Exploring the physiology and architecture of *Salmonella enteritidis* biofilms under alkaline conditions

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**Background**

Formation of bacterial biofilms is an important survival strategy in multiple adverse environments. It is often affected by the nature of the attachment surface, the bacterial strain and the surrounding physicochemical conditions. *Salmonella enteritidis* is a Gram-negative, rod-shaped facultative anaerobic pathogen of humans, which is responsible for causing serious food-borne infections around the world. In 2016, 94,530 cases of salmonellosis were reported in Europe with *Salmonella enteritidis* being responsible for the majority of human cases to show an increase during 2016. It comes second after *Campylobacter* which is the most commonly reported gastrointestinal pathogenic bacterium (EFSA and ECDC, 2017). The ability of *Salmonella* to produce biofilms on material commonly encountered in the food industry is considered the principal contamination reason of food-borne outbreaks (Wang et al., 2013). The effect of low pH on biofilm formation has been well studied, in contrast to the alkaline range. The aim of this project was to study the effect of alkaline stress on the formation of biofilm by *Salmonella enteritidis* and to examine the biofilm architecture patterns under different conditions, by use of confocal microscopy.

**Materials and Methods**

The biofilm formed after each experiment was estimated using the crystal violet absorption method. After growing *S. enterica* serovar Enteritidis (NCTC4444) in Tryptone Soy Broth at 37°C, in 24-well plates, the planktonic bacteria were removed and three washing steps using 1 mL of sterile water were made in every well. Then, the remaining cells were stained using 1% mL of crystal violet. After 10 min, the excess stain was removed with several washes and the attached bacteria were then solubilised in 1 mL of 70% ethanol and 30% acetone per well. The absorbance of each solution was measured at 550 nm using the Ultraspec™ 3100pro UV/VISIBLE spectro-photometer (Amersham Biosciences). Leica SP5 Confocal laser scanning microscope (LEICA Microsystems, France) was used in order to obtain images of the biofilms. All biofilms were scanned using an oil-immersion lens with 20x magnitude and an argon laser set at 30% intensity. A paired t-test was performed using MiniTab 17 (Pennsylvania State University, USA).

**Key findings**

1. The optimal pH for *Salmonella* biofilm formation was found to be pH 7.0, while pH 10.0 reduces it significantly (p-value=0.015) (Figure 1A). The same happened to planktonic growth at pH 10.0 (Figure 1B).
2. Biofilm formation was hindered due to the alkaline pH, yet the number of viable cells remained high (Table 1, Figure 4).
3. The effect of alkaline stress on biofilm formation is bigger when it is applied during stationary phase of growth (Figure 2A).
4. Household and commercial detergents at low amounts decreased viability and biofilm formation, but not completely (Figure 3).
5. The biofilm architecture at pH 7.0 was characterized by small cell clusters, whereas at pH 10.0 a slightly thinner layer of individual cells was observed (Figure 4, Table 2).

**Conclusions**

- Our findings indicate that although the ability of *Salmonella* to form biofilm is impaired at alkaline pH, most cells survive the alkaline stress. This highlights the need for new disinfectant strategies involving alkaline reagents.
- These results are novel as there are currently no similar studies comparing pH 7.0 and pH 10.0 for this serovar, which is a very important hazard for the food industry.

**References**


**Acknowledgements**

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![Figure 1](image1.png) Biofilm formation (A) and planktonic cell growth (B) at a range of pH (4-10) after 24 h in TSB. N=3. p-values are indicated in the graphs when statistical differences are present, comparing to the control pH 7.0 (p ≤ 0.05; * is used when p ≤ 0.05, ** when p ≤ 0.01, and *** when p ≤ 0.001).

![Figure 2](image2.png) Biofilm formation (A) and planktonic cell growth (B) after the addition of sodium hydroxide (NaOH) after 24 h. Set volumes of sodium hydroxide were added in TSB (pH 7.0), reaching pH 10.0. The statistical differences resulted from comparison with the control pH 7.0 in each case, for the three replicates that were tested (N = 3). p-values are indicated in the graphs when statistical differences are present, comparing to the control pH 7.0 (p ≤ 0.05; * is used when p ≤ 0.05, ** when p ≤ 0.01, and *** when p ≤ 0.001).

![Figure 3](image3.png) Biofilm formation and planktonic cell growth after the adjustment of the pH using sodium carbonate (A)(B) and Elma™ 70 (C)(D), respectively, after 24 h. Sodium carbonate was used as an alkaline pH buffer in 0.025% in TSB media. Elma™ 70 was used as an alkaline detergent in different concentrations (0-20%). The statistical differences resulted from comparison with the solution 0% in each case, for the three replicates that were tested (N = 3). p-values are indicated in the graphs when statistical differences are present, comparing to the control pH 7.0 (p ≤ 0.05, * is used when p ≤ 0.05, ** when p ≤ 0.01, and *** when p ≤ 0.001).

![Figure 4](image4.png) Confocal laser scanning microscopy images S. enteritidis biofilms formed under pH 7.0 (A) and pH 10.0 (B) conditions. A lens of 40x magnification was used to provide a field-of-view feature. The viable cells are labeled with Syto9 (green fluorescent dye) and the dead ones with PI (red fluorescent dye). Two replicates were prepared for the study of the biofilm architecture.

![Table 1](image5.png) Table 1. Cell viability of biofilm-linked cells. N=3.

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<th>Average % of steps</th>
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Figure 3. Biofilm formation and planktonic cell growth after the adjustment of the pH using sodium carbonate (A)(B) and Elma™ 70 (C)(D), respectively, after 24 h. Sodium carbonate was used as an alkaline pH buffer in 0.025% in TSB media. Elma™ 70 was used as an alkaline detergent in different concentrations (0-20%). The statistical differences resulted from comparison with the solution 0% in each case, for the three replicates that were tested (N = 3). p-values are indicated in the graphs when statistical differences are present, comparing to the control pH 7.0 (p ≤ 0.05, * is used when p ≤ 0.05, ** when p ≤ 0.01, and *** when p ≤ 0.001).

![Table 2](image6.png) Table 2. Biofilm thickness measurements. N=2.

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