by:

175

176 $\tau = \eta\dot{\gamma}$

177 where:

178 τ = shear stress (Pa)

179 $\dot{\gamma}$ = shear rate (s^{-1})

180 η = viscosity (Pa s)

181

182 and

183

184 $\dot{\gamma} = nM$
 $\tau = fM_d$

185

186 where:

187 n = rotor speed (rpm)

188 M_d = torque (N cm)

189 M, f = dimensionless instrument-dependent system parameters, 0.245 and 471.6

190 respectively.

191

192 *2.6 Statistical Methods*

193 The cell counts obtained at each exposure time at various shears were compared using
194 the ANOVA test followed post hoc by Tukey's test for individual pair of data sets.

195 Statistical analyses were performed using SPSS 14.0 software (SPSS Inc., Chicago,
196 IL, USA)

197

198

3. Results and discussion

The procedures developed here enabled cores to be consistently produced within close physical tolerances, and this coupled with the electronically controlled speed at which the cores were rotated, meant that the shear forces at the surface were maintained constant over time and were solely functions of the rotational speed. Figure 2 displays the theoretical relationship between rotor speed, shear rate and shear stress for a conventional steel rotor rotating in glycerol (a Newtonian fluid) and that obtained by replacing the steel rotor with one made of raw potato of identical dimensions. The data was obtained by taking instrument readings over the range 12.5 to 775 rpm corresponding to shears forces between 1.3 and 125 Pa. The close agreement between the two enables us to quote below with high precision the shear forces acting on the surface of the agar and potato cores.

Figure 3a shows the percentage detachment of *L. innocua* from agar cores. The point shown at time zero in this and subsequent figures, and indicated by an 'x', was obtained by allowing cells to attach to the core in the normal way and then immersing it at a steady rate into the cup containing the glycerol whereupon the latter was immediately sampled without rotation of the core. This was done to allow an estimate to be made of cell detachment caused by passage of the core through an air-liquid interface. At the lowest shear force of 1.3 Pa, a gradual detachment of cells occurs until 20 s had elapsed when a value of 98 % detachment was achieved, and after which there was apparently no further loss of cells. Detachment at all shear forces above this value (i.e. 2.8, 11.8, 125 Pa) was very much more rapid and all the profiles are very similar. By 30 s approximately 100 % detachment had occurred regardless of

applied shear force. The results for 1.3 Pa were significantly different ($P < 0.5$) from those at 2.8 and 11.8 Pa at 5 and 10 sec. However, after 20 and 30 sec there were no significant differences between all three applied stresses. There were no significant differences in cell detachments brought about by shear stresses of 2.8 and 11.8 Pa

The results obtained for agar cores with *P. agglomerans* are shown in Figure 3b. For an applied shear force of 11.8 Pa the profile displays a slight peak at 10 s but the detachment remains low at below 10 %. At the higher shear force of 125 Pa a steady loss of cells is observed and at 30 s 85 % detachment was attained. There were no significant differences in cell detachments after 5 sec. After 10, 20 and 30 the counts at 11.8 Pa were significantly different from those at 125 Pa

The detachment of *L. innocua* from potato cores (Figure 4a) shows clear differences at shear forces of 1.3 and 2.8 Pa. At a shear force of 1.3 Pa the detachment achieved after 30 s was 36 % whereas at 2.8 Pa it was 106 %. The profile obtained at 125 Pa appears anomalous: detachment was initially rapid and after 5s had reached a value of 56 %, thereafter, an apparent decrease was observed with detachment remaining approximately constant at 47 %. The value of detachment at 30 s was actually lower than was achieved under the influence of a shear force of 2.8 Pa even though the shear force acting at the surface of the core was over 4 times greater. There were no significant differences between the number of cells detached at all three applied shear stresses at 10, 20 and 30 sec. Cell counts at 5 sec at 1.3 Pa were significantly different

from those at 2.8 and 11.8. There were, however, no significant differences in the number of cells shed at the latter two applied stresses.

The detachment profiles for *P. agglomerans* from potato (Figure 4b) show the rate at 2.8 Pa to be relatively low reaching a value of 9 % after 30 s. When the shear force was increased to 11.8 Pa a peak detachment of 18 % was achieved at 20 s and this is followed by a decline to a value (7 %) which is close to that achieved at 2.8 Pa. The results at 125 Pa also reveal a marked anomaly with an initial value of 81 % which actually decreases quite markedly to 13 % by 30 s. All counts were statistically different at 5, 10 and 20 sec. but not at 30 sec.

The procedures developed here enabled cores to be consistently produced within close physical tolerances, and this coupled with the electronically controlled speed at which the cores were rotated, meant that the shear forces at the surface were maintained constant over time and were solely functions of the rotational speed. Moreover, these were mathematically predictable with a high degree of precision. However, it was necessary to account for the shear forces generated when an object is transferred across an air-liquid interface as in our case inevitably occurred when the cores were lowered into the glycerol. Meinders et al. (1992) have argued that these forces can be significant and our results show that this action did indeed result in measurable detachment. The shear forces that were generated at the surface of the potato and agar as a result of rotation in glycerol were too low to cause deformation of the cores

themselves as confirmed by measurements made by Alvarez and Canet (1998) for potato and Barrangou et al. (2006) for agar.

The results presented here show that *P. agglomerans* required a greater force to detach it from the surfaces of both potato and agar than did *L. innocua*. In the case of potato this was not unexpected given that the *P. agglomerans* is not only a plant pathogen, but a known biofilm former and therefore likely to be specially adapted to colonize plant tissue. However, studies of the complex interactions that occur between micro-organisms and plant tissues are primarily confined to the surfaces of leaves (Frank, 2001) and no specific biochemical studies on the particular combination of *P. agglomerans* – potato currently appear to exist. Similar findings were reported by Garrood et al. (2004) who compared, amongst other organisms, the attachment and detachment of *P. agglomerans* and *L. monocytogenes* to discs of raw potato. Although these workers did not attempt to estimate shear forces, they modelled the processes of detachment on a probabilistic basis.

The method employed here to monitor detachment was based on the detection of viable bacteria that had been shed from the surface of the cores into the glycerol. Therefore, any apparent declines in detachment – as were observed with *P. agglomerans* from potato at high shears (Figure 4b) – are suggestive of other phenomena acting on detached cells in suspension. The fate of detached cells may have been affected by a number of factors (both chemical and physical) that, although were not directly related to shear, are a consequence of it.

One possibility is loss of viability whilst in suspension. It is highly unlikely that glycerol itself would have contributed to this. Although Saegeman et al. (2007) investigated the effect of concentrated glycerol solutions (85 %) on the viability of both Gram positive and Gram negative bacteria, the times at which they achieved reductions were of the order of days, rather than the short contact times measured in seconds employed in this work when detached cells were actually in contact with glycerol prior to plating onto agar. Another possibility is the release of toxic compounds from the potato tissue. Potato tubers have been shown to contain a diverse range of constitutive and inducible anti-microbial compounds that include peptides, enzymes and phytoalexins (Berrocal-Lobo et al., 2002; Guevara et al., 2002; Lopez-Solanilla et al., 2003). At high surface shears rapid mass transfer of such compounds could be expected to take place followed by efficient dissipation throughout the mass of glycerol present in the cup. However, given the short contact times and low concentrations, any such compounds would need to have considerably higher activity than any that have so far been isolated from potatoes by previous workers.

A more likely explanation for the apparent decrease in viability of detached cells are purely physical phenomena which are faster acting. Under the influence of high surface shears, starch and possibly other constituents of potato, could have been caused to be shed into the glycerol along with bacteria. Starch has been used to bring about the efficient flocculation of bacterial cells in order to harvest them (Ferenci and Lee, 1991). The apparent declines in viability observed here could be explained by only a low degree of flocculation or cell aggregation occurring due to starch or other constituents of potato.

There are obvious limitations in employing an instrument for a purpose for which it was not designed: the data presented here, in common with that of others (Garrood et al., 2004) reveals that micro-organism - surface interactions are rapid, and therefore correspondingly rapid sampling is called for. We encountered sampling difficulties primarily because of the viscosity of the glycerol, and it was not possible to obtain a sample of annular fluid earlier than 5 sec. after rotation of the core was initiated. Furthermore, the dimensions of the annulus between the rotor and the edge of the cup was only 13 mm. Every effort was made to sample from the mid point of the annulus but even this was rather imprecise. We believe that these difficulties can be overcome and indeed, are in the process of designing and fabricating an improved device that will feature automated sampling coupled to a fraction collector that will enable more extensive studies to be performed including that of leafy produce such as lettuce.

4. Conclusions

The equipment described here was successfully used to obtain quantitative data that could ultimately be used in the rational design of washing processes for microbially decontaminating fresh produce. The advantages of employing water rather than solutions of chemical agents are obvious however apparently benign such agents may prove to be. Even if recourse has to be made to such agents, it would seem logical that the design of water-washing processes should be made as efficacious as possible so that any subsequent chemical treatment can be brief and employ only dilute concentrations.

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Figure Legends

Figure 1. Procedure for Preparing Potato Cores

468 a) Prong inserted into cut potato. b) Coring device located over prong. c) Completed
469 core.

470

471 Figure 2. Dependence of Shear Stress at Rotor surface on Speed and Shear Rate.

472 Solid line denotes theoretical relationship. (●) Experimentally obtained values for
473 potato cores.

474

475 Figure 3. Detachment of *L. innocua* (a) and *P. agglomerans* (b) from Agar Cores

476 × initial detachment ● 1.3 Pa ■ 2.8 Pa Δ 11.8 Pa ◇ 125 Pa

477

478 Figure 4. Detachment of *L. innocua* (a) and *P. agglomerans* (b) from Potato Cores

479 × initial detachment ● 1.3 Pa ■ 2.8 Pa Δ 11.8 Pa ◇ 125 Pa

480

a

b

c

481

482 Figure 1

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485 Figure 2

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487

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489 Figure 3

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491

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493 Figure 4

a

b