# THE INFLUENCE OF EXTRINSIC STRESSES ON THE GROWTH AND ENDOTOXIN PROFILES OF ESCHERICHIA COLI AND PSEUDOMONAS AERUGINOSA

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## ABSTRACT

The LPS, endotoxin of Gram-negative organisms in communal growth as compared to pure culture was the focus of this research. The experiment aimed to show pure and communal samples grown in the presence of the extrinsic stresses. The change in toxicity was measured using the Limulus Amoebocyte Lysate (LAL) test. The overall sensitivity of organisms was similar for the same sanitiser and the same detergent. Growth in community was found not to be the arithmetic sum of the individual growth patterns. The detergents had a marked effect on the growth of all samples throughout the growth cycle. This finding reveals that the acceptable refrigeration temperatures still allows for pathogen growth and thus for biofilm formation. The quantification by LAL showed hat the enumeration of the food-borne pathogens isolated from households might not be indicative of acclimatisation obtained over short periods of time and the causal stress could turn these organisms into more or less toxic pathogens.

## 1. INTRODUCTION

Several inquiries have reported the inability of sanitisers to completely eliminate Gram-negative bacteria on fresh produce (Kim et al., 2000). Among these are the shiga toxin producing Escherichia coli (STEC) strains which have emerged as important human enteric pathogens. Strains which express, for instance, the lipopolysaccharides (LPS) O-antigen 157 (E. coli O157 strains) are commonly associated with severe clinical manifestations, including bloody diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome (Tarr 1994). Illness caused by E. coli occurs sporadically, in small clusters and outbreaks. The transmission occurs in a variety of ways, including through food and water and through person- to- person and animal-to–person contact (Armstrong et al., 1996). In addition to Escherichia coli, Pseudomonas aeruginosa, is also an opportunistic Gram- negative food borne pathogen, nosocomial and widespread in the environment. Particular features of P. aeruginosa include its ability to grow in low nutrient environments, and to cause both acute and chronic infections (Sadikot et al., 2005). The toxicity of pseudomonades results from the production of Toxin A which ADP ribosylates into elongation factor-2 (EF2-used in protein synthesis).

Apart from being an endotoxin, the LPS is the major constituent of the cell wall of Gram-negative bacteria. Several papers on the O-antigen ultra-structure and the influence of extrinsic and intrinsic factors thereon have been published in the last few decades (Vuddhakul et al., 2000, Venter et al., 2006; Abraham et al., 2009a; Abraham et al., 2009b).

Little mention has, however, been made of the influence of sanitisers and detergents applied in the food industry on the LPS structure of micro-organisms grown in mixed cultures. Quantitative analyses into LPS have been hampered due to their uncharacteristic structure, their miscellany chemically and physically and their expansive spectrum of biological activities has affected worldwide research in this field. Questions remain about the role of endotoxins in human health, especially pathophysiology (Petch et al., 2000). The Limulus Amoebocyte Lysate (LAL) assay has been extensively applied for the detection of endotoxins in quality control of injectable drugs and medical devices. The LAL tests detect the presence and concentrations of intact LPS and is taken as a comprehensive gauge signaling the presence (or assuring the absence) of pyrogens and/or pro-inflammatory species (Schindler, 2009).

This study aimed to investigate the effect of extrinsic stresses such as sanitisers, detergents and temperature on the growth and toxicity of Escherichia coli and Pseudomonas aeruginosa when grown as pure cultures or in combination.

## 2. MATERIALS AND METHODS

### 2.1 Strains, growth medium and growth conditions

Escherichia coli 0111 (ATCC 25093) and Pseudomonas aeruginosa ATCC (ATCC 27853) were obtained from an internal bacterial culture collection. Organisms were grown on PCA (Plate count Agar) overnight and then used to inoculate media in Erlenmeyer flasks. Al flasks were inoculated from an agar plate using inoculum wire and then grown in duplicate in 200 ml chemically defined Tryptone soy broth (Merck, SA) in 500 ml Erlenmeyer flasks. The choice for making use of this media is that Shiga toxin-producing strain detection is rapid, reliable and sensitive (Hussein and Bollinger, 2008), and this media is chemically defined which, rules out the possibility of any interfering variables. Growth (pure and communal), was done at 28°C, shaking for 30 hours and monitored by measuring optical density (OD620nm). Samples were collected at 0, 15 and 30 hours.

### 2.2 Sanitiser and detergent concentrations

Stock solutions of two sanitisers and detergents commonly used in the food industry were prepared. The active ingredients of these sanitisers and detergents were as follows: sanitiser one and two – chlorine based; detergent one and two – ammonium based. The stock solutions were used to supplement the experimental cultivation broth to a final concentration of 50% of the lethal dose which permitted growth of the organisms. This was determined by performing a growth study of each pathogen individually, combined and with each sanitiser and detergent in turn. A range of concentrations of sanitiser and detergent solutions were prepared and all experimental samples were incubated at all concentrations.

By plotting the percentage difference between the samples with sanitiser and detergent and those without against the concentration, the LD100 and the LD50 was determined.

### 2.3 Temperature

Growth conditions were as previously mentioned whereas temperature was varied to 4°C and 10°C respectively. These temperatures were selected as they represent the most common household storage temperatures.

### 2.4 Calculations

Absorbance readings of cultures with or without sanitiser and detergent (stressed and unstressed) were converted to percentage differences and calculated as follows:

$$\frac{[\text{OD 620 CONTROL}] - [\text{OD 620TEST}]}{\text{OD 620}} \times \frac{100}{\text{TOTAL}} \quad \frac{}{1}$$

### 2.5 LAL

The endotoxin concentration or the resulting toxicity was determined using the quantitative chromogenic LAL assay (end-point method) using the LAL Kit 50-648U (Cambrex). This test was performed by the microtitre plate method as described by Laitinen (1999) and Rhee and Kang (2002). The kit included vials of standard E. coli 0111 containing a defined number of endotoxin units (EU). A standard curve ranging from 0 – 0,12 EU/ml was constructed by plotting OD410nm versus EU per absorbance unit (Hansen et al., 1999). Sample preparations (with exact same cell density) were also assayed in duplicate and the concentration plotted against OD410nm. The absorbance of p-nitroalinine released from the substrate was measured using the BioRad 680 Micro plate Reader. All equipment used was pyrogen-free.

The LAL readings in EU, indicating degree of toxicity was then quantified in conjunction with OD readings for growth. This was analysed as follows: For the growth analysis the OD readings were processed to represent % difference in growth as follows:

$$\left(1 - \frac{OD_{test}}{OD_{control}}\right) X \frac{100}{1}$$

A positive reading indicates better growth by the control or unstressed sample; whereas a negative reading shows that the stressed samples exhibited better growth. Change in toxicity was calculated as follows:

$$\left(\frac{LAL_{TEST}}{OD_{TEST}}\right)-\left(\frac{LAL_{CONTROL}}{OD_{CONTROL}}\right)$$

Where a positive value indicated that there was a decrease in the toxicity of the cells after exposed to the stress, and a negative reading signifies an increase in toxicity.

## 3. RESULTS AND DISCUSSION

Similar growth profiles were observed for both E. coli and P. aeruginosa under identical growth conditions. The working (LD50) concentration for the sanitisers was determined as 3.2 x 104 PPM and that of the detergents as 1.6 x 104 PPM (results not shown).

Sanitiser 1: A small difference in percentage growth is detected for the comparison between stressed and unstressed samples. Sanitiser 1 did not influence the growth of E. coli significantly (Table 1). For P. aeruginosa on the other hand, there was a 94.4% difference in growth after the first 15 hours when compared to the control (Table 1). Sanitiser 2: This sanitizer did not influence the growth of E. coli in pure culture. P. aeruginosa on the other hand grew better when subjected to sanitiser 2 for 15 hours. Detergent 1: A large percentage difference in growth was observed for E. coli and P. aeruginosa over 15 – 30 hrs of exposure; in fact almost no growth was observed. Detergent 2: For E. coli the effect of detergent 1 and detergent 2 was similar. The mode of action of these stresses is related. The effect on P. aeruginosa was severe over 15 -30 hours of growth, with almost no growth observed in the test sample after 30 hours. Temperature effect: The growth of both E. coli and P. aeruginosa was affected at the storage temperature of 4ºC and 10ºC. The lower temperatures had a more pronounced effect on P. aeruginosa than E. coli (Table 1).

Endotoxin concentrations involved in biotechnology processes greatly depend upon the source of the product. They range from much less than 100 EU/ml in culture supernatants to more than 1 000 000 EU/ml in supernatants of high density bacterial cultivations (Petch et al., 2000). The stresses varied in their influence on the pure cultures. When growth occurred in combination or community a shock response was seen as famine or feast metabolic behaviour. When grown in combination, the two organisms could possibly have competed for nutrients or have worked together, subsequently the feast of famine metabolic response. When stressed with sanitiser 1, E. coli exhibited healthy growth during early growth which is indicative of a feast scenario. A marked slight decrease in toxicity was simultaneously observed (Table 2). P. aeruginosa showed weaker growth when stressed with a concomitant decrease in toxicity, at 15 hours of growth.

This reduction in toxicity may possibly be ascribed to an increase in lipid synthesis with a decrease in endotoxin (LPS) production as a defense mechanism. During communal growth the pattern mimics that of E. coli grown in pure culture, which leans towards this being an E. coli response rather than P. aeruginosa.

**Table 1: The percentage difference in growth between stressed and unstressed samples at 0, 15 and 30 hours. S1 –sanitiser 1; S2 – sanitiser 2; D1 – detergent 1; D2 – detergent 2**
**4º C and 10º C data expressed as log of absorbance at $620_{nm}$**

| STRESS | 0 HRS | 15 HRS | 30HRS |
|---|---|---|---|
| ***Escherichia coli*** | | | |
| S1 | 0 | 18.4 | 8 |
| S2 | 0 | 1.3 | 1.6 |
| D1 | 0 | 93.1 | 99.4 |
| D2 | 0 | 92.9 | 96.8 |
| 4º C | 0.003 | 0.01 | 0.012 |
| 10º C | 0.01 | 0.016 | 0.036 |
| ***Pseudomonas aeruginosa*** | | | |
| S1 | 0.8 | 94.4 | 35.3 |
| S2 | 45 | -18.6 | 4.4 |
| D1 | -29.7 | 79.9 | 51.9 |
| D2 | -60.4 | 79.1 | 98.1 |
| 4º C | 0.005 | 0.011 | 0.009 |
| 10º C | 0.002 | 0.016 | 0.018 |
| ***Escherichia coli + Pseudomon as aeruginosa*** | | | |
| S1 | -77.1 | 24.4 | 10.9 |
| S2 | 30.7 | 0.3 | 98.1 |
| D1 | -39.5 | 93.5 | 98.1 |
| D2 | -92.8 | 90.4 | 83.4 |
| 4º C | 0.004 | 0.011 | 0.004 |
| 10º C | 0.001 | 0.013 | 0.018 |

**Table 2: The change in toxicity measured by LAL assay due to extrinsic stresses over 0, 15 and 30 hours of growth expressed in endotoxin units (EU). S1 –sanitiser 1; S2 – sanitiser 2; D1 – detergent 1; D2 – detergent 2.**

| STRESS | 0 HRS | 15 HRS | 30HRS |
|---|---|---|---|
| ***Escherichia coli*** | | | |
| S1 | -300 | 33 | 6 |
| S2 | 402 | -17 | -9 |
| D1 | -41 | 96 | 99 |
| D2 | -553 | 97 | 98 |
| 4º C | -200 | 99 | 99 |
| 10º C | 0 | 99 | 98 |
| ***Pseudomonas aeruginosa*** | | | |
| S1 | 33 | 87 | 56 |
| S2 | 50 | -85 | 4 |
| D1 | -137 | 71 | 99 |
| D2 | -563 | 87 | 99 |
| 4º C | 17 | 96 | 99 |
| 10º C | 67 | 95 | 99 |
| ***Escherichia coli + Pseudomonas aeruginosa*** | | | |
| S1 | -200 | 55 | 0.09 |
| S2 | 58 | -2 | -10 |
| D1 | -133 | 91 | 99 |
| D2 | -310 | 88 | 98 |
| 4º C | -100 | 99 | 100 |
| 10º C | 50 | 99 | 99 |

Sanitiser 2 caused a small difference in percentage growth of E. coli with a more marked increase in toxicity when stressed. A possible increase in lipid A concentration, which is associated with a increase in 3-hydroxy fatty acid concentration implies a greater presence of LPS. At 15 and 30 hours of growth no noticeable difference in growth was observable and the change in toxicity generally resembles this trend, except for P. aeruginosa which showed a notable increase in toxicity at 15 hours. A possible reason for this might be that no lipid synthesis took place during this time and endotoxin release occurred due to the stress. No noteworthy change in toxicity was observed at 30 hours of growth. A most likely reason could be cell death and no endotoxin activity.

Detergent 1 initially had little effect on the samples as this can be seen by the slightly better growth by all samples.

The difference in toxicity manifested as an increase for the stressed sample. At 15 and 30 hours no significant difference in growth percentage could be observed except for E. coli at 30 hours. At the same time, all the samples showed a similar reduction in toxicity, with the change at 30 hours exactly the same (Table 2). When compared to detergent 1, detergent 2 initially caused a feast scenario with the cultures possibly engaged in lipid biosynthesis. It would be reasonable to assume increased 3 –Hydroxy fatty acids concentration.

Growth difference at 4o C was initially characterised by better growth by E. coli and the community, which most likely represented E. coli rather than P. aeruginosa. At 10 ºC samples surprisingly did not respond well to the stress and a very large difference in growth percentage was initially observed (Table 1). The corresponding change in toxicity did not mirror this change, but rather a moderate reduction was observed for P. aeruginosa and the community. At 15 and 30 hours a small reduction in growth an almost even reduction in toxicity can be seen (Table 2).

## 4. CONCLUSION

The sensitivity of E. coli, a typical food borne pathogen and P. aeruginosa, a typical spoilage organism varied to the sanitisers and detergents. A noteworthy finding is that the sensitivity was very similar for the same sanitiser and the same detergent. The sensitivity in communal growth was slightly varied, but generally followed that of the sensitivity of the individual organisms. The household storage temperatures inhibited the growth of the organisms, but did not prevent it. E. coli in particular thrived at 10ºC between 15 – 30 hours. P. aeruginosa showed an uncharacteristic difficulty to grow at these storage temperatures. The result for communal growth, nonetheless, is not at all a reflection of the combination of the growth patterns of the individual organisms. Considering the LAL test for the change in toxicity provides evidence that enumeration of food borne pathogens isolated from households might not be indicative of adaptation (acquired in shorts periods of time) and underlying stress induced “threats” rendering these organisms more/less toxic. Sanitisers and detergents influenced growth and competition in communal fashion. The sanitisers and detergents had a greater effect on older biofilm formation shown by a decrease in toxicity. The response to household temperatures was shock to adaptation at 10ºC and no evening out at low temperature.

## 5. REFERENCES

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