



## Surface behaviour of *S. Typhimurium*, *S. Derby*, *S. Brandenburg* and *S. Infantis*

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### ABSTRACT

Cross-contamination due to *Salmonella* on the surface of processing equipment greatly contributes to contamination of pork products. Therefore, a clear understanding of surface and survival behaviour of relevant *Salmonella* serovars in pork processing environments is needed to develop better strategies for *Salmonella* control. Within this study the biofilm forming behaviour of *S. Typhimurium*, *S. Derby*, *S. Brandenburg* and *S. Infantis* isolates was analysed using the crystal violet assay. This assay, commonly used to analyse total biofilm formation, revealed variation in biofilm forming capacity between and within serovars. This has not been shown before for *S. Derby*, *S. Brandenburg* and *S. Infantis*. From each serovar, isolates with different biofilm forming capacity were selected to analyse biofilm formation on stainless steel. This revealed no significant differences between biofilm formation on polystyrene compared to stainless steel. Furthermore a relation was observed between biofilm forming capacity of an isolate and survival on stainless steel surfaces. On such surfaces, biofilms showed greater and longer survival than planktonic cells, and they were less susceptible to peracetic acid disinfection treatments. However, the latter effect was marginal and only observed in the presence of organic material, which drastically decreased the activity of peracetic acid. With the obtained results a hierarchical cluster was also performed to identify differences and similarities between the four different serovars. This indicated that the surface behaviour of *S. Typhimurium* was more comparable to *S. Infantis* than to *S. Derby* or *S. Brandenburg*.

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## 1. Introduction

*Salmonella* infections in humans are in general food borne, with an important source of risk being food from animal origin. The most frequently implicated food sources are eggs, poultry meat and pork (EFSA, 2011a,b). As shown by epidemiological studies in the European Union (EU),

consumption of pork is associated with 15–20% of all human cases of salmonellosis and with 7.8% of all foodborne outbreaks (Botteldoorn et al., 2003; ECDC, 2011).

Within the EU, the prevalence of *Salmonella* on slaughter pigs and in pork processing environments is well monitored. This reveals that at point of slaughter on average 10.3% of the slaughter pigs and after the slaughter process 8.3% of the carcasses are positive for *Salmonella* (EFSA, 2008). *Salmonella* serovars that are repeatedly found on farm animals within the pork processing environments and on carcasses at the end of the slaughter process are *S. Typhimurium*, *S. Derby*, *S. Infantis* and *S. Brandenburg*

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(Botteldoorn et al., 2003; De Busser et al., 2011; EFSA, 2008; Volf et al., 2010).

Contamination of carcasses during the slaughter process may occur in two ways. One route is direct contamination by the contents of the intestine or lymph nodes. The other one is indirect by contact with dirty processing equipment, so-called cross-contamination. Cross-contamination is common within the slaughter process, accounting for about 29% of the total amount of contaminated carcasses (Botteldoorn et al., 2003; EFSA, 2008; BIOHAZ, 2010; Van Hoek et al., 2012). This indicates that the presence of *Salmonella* on surfaces of processing equipment is a problem in pork processing environments and that better strategies for *Salmonella* control require a clear understanding of the surface and survival behaviour of *Salmonella*. Since *Salmonella* is exposed to a variety of different conditions within the processing environment different characteristics contribute to the survival and persistence of *Salmonella* in slaughterhouses. In this study the biofilm forming capacity and its relation with survival of *Salmonella* on stainless steel surfaces and susceptibility for peracetic acid (PAA) were analysed and compared for *S. Typhimurium*, *S. Derby*, *S. Brandenburg* and *S. Infantis*. Also the influence of organic matter (3% BSA) on the effectiveness of PAA was investigated.

## 2. Materials and methods

### 2.1. Bacterial isolates and media

A total of 23 *S. Typhimurium*, 13 *S. Derby*, 29 *S. Brandenburg* and 55 *S. Infantis* isolates were used in this study. All isolates were obtained from multiple pork processing plants and were isolated from processing equipment surfaces, food products, raw materials or the processing environment. Isolates from different origin within a pork processing environment were used to get a broad view of *Salmonella* isolates present in these settings. Stock cultures of these isolates were stored at  $-80^{\circ}\text{C}$  in Brain Heart Infusion Broth (BHI, Becton Dickson) supplemented with 15% glycerol (Sigma). Isolates were cultivated in Luria Bertani broth (LB, Merck) for 18 h at  $37^{\circ}\text{C}$  with agitation (200 rpm), after which they were used in the different experiments. To mimic the conditions in a pork processing environment, the experiments were performed at  $25^{\circ}\text{C}$  in the meat-based medium BHI.

### 2.2. Preparation of surfaces

Coupons (18 mm  $\times$  22 mm) of polystyrene (PS) and stainless steel (SS) (AISI type 304L) were used. Before use, the PS coupons were soaked for  $>2$  h in 70% ethanol, after which they were washed three times with sterile deionized water and dried in a safety hood. The SS coupons were soaked in 1 M NaOH (Sigma) for 30 min at  $50^{\circ}\text{C}$ , after which they were rinsed with water. Subsequently, the SS coupons were soaked in acetone (BDH, Prolabo) for 15 min at room temperature, washed four times with deionized water, autoclaved and dried overnight at  $50^{\circ}\text{C}$ .

### 2.3. Crystal violet assay

Biofilm forming capacity was examined using the crystal violet (CV) assay for analysis of biofilms in polystyrene 96 well microtiter plates (Greiner Bio-one) or on PS and SS coupons. For the microtiter plate CV assay, wells were filled with 200  $\mu\text{l}$  BHI medium. For the coupon CV assay, a coupon was vertically placed in a well from a 12 well plate (Greiner Bio-one) filled with 3 ml of BHI medium. For both CV assays, the medium was inoculated with 1.5% (v/v) overnight cultures and the plates were incubated for 24 h at  $25^{\circ}\text{C}$ . After incubation, biofilms were washed twice with water and stained with 0.1% (w/v) CV (Merck) for 30 min. Subsequently, the biofilms were washed three times with water and the attached CV was dissolved in 250  $\mu\text{l}$  per well for the microtiter plate and 5 ml for the coupon CV assay of 96% ethanol. The absorbance was measured at 595 nm (SpectraMax, Molecular Devices). Both CV assays were performed in at least three biologically independent experiments.

### 2.4. Enumeration of biofilm cells

For enumeration of biofilm cells by plate counting, biofilms were grown as described above on PS and SS coupons. After incubation, the biofilms on the coupons were washed four times with peptone physiological salt solution (PPS, 0.8% NaCl and 0.1% neutralised bacteriological peptone (Oxoid) to remove unbound cells. Then each coupon was transferred to a tube containing 5 ml of PPS and glass beads ( $\leq 106 \mu\text{m}$ , Sigma) and vortexed for 1 min to detach the biofilm and obtain single cells. Next, appropriate dilutions were made in PPS and the cells were enumerated by plating on tryptone soy agar (TSA, Oxoid). After overnight incubation at  $37^{\circ}\text{C}$ , the colonies were counted.

### 2.5. Curli fimbriae and cellulose production

Curli fimbriae and cellulose production was analysed as described previously (Römling et al., 2003) on LB without NaCl agar plates supplemented with 40  $\mu\text{g/ml}$  Congo red (Sigma) and 20  $\mu\text{g/ml}$  Coomassie brilliant blue (Merck) or with 40  $\mu\text{g/ml}$  Calcofluor (Fluorescent Brightner 28, Sigma). After 48 h of incubation at  $25^{\circ}\text{C}$ , the colony morphology on the agar plates was examined.

### 2.6. Survival of *Salmonella* on dry SS surfaces

Survival on dry SS surfaces was examined for both planktonic cells and biofilms. For analysis of planktonic cells, the isolates were cultured statically in tubes containing 10 ml of BHI for 24 h at  $25^{\circ}\text{C}$ . Of this overnight culture 30  $\mu\text{l}$  ( $\sim 8.0 \log \text{CFU}$ ) was applied to a SS coupon which was subsequently dried in a safety hood for 30 min. For analysis of biofilms, SS coupons were used to grow biofilms as described above, after which the coupons were washed three times with phosphate buffered saline (PBS), dried for 30 min in a safety hood and placed in a 12 well plate. After incubation for 1, 2, 5, 7 or 14 days at  $25^{\circ}\text{C}$  at 35–65% RH, the coupons were transferred to tubes

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