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

By

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DECLARATION OF INDEPENDENT WORK

I, ELVINA MELINDA SMITH, student number 206071523 do hereby declare that the research project submitted to the Central University of Technology, Free State for the Degree MAGISTER TECHNOLOGIAE: ENVIRONMENTAL HEALTH is my own independent work. This research study has not been submitted by me or any other person in fulfillment of the requirements for the attainment of any qualification.

…… ……

SIGNATURE OF STUDENT **DATE**

I dedicate this with LOVE to my parents,
the late Jimmy and Joan Smith.

THANK YOU……..

“I CAN DO ALL THINGS THROUGH CHRIST WHO STRENGHTHENS ME”
PHIL.4:13

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SUMMARY

The threat to the world food supply and the concern for public health as a result of food-borne diseases has been established as a constant global problem. The safety of food, in particular, is of significance to consumers and producers alike. Regarding the diseases related to food-borne pathogens, the disease syndromes affecting the entire human body has become inestimable. The focus of the study was to establish the effect of sanitisers, detergents and household storage temperatures on the growth profiles and toxicity of typical food related organisms. The endotoxin, LPS of these Gram-negative organisms in communal growth as compared to pure culture was the focus of the investigation. Pure and communal samples were grown in the presence of the extrinsic stresses including storage temperature. The change in toxicity was measured using the *Limulus* amoebocyte lysate test and the possible change in the immune response was determined using the porcine-IL-6 test. The first obvious finding was that the overall sensitivity of organisms was similar for the same sanitiser and the same detergent. The sensitivity of the community varied slightly but in principle followed the same pattern as the individual organisms. The LD_{50} for all growth samples were as follows: 32 X 10^4 PPM for sanitiser 1 and sanitiser 2, and 16X 10^4 PPM for detergent 1 and detergent 2. 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. The sub-optimum household storage temperatures inhibited the growth throughout the cycle but growth did not cease entirely. This finding may have revealed that the acceptable refrigeration temperatures still allows for pathogen growth and thus for biofilm formation. Furthermore, the response of the community to the extrinsic stresses appears to be entirely different to the pure culture and therefore needs further exploration to address the problem. Regarding the quantification by LAL, it was found that 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 turning these organisms into more or less toxic pathogens. The sanitisers and detergents induced competition in colonial fashion and the growth varied between feast and famine. The extrinsic stresses had a more observable effect on the older biofilm as this was shown by a decrease in toxicity. The toxicity as quantified by porcine-IL-6 yielded a mixture of stimulation levels for the cytokine. The toxicity change indicated by the test showed a variation between lowering and noticeable elevation for pure cultures. A marked elevation in toxicity was detected in community at storage temperature 4°C. The study would suggest that porcine IL-6 is not an accurate biomarker for pyrogenicity since its sensitivity is questionable and its inability to indicate toxicity if there is a possible change in the LPS structure. It should be said that further elucidation is needed to support this finding. Having said all that, it is no surprise that the validation for the two tests favours the LAL procedure. The large room for pre-test stimulation in pigs' blood also tends to cast a shadow on the IL-6 findings. The findings of the study contribute to the body of knowledge covering the effects and quantitative analysis of toxins in food. This should add to safety assurance by sensitizing the industry regarding the most suitable analytical methodologies to apply.

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LITERATURE REVIEW

1.1 GENERAL BACKROUND

The sustainability of life on earth is anchored in the occurrence and growth of micro-organisms. These micro-organisms, their growth cycles, influences on their environment and their biochemical pathways form an essential research zone towards achieving the sustainable life on earth. These facets of their being are confined to the environmental conditions under which micro-organisms can grow, and this is collectively termed its "habitat domain" (Wimpenny, 1981). The range of environmental conditions can be classified as intrinsic and extrinsic, which include such variables as temperature, pH, water potential ($à_w$), oxygen tension and antimicrobial compounds (Wimpenny, 1981). It is within this habitat domain where micro-organisms exercise their influence on our everyday lives. These influences may affect human health, food quality and supply and ultimately the sustainability of life. Over and above these, other factors having an influence on whether micro-organisms can exercise their effect or not happens during the research procedure and these may include the collecting of samples, transporting them to the laboratory, taking sub samples, and preparing a suspension. These are the

processes involved in the microbiological examination of food and the variability and inconsistency at each stage adds to the changeability of the result (Corry *et al.,* 2007).

So, in essence the growth of these micro-organisms plays a central role in their influence but it is also imperative that the exploration of other influencing factors should also be considered. Microbial scrutinisation could ultimately lead to the prevention of harm caused by microbes. It is regarded as a hygienic index of food production processes and is based on the sampling of raw materials, the product under process and the final product. Such sampling and scrutinisation facilitates the establishment of the effectiveness of good manufacturing, handling and delivery practices (Mossel, 1995). Microbiological information required for the quality assurance systems and, particularly, those concerned with food safety, provides the ability to make accurate decisions when harm arises and, even, to prevent its incidence (Buchanan, 1990). The prevention and monitoring of the possible harm which micro-organisms can cause should be a vigilant process focusing on all stages of the production/preparation process.

1.2 FOOD BORNE DISEASES

1.2.1 What is a pathogen?

A pathogen, according to Willey *et al*., (2008), is any organism that produces a change from a health state in which part or all of the host body becomes incapable of carrying out its normal functions. The ability of the pathogen to cause disease is known as pathogenicity. Furthermore, a primary pathogen is one which causes disease by invading a healthy host and interacts directly. This is unlike an opportunistic pathogen which forms part of the host's natural microbiota, but may cause disease when the host's immune system becomes compromised or when it accesses tissues in other sites. These would include prokaryotic and eukaryotic organisms with significance in genotypic and phenotypic characteristics. Table 1.1 lists some of the most prominent food borne pathogens, the foodtypes they are mostly associated with and the relevant local legislation.

Preservation of foods has, since the beginning of mankind been essential for our survival. The preservation techniques used in early days relied, without any understanding of microbiology, on the inactivation of the spoiling micro-organisms

Table 1.1 Major Food- borne pathogenic micro- organisms, the associated food and the South African Regulation

MICRO-ORGANISM	ASSOCIATED FOODS	REGULATION(RSA)
Clostridium botulinum	Low-acid foods, meat, fish, vegetables,marine products	R 692 Absent in 0,1g
Salmonella spp.	Beef, turkey, pork, chicken, eggs, shellfish	R 692 Absent in 1g
Listeria monocytogenes	Raw milk, soft cheese, ice cream, raw meat sausage	R 692 Absent in 1g
Staphylococcus aureus	Ham, turkey, chicken, pork, roast beef, eggs, salad	R 692 Absent in 20g
Bacillus cereus	Meat, vegetable dishes, milk, cream pastries, soup	R 692 Absent in 1g
Yersinia enterocolitica	Fresh meat, pork, vegetables, milk	R 692 Absent in 1g
Escherichia coli	Raw and rare meat, poultry, raw milk, unprocessed cheese	R 692 Absent in 20g of sugar

through drying, salting, heating and fermentation (Gram *et al.*, 2002). This kind of preservation, to a certain extent could guarantee a limited food supply beyond subsistence. Food spoilage is not a simplistic process and large amounts of food are lost as a result of microbial spoilage even when modern day preservation techniques are employed. Notwithstanding the heterogeneous nature of raw materials and processing environments, the microflora that develops during storage in spoiling foods can be predicted by taking note of the origin of food, the substrate base and a few fundamental preservation parameters. Spoilage of food through microbial means is by far the most common cause of food wastage and may reveal itself as visible growth (slime, colonies), as textural alteration (degradation of polymers) or as off –smell and off-taste.

1.2.2 Occurrence of typical food borne pathogens

The occurrence of food borne pathogens is usually established by following the incidence of the disease, although it is challenging to obtain accurate approximations of the incidence of microbial foodborne disease. An example of a recent outbreak is that of *Escherichia coli 0104*:H4 in Germany, Europe first detected on 6 June 2011. By 8 June 17 deaths had been reported and 1500 patients reported with hemolytic uremic

syndrome (HUS). This particular strain is a close relative of the strain investigated in this study. This strain (104:H4) has never been seen in the human population and may be the deadliest ever (Reuters, June 2011). When the ratio of this data is applied to the rest of the world, the problem is likely, that during a similar outbreak it could be expected to be even more extensive in developing countries. The disadvantaged and needy are the most vulnerable to ill health. Food and waterborne diarrhoeal diseases, for example, are of the principal causes of illness and death in less developed countries, most of whom are children (FAO/WHO, 1997). A recent example is what happened in the Eastern Cape, South Africa during 2008/9 where faecal matter had contaminated the water and a number of infants succumbed to this. Therefore, the occurrence of foodborne pathogens goes hand in hand with resulting disease and is predominantly there where conditions are unsanitary and sullied. This is clearly a global problem that requires continued attention.

1.2.3 The control of food borne pathogens

The safety of food products is of key importance to consumers and producers (Zwiettering and Hasting, 1997). Amongst others, a major part of the management of

food safety is decided by processing, hygienic processing lines are of significant importance to the manufacture of safe food (Zwiettering, Hasting, 1997). In addition many variables such as the composition, the process hygiene and the storage and distribution conditions could also determine the microbial quality and safety of a food product. When looking at these conditions collectively, a way of reducing the incidence of food loss can be derived.

A necessity for a system to manage the occurrence of foodborne pathogens and the subsequent disease became apparent. The Hazard Analysis Critical Control Points system (HACCP) was developed in the early 1970s. This system is used to manage the safety of food products methodically by paying special attention to the steps essential in the production of acceptable safe foods. Many food processing companies have introduced safety management systems based on HACCP principles. Application of the principles of HACCP has become mandatory for all exporting and importing food companies in South Africa (Regulation 908). To assist with the HACCP system further research in food safety is mandatory.

Subsequently, quantitative risk assessment to estimate the probability of adverse health consequences from microbial pathogens in food has developed from techniques used to assess the risk associated with chemicals. The procedure is complicated by numerous variabilities and unknowns. The scope of risk assessment is hampered or limited by the fact that microbial populations are not static in foods (Bernard and Scott, 1995). Over and above that, it is still necessary to have a methodical way to control the safety of food.

1.3 FOOD SPOILAGE

An estimated 25% of all foods grown world wide is lost post harvest or post slaughter due to microbial spoilage (Gram *et al.*, 2002). The cost of important agricultural products has hit the ceiling and in recent years has reached historical records. This trend seems threatening for poor households, as food covers 75% of their budget (Gram *et al.*, 2002). It is therefore evident that the significance of the food spoilage organisms cannot be underestimated.

1.3.1 Typical food spoilage organisms

The types of organisms which can be classified as food spoilage agents can come from a number of Phyla, Genera or families of micro-organisms. Each and every food product can harbour its own specific and characteristic microbiota at any given time during manufacturing and storage. The metabolite production associated with the spoilage of a particular product is indicative of the spoilage potential of a micro-organism in pure culture. In general, numerous organisms isolated from a food product will be able to produce spoilage metabolites when allowed to grow without any limitations (Gram *et al.*, 2002). As seen from Table 1.2, a large array of micro-organisms can, under certain conditions, be a contributing factor to the spoilage of food. In theory, it can be assumed that all micro-organisms are initially present on a food product and the nutrient composition along with the chemical and physical parameters primarily determine the selection of a particular microorganism(s) (Gram *et al.*, 2002).

1.3.2 Role played by *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a typical example of a food spoilage organism. This organism is an ever-present Gram-negative bacteria which can be found in water, soil and organic waste. This psychotropic bacterium is habitually found in domestic refrigerators, milk and dairy products (Cousin, 1982). The magnitude of psychotropic bacteria is growing with the requirement for dairy products with an increased shelf life. Initially, the world wide trend towards the merging of smaller dairy companies into larger production units has resulted in extended phases of the storage of raw milk until the final products are obtained. These longer storage periods provide an opportunity for the growth of *Pseudomonas,* which usually produce extracellular thermostable lipases and proteinases which subsequently cause depreciation of milk quality before and after processing (Garcia *et al.*, 1989). The answer to this dilemma is that recently companies have invested in more trucks, better storage facilities and modern, state of the art equipment.

1.4 HABITAT OF *ESCHERICHIA COLI* AND *PSEUDOMONAS AERUGINOSA*

1.4.1 Natural communal habitat

Bacteria can exist in the planktonic state and/or in communities. A large number of bacterial populations exist in liquid milieus, nevertheless, at the same time a significant number is capable of managing their behaviour to form sessile communities made up of a large number of densely packed cells. These well designed or orchestrated complex communities, called biofilms, form on the surface or at air-liquid interfaces. An extracellular polymeric matrix (EPS), compiled of polysaccharides, proteins and often DNA holds all cells together within the biofilms. Within these communities, all the members cooperate in the assembly of the biofilm by supplying matrix components (Bassler and Losick, 2006).

Table 1.2: Typical spoilage organisms of food products depending on physical/chemical preservation profile. (Adapted from Gram *et al.,* 2002)

Temperature		Atmosphere		pH		Aw		Substrate-base				
Low	**High**	**Aerobic**	**Non-aerobic**	**Low**	**High**	**Low**	**High**	**Amino acids**	**Simple CHO**	**Complex CHO**	**Typical product**	**Typical spoilage organism**
X		X			X		X	X			Fish	*Shewanella, Pseudomonas*
X			X		X		X	X			Fish	*Photobacteria, Shewanella*
X			X		X	X		X			Smoked fish	LAB, *Enterobacteriaceae, Photobacterium*
X			X	X		X		X	X		Marinated fish	LAB, yeasts
X		X		X			X	X	X		Meat	*Pseudomonas*
X			X	X			X	X	X		Meat	LAC, *Enterobacteriaceae, Brochotix, Clostridia*
X			X	X		X		X	X		Meat products	LAC, *Enterobacteriaceae, Brochotix*
	X	X			X		X		X		Milk	*Pseudomonas, Bacillus*
	X	X			X		X			X	Raw vegetables	*Erwinia, Pseudomonas*, Fungi
	X		X		X	X		X			Eggs	*Pseudomonas, Enterobacteriaceae*
							X		X	X	Fruits	Yeasts, Filamentous, Fungi
											Mayonnaise salads	Yeasts, LAB
	X			X			X		X		Beer	LAB, Yeasts
	X			X			X		X		Wine	LAB, Yeasts
	X		X							X	Cereals	Filamentous fungi
	X		X							X	Nuts	Filamentous fungi

1.4.2 Biofilm formation

A biofilm is a population of microbes, aggregated on surfaces, embedded in a matrix of extracellular polymers. These communities can be made up of a single species or, often, of numerous species of bacteria or fungi and bacteria. Bacterial cells in these biofilms are in their common natural growth state wherever moisture and sufficient nutrients are available (Nobile and Mitchell, 2009). The formation of this communal state or biofilm may be seen as a developmental process consisting of four common traits: attachment and aggregation; extracellular matrix production; orchestrated behaviour and communication and the generation of diversity (Fig. 1.1). The methodology governing these traits are immeasurably different from species to species, but they come together to produce very similar results (Nobile and Mitchell, 2009). The diversity generated manifests itself in the way bacteria use a variety of means to communicate with one another and with their eukaryotic hosts. In various cases, community interactions allow bacteria to orchestrate their behaviour and thereby act like multicellular organisms. In contrast though, some bacterial communal connections promote individuality among

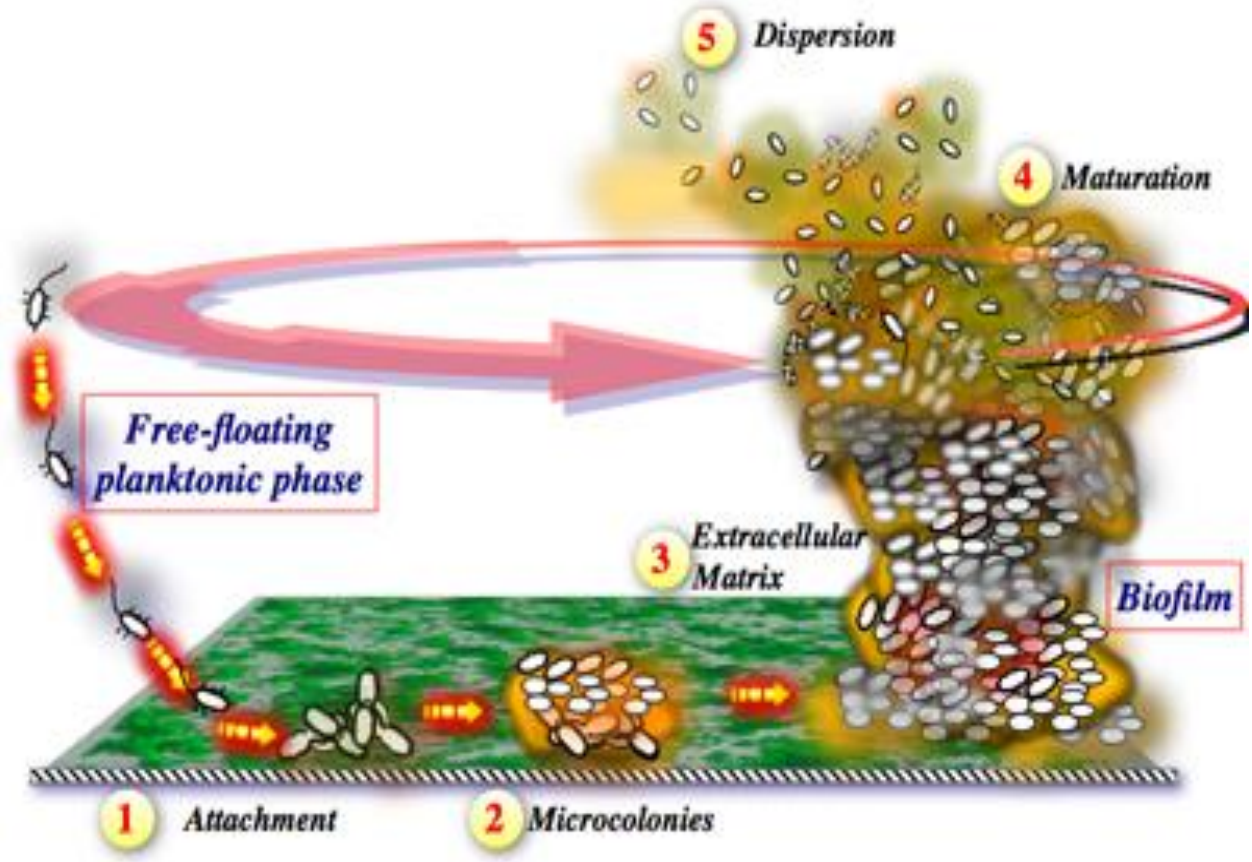

Figure 1.1: An outline of the mechanism of biofilm formation

(Adapted from Ghigo, 2006)

members within the group and thereby advance heterogeneity (Bassler and Losick, 2006). The discovery of quorum sensing, made it apparent that bacteria possess stylish and complicated systems of communication that enable them to send and receive chemical signals to and from other cells. In its uncomplicated form, quorum sensing is a cell-cell conversation mechanism by cells in their environment, by which they tally their own numbers through manufacturing and perceiving the accumulation of a signaling molecule that they export (Bassler and Losick, 2006).

The EPS, mentioned before, constructed by all the members of these bacterial communities, cooperate in the assembly of the biofilm by donating matrix components (Bassler and Losick, 2006). The extracellular polymeric matrix acts as a blockade through which diffusive transport occurs by diffusion rather than in a convective fashion. A common function assigned to the EPS is their broad-spectrum protective action on biofilm micro-organisms against undesirable conditions. The EPS matrix is able to slow down or preclude antimicrobials from reaching target micro-organisms within the biofilm by curbing diffusion and/or chemical cooperation with the extracellular proteins and

polysaccharides (Simões *et al.*, 2010). In support of that, it has repeatedly been observed that biofilm cells can tolerate higher concentrations of biocides than their planktonic counterparts (Simões *et al.,* 2010).

It has been observed in many bacterial species that biofilm formation appears to be promoted by non-optimal or unfavourable growth conditions or any stress experienced by the cell. The resulting growth in a chemically defined medium produces increased biofilm formation as compared to isolates of *E. coli* grown in a rich medium. The observed growth at temperatures much lower than the optimum is characterised by the trigger of the production of adhesion factors such as curly fibers in enterobacteriaceae (Landini, 2009). The most descriptive, notwithstanding indirect evidence for the activation of stress responses in biofilm cells comes from the study of prophages which are made in mature *P. aeruginosa* biofilms, leading to sloughing and dispersal of the biofilm itself (Landini, 2009). Collectively, factors such as environmental conditions, the availability of nutrients, ionic strength and osmotic pressure could have an effect on biofilm formation (Kawarai *et al.*, 2009).

1.4.3 Significance of biofilms to food Industry

The presence of biofilms can be hazardous to both human life and industrial practices. The occurrence may cause infection, pathogen contamination and slime development. In contrast, these may be advantageous in environmental technologies and bioprocesses (Hori and Matsumoto, 2010). In any food processing location, microbial biofilms could lead to unfavourable and undesirable outcomes. They could be an incessant source of contamination to foods coming in contact with them when formed on contact surfaces (Joseph *et al.*, 2001). The biofilms formed in the pipelines, in particular, in the food-processing environment are identified as a latent source for food poisoning. Biofilms here are typically removed by cleaning in place (CIP) washing or by hand washing after breaking down or disassembling the machinery but this modus operandi is very labour intensive. In light of this, the inhibition or prevention of biofilm formation may be the paramount way for controlling biofilms in food processing equipment (Furukawa *et al.*, 2010).

When considering the dairy industry, it is reported that biofilms may be a source of refractory contaminations, causing food spoilage and representing possible sources of public health problems such as outbreaks by foodborne pathogens. The main basis of contamination of milk and milk related products are regularly as a result of unacceptable cleaning and disinfection of equipment. Biofilms found in the dairy industry are primarily by bacterial extracellular polymeric substances (EPS) and milk residues (Flint and Hartley, 1996).

Pseudomonas spp. are one of the most crucial bacteria causing spoilage of conventionally pasteurised liquid milk products, acting in two different ways. In the first instance, they produce the majority of lipolytic and proteolytic enzymes secreted in raw milk during storage before processing, even in psychotropic environments. Secondly, after pasteurisation, *Pseudomonas spp*. can cause spoilage of conventionally pasteurised milk during refrigerated storage (Flint and Hartley, 1996). The impact of contamination by micro-organisms or the formation of biofilm is clearly a significant consideration in industry.

1.4.4 Domestic significance

Bacteria are able to colonise/survive on most food preparations surfaces, utensils, domestic dishcloths, sponges and other cleaning materials, from where they can contaminate food (Jackson *et al.*, 2005). Reported statistics indicate that the incidence of foodborne illness in Europe is unacceptably high, even though it is a noteworthy underestimation of the true enormity of the problem. For the period 2005 – 2009, 16263 confirmed cases of verotoxin - producing *Escherichia coli* in humans and food were reported throughout Europe (EFSA Technical report, 2011). The most frequently cited sites of outbreaks of foodborne disease are restaurants, hotels and take-aways but, it has been suggested that foodborne illness is encountered in private homes three times more regularly than in commercial operations (Borneff *et al.*, 1988). Up to 50% of these incidents are ascribed to inappropriate food storage including ineffective cold storage and refrigerator handling. Adding to the gravity of this scenario is the increasing importance of frozen “ready- to- eat” products, which have now become more than 60% of the average European shopping basket, and is on the rise in developing countries. This fact would imply that the refrigeration practices will continue to be major

determinant in domestic food safety. In the event of failure to follow correct practices in the adjustment, maintenance, use or cleaning of domestic refrigerators a number of risks could arise for consumers. This is significant because refrigerators form an important linkage in the extensive chain of cross-contamination, and causes 28% of outbreaks of domestic foodborne disease (Ryan *et al.*, 1996). This situation is worsened by bacteria contaminating unwashed raw foods, leaking packages and hands in refrigerators which may directly or indirectly contaminate other stored foods. Alternatively, biofilms may form, attach to and persist on the internal surface of the refrigerator posing risks of indirect longer term contamination during ensuing food preparation activities (Michaels *et al.*, 2001). Countless domestic refrigerator temperatures are incorrectly adjusted, operating above the advised temperature and are therefore capable of supporting sub-optimum but significant growth of mesophilic organisms (Jackson *et al.*, 2005). The possibility of fungal growth at storage temperatures should also be mentioned. Even though most molds prefer warmer temperatures, they are able to grow at storage temperatures. They are able to grow on

refrigerated jams and jellies as well as on salty meats such as ham, bacon and salami (FSIS, 2011).

There is, nonetheless very little information on the spread to, and persistence on the interior surfaces of domestic refrigerators, making it difficult to quantify the affliction of such pathogens in these environments, and at the same time to assess the threat they pose to consumers (Jackson *et al*., 2005). Therefore, further elucidation of the threat of contamination through growth and transfer inside refrigerators is needed.

1.5 GROWTH OF MICRO-ORGANISMS

1.5.1 General background

Micro-organisms grow and thrive under favourable conditions. In general the growth time of an organism in culture depends on the growth medium and the incubation conditions. When a microbial population is inoculated into a fresh medium, growth usually commences only after a period of time called the lag phase (Fig. 1.2). The phase may be brief or lengthy, depending on the size of the inoculum and the growth

conditions responsible for carrying out the growth reactions. When a micro-organism is transferred to a medium where essential metabolites must be biosynthesised, time is needed for the production of the new enzymes responsible for catalysing these reactions (Riley *et al.*, 2008). During the exponential phase (Fig. 1.2), which follows the lag phase, the growth of each cell is characterised by the division to form two cells, each of which also divides to form two more cells, repeatedly. The time period for this, depends on the availability of resources and environmental factors. Cells during this phase are typically in their healthiest state. As the cell growth goes through the growth phase, the nutrient to mass ratio decreases. The rate or pace of exponential growth is affected by environmental conditions such as temperature, composition of the culture medium, as well as the genetic characteristics of the organism itself. The exponential growth phase is limited by the essential nutrients of the medium being used up or by the cells and the accumulation of waste products in the medium, hindering exponential growth.

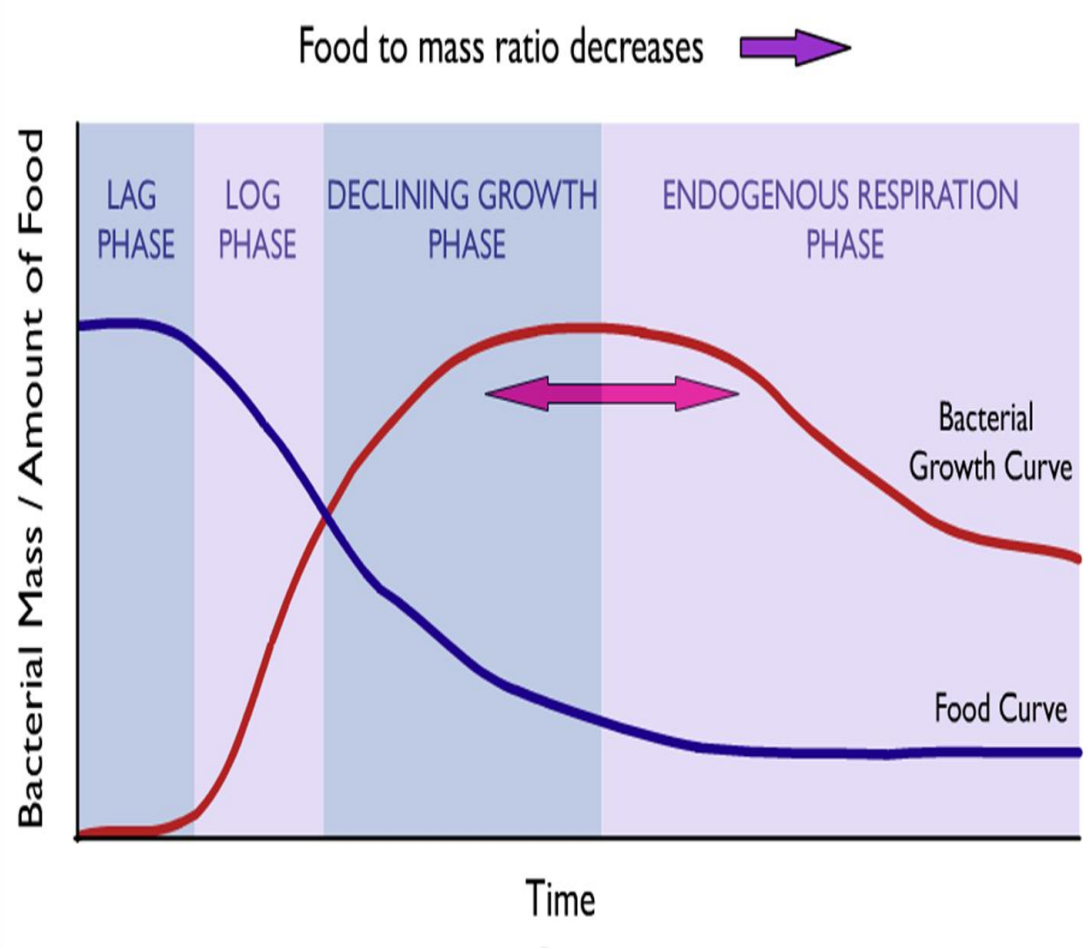

Figure 1.2: A general outline of the growth phases of micro-organisms and the food to mass ratio (Surgi, 2010)

Growth is subsequently terminated and the population growth reaches the stationary phase (Fig. 1.2). During the stationary phase there is no net change in cell number and thus the growth rate of the population is nil. Most of the cell functions such as energy metabolism and biosynthesis continue during this phase. In the event of incubation with continued growth beyond the stationary phase, the cells will eventually die. The death phase of the growth cycle is now reached. Although most research is not conducted in or on micro-organisms in their natural habitat, a batch culture does provide crucial information on microbiological interaction and related toxicity which should be taken into consideration in future research.

Temperature, or more correctly cardinal temperature, is almost certainly the most important environmental factor affecting growth and the subsequent survival of micro-organisms. At this point it should be mentioned that not much is known about the cardinal temperatures on communal growth. Either too low or too high a temperature will inhibit the growth of micro-organisms and may even cause death (Riley *et al.*, 2008).

When there is an increase in temperature, chemical and enzymatic reactions are accelerated and the resulting growth proceeds faster, however at and above a certain temperature, cell components may be irreversibly destroyed. Each type of micro-organism has a minimum temperature below which growth is not possible. Four classes of micro-organisms can be identified according to their growth temperature optima: psychrophiles, with low temperature optima, mesophiles with midrange temperature optima, thermophiles, with high temperature optima and hyperthermophiles, with very high temperature optima (Riley *et al.*, 2008). Interestingly, a number of models for predicting the growth rate of micro-organisms, as a function of either temperature alone or of temperature combined with other factors have been put forward recently (Kovářová *et al.,* 1996). In addition, other factors which could have a significant effect on bacterial growth rate are pH, osmotic pressure and oxygen tension. Every micro-organism has a range within which growth is possible and typically shows a well-defined growth optimum for all these (Riley *et al.*, 2008).

1.6 TOXINS

1.6.1 TYPES OF TOXINS

1.6.1 .1 Exotoxins

Exotoxins are proteins excreted from the microbial pathogen as they grow. Exotoxins migrate from a site of infection and cause injury at distant sites. These toxins fall into one of three categories: the cytolytic toxins, the AB toxins and the superantigen toxins (Riley *et al*., 2008). These exotoxins may be secreted by a number of micro-organisms, *Pseudomonas aeruginosa* being one. *P. aeruginosa* is a Gram-negative, aerobic, non-lactose-fermenting extracellular bacterium (Orbritsch *et al.*, 2004).

When conditions are favourable, it acts as an opportunistic pathogen, but in an immunosuppressed host it may bring about serious infections. *Pseudomonas* genus produces numerous chemicals which may influence its pathogenicity (Nicas and Iglewski, 1985). Among them, exotoxin A (ETA) may be considered the foremost and the most lethal virulent factor fabricated by the majority of clinical isolates (Hamood *et al*., 1996). It is widely acknowledged that ETA inhibits protein synthesis in mammalian

cells through the transference of the ADP-ribose moiety of NAD^+ to the elongation factor 2, leading to subsequent cell death (Iglewski *et al.,* 1977). In contrast to exotoxins, which are the secreted products in living cells, endotoxins are cell bound and released in large amounts only when the cells lyse (Riley *et al.*, 2008).

1.6.1.2 Endotoxins

The name endotoxin acclaimed to Pfeiffer 1904 (Westphal *et al.*, 1984), was established to differentiate heat stable toxin release during bacterial lyses from secreted, heat sensitive bacterial exotoxins. When endotoxins are in pure chemical form it is known as lipopolysaccharides (LPS) (Hodgson, 2006). Bacteria which may bring about alterations in their LPS structure can play a role in the avoidance of immune detection through molecular mimicry of host structures (Moran and Prendergast, 2001). Numerous Gram-negative bacteria construct lipopolysaccharides as part of the outer layer of their cell envelope (Fig. 1.3). It is made up of lipid a, a oligosaccharide which forms the core and an O-antigen with repeated units. The lipid A is non-polar and it anchors the molecule in

the outer membrane, while oligosaccharide core joins the lipid A to the O-antigen (Abraham *et al.,* 2009; Fig. 1.3).

The peculiar permeability properties that distinguish this outer membrane from the inner membrane are mainly as a result of the presence of LPS in its outer edge of the outer membrane. More than a few distinctive structural features of LPS contribute to the effective permeability barrier of the outer membrane (Sperando *et al*., 2009). A feature of the cell membrane is that it is trilaminar made up of a cytoplasmic membrane, peptidoglycan layer and outer membrane. The LPS molecules are found mainly in the outermost monolayer of the outer membrane (Seltman and Holst, 2002). The properties of exotoxins versus endotoxins are summarised in Table 1.3. Endotoxins are consequently an essential component of the outer membrane and are a key example of unique and highly conserved bacterial surface molecules that connect with the inborn immune system of the mammalian host (often human) by means of pattern recognition receptors on a range of host cells (Hodgson, 2006). Human beings are exceedingly sensitive to LPS. Pico gram quantities of LPS are usually sufficient to trigger a shock

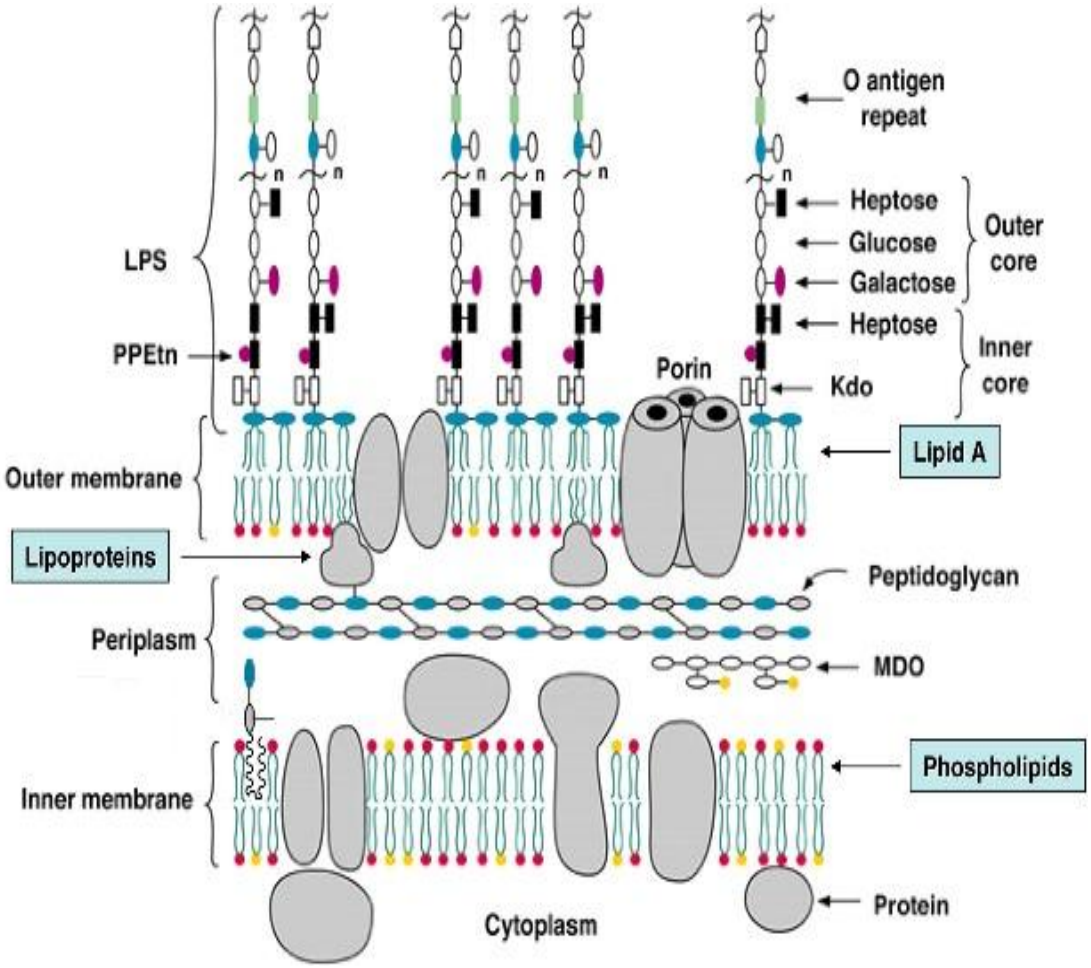

Figure 1.3: Ultra-structure of the LPS of Gram-negative bacteria.

Kubelt, J. 2004

response. This sensitivity to LPS has a lot to do with the catalytic upshot of the LPS binding protein and is shared with other animals (Hodgson, 2006).

Table 1.3: A comparison between the characteristics of endotoxins and exotoxins.

A COMPARISON:	
EXOTOXINS	ENDOTOXINS
• Secreted actively from viable microbes	• Part of the bacterial architecture
• Heat labile; Protein	• Heat stable; Lipid/sugar
• Very specific activity	• Low specific activity
• Very toxic	• Variable toxicity
• Toxoidable: can be denatured to remove toxicity and retain antigenicity	• Nontoxoidable: chemical composition prohibits molecular modification

The foundation for differences in sensitivity may lie in the expression of surface LPS receptors by immunologically relevant cells (Roeder *et al.*, 1989). In conclusion, LPS plays a major role in pathogen-associated molecular patterns that not only alert the host

to infection, but also prevents unsuitable autoimmune reactions (Krug *et al*., 2001). LPS infection is often referred to sepsis.

During Gram-negative sepsis, the endotoxin stimulates host macrophages to release inflammatory cytokines and excessive inflammation which could cause multiple organ failure and death. *Limulus* Amoebocyte Lysate (LAL) detection has been widely used for over 25 years for the detection of endotoxins. Recombinant Factor (rFC), the endotoxin inducible coagulation enzyme in LAL, forms the bases of a novel micro-enzyme assay for high throughput screens of endotoxin and opens up a new era in endotoxin testing (Ding and Ho, 2001). Other innovative diagnostic biomarkers are in development to assist in rapid diagnoses and therapy for sepsis. One such measure is procalcitonin (PCT), a non-specific indicator of systemic inflammation. This marker compares favourably with a number of other standard and experimental biomarkers (Opal, 2007). One such procedure for detection is the Porcine-IL-6 detection test. This assay detects the presence of interleukin-6, which is one of the cascades of biomarkers in the immune response in humans. Focus on this type of research may open more avenues to a

greater understanding of the complexities of the mammalian immune system (Hodgson, 2006).

The endotoxin molecule is a non-constant and may change form depending on intrinsic and extrinsic factors. The intrinsic factors are determined by the type of bacteria and the extrinsic factors are as a result of a changing environment (Abraham, 2009). Phenotypic variation in the LPS structure of Gram-negative bacteria may be influenced by sub-optimum temperature, unfavourable growth conditions, biofilm formation, limitations of salt, sucrose and glycerol, limiting concentrations of phosphate and variation in pH. The possible variations may include an increased production of the O-chain, loss of the O-chain, incorporation of some palimtoleic acid [16:1] into lipid A, non-hydroxylated fatty acids is decreased , hydroxylated fatty acids increase and an absent polymeric side chain (Abraham, 2009).

1.6.2 Toxins in food

Food poisoning is mostly induced by enterotoxins food poisoning can universally be divided into infections and intoxications (Granum *et al*., 1995). The bacterial toxins produced in a food chain are of special importance when it comes to human food production for human consumption. The entry mechanisms of these bacteria into the food chain vary and they may accumulate reaching high, often lethal concentrations (Mebs, 1998).

E. coli, a Gram-negative bacterium, is autochthonous to intestinal tract of humans and other mammals. Hitherto, there are a number of *E. coli* strains which represent primary pathogens with augmented potential to cause diseases, specifically diarrhea and urinary tract infection (Cohen and Gianella, 1995). A great assortment of other strains belonging to many dissimilar serotypes can be isolated from the environment and these may contaminate food (Chinen *et al*., 1993). Shiga toxin producing *E. coli* (STEC) were first distinguished as human pathogens in 1982 when *E. coli* 0157:H7 was responsible for two outbreaks of hemorrhagic colitis related to the consumption of undercooked beef

(Doyle and Padhye, 1989). As of then, an excess of 100 serotypes of STEC have been isolated from animals, food and other sources. Outbreaks caused by 0111:NM, in particular, has been reported in Italy (Caprioli *et al.,* 1994). More recently the outbreak in Northern Germany of serotype 0104:H4 during June 2011 (Reuters, June 2011). The study of Shiga toxins is extensive and they are viewed as well established virulence factors and food safety threats (Meng *et al*., 1998).

1.6.3 Antimicrobial agents

Unlike biofilm and communal growth, antimicrobial agents may also influence the action of microbes. Since Sir Alexander Fleming discovered penicillin in 1928, it has become broadly recognised that micro-organisms engage in chemical warfare in which one species wards off other species through the secretion and discharge of antibiotics and other antimicrobial agents. Often, as is seen in the case of bacteriocins, bacteria may produce chemicals that kill/hamper other strains of the same species (Bassler and Losick, 2006). Micro-organisms adhere to surfaces by every possible means to form biofilm of community to effectively arrive at resistance to antibiotics and infection.

Alternatively, they have evolved with the mechanism for adhesion under various environmental conditions. Any inhibition of adhesion and/or biofilm formation is quite difficult against all micro-organisms in environments even when it is possible against specific species (Hori and Matsumoto, 2010).

A search for combating adhesion and community formation is ongoing. The use of sanitisers is an effortless and frequently used way to control biofilms. Specifically, the resistance of pathogenic bacteria in biofilms to antimicrobials is at the root of many persistent and chronic bacterial infections (Hori and Matsumoto, 2010). It is because of this obstacle that a wider understanding of communal growth is necessary.

Anti-microbial agents can be divided into three categories, namely, antibiotics, oxidants and biocides. The effectiveness of these was examined on planktonic cells and biofilms. It was found that chlorine, an oxidant, the most common disinfectant, reacts with various components within bacterial cells. Chlorine forms part of the most common and abundant domestic sanitisers. Having said that, the effectiveness of chlorine against

biofilms is three times lower than against planktonic cells. The difficulty now is that higher concentrations of chlorine are required to effect disinfection against biofilms, but the use of chlorine produces unsafe disinfectant by-products (Hori and Matsumoto, 2010). Furthermore, the rate of increase in resistance to antimicrobials in enteric bacteria has been found to be much higher than in pathogens found elsewhere. (Mayrhofer *et al*., 2004). There is much debate about the development of resistance to antimicrobials human medicine, as well as in agriculture (Mayrhofer *et al*., 2004). This could possibly be attributed to the fact that many micro-organisms in biofilms or in a sessile condition express different physiological phenotypes from those in the planktonic state. It now follows that the effective control of unwanted biofilms can be attained by understanding the type and nature of the contaminating residue materials (carbohydrates, fat, protein, mineral salts) and the type of unwelcome micro-organisms on the surfaces. The selection of the required sanitisers and detergents will then play a pivotal role (Mayrhofer *et al*., 2004).

The selection of detergents and disinfectants depends on their efficacy, safety and ease of removal. This is especially true when considering the corrosive nature of the chemical treatments and the subsequent effects it could have on the final products. The most effective sanitation programme cannot make up for basic deficiencies in equipment design. As long as the equipment and processing environment are hygienically designed, having no fissures and dead space, an effective cleaning and disinfection programme is the main line of attack to control surface contaminations. Undesirable material from the surfaces, including micro-organisms, scum, foreign bodies and residual cleaning products may be removed by an effective sanitation programme (Simões *et. al.*, 2010), such as CIP where the contact time, type of dirt and the concentration of the sanitiser may vary. A number of studies have been done on the chemicals with strong biofilm-specific inhibitor action and in the food-processing milieu; safety concerns exist around the use of some of these anti-biofilm compounds. Food additives, an example of these, are safe and if some of these additives had biofilm inhibitory activity, it stands to reason that they would be sought-after for the use in

controlling biofilm in the food environment. This would be especially so to eliminate the toxins compromising food safety (Furukawa *et al*., 2010).

1.7 RATIONALE

The threat of a reduction in the world's food supply of the world remains a constant concern. The safety of food is of significance to consumers and producers. Therefore, an all encompassing approach is necessary to reduce food loss. This problem is greatly enhanced in developing countries where the food supply is already under extreme pressure. Notwithstanding the global food supply, the concern for public health as a result of food-borne diseases has become a larger problem with the advent of the discovery of an increasing number of biocide and other antimicrobials resistant micro-organisms. The current mortality rate remains unacceptably high for patients who develop severe sepsis or septic shock as a result of Gram-negative pathogens. In response, most scientific studies have focused on the planktonic existence of typical food-borne micro-organisms as a controllable and easily monitored environment.

In view of the knowledge regarding biofilms, it is crucial to ask to following: What is the practical implications surrounding the introduction, presence, spread and eradication of food-borne pathogens in the home? Is the incidence of food poisoning in the home due to ignorance and negligence regarding a proper cleaning regime, use of sanitisers and detergents? Due to microbial populations not being static in food, does the storing, cleaning and sanitising routine within the kitchen always prevent the contamination or spread food-borne toxins, especially in community? Is the selection of detergents and sanitisers by consumers affected by the media, price and/or safety? Are these effective for the reason they were purchased and are they used as is required and does this also affects the efficacy of these products? Also, how the resistance of the pathogens is influenced by communal growth, especially on surfaces in the fridge.

To try and answer some of these questions the aim of this study was to determine the influence of extrinsic stresses, which include household sanitisers and detergents and storage temperature on the communal growth of typical food-borne bacteria (*E. coli* and

P. aeruginosa), with emphasis on changes in endotoxicity of LPS. The objectives of the study have been addressed as aims in different chapters as follows:

- CHAPTER 2: The influence of extrinsic stresses on the communal growth of typical food related organisms.

  Objectives:

  - to determine how sensitive a typical spoilage organism (*P. aeruginosa)* and food borne pathogen *(E. coli*) are to commercial sanitisers, detergents and storage temperature

  - to determine if there was any change in sensitivity of the micro-organisms to the sanitisers, detergents and storage temperatures when it occurs in community autochthonous.

  - to determine any change in growth patterns in communal growth.

- CHAPTER 3: The Influence of extrinsic stresses on the toxicity of *Escherichia coli* and *Pseudomonas aeruginosa* as quantified by *Limulus* Amoeboicyte Lysate Test.

Objectives:

- Determine the influence of household sanitisers, detergents and storage temperature on the toxicity of *E. coli* and *P. aeruginosa*

- Compare the possible changes in toxicity between the stressed pure culture and the stressed communal growth.

- CHAPTER 4: The influence of extrinsic stresses on the toxicity of *Escherichia coli* and *Pseudomonas aerigunosa* as quantified by Porcine – IL-6 production.

  Objectives:

  - Quantify the influence of sanitisers, detergents and household storage temperature on the toxicity of *E. coli* and *P. aeruginosa*.

  - Quantify the change in toxicity using Porcine-il-6 production

- CHAPTER 5: A comparison of the validity, accuracy, shortcomings of the *Limulus* Ameoboicyte Lysate Test and Porcine–IL-6 production, for the quantification of change in toxicity.

- CHAPTER 6: Conclusion

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CHAPTER 2

THE INFLUENCE OF EXTRINSIC STRESSES ON THE COMMUNAL GROWTH OF TYPICAL FOOD RELATED ORGANISMS

2.1 ABSTRACT

The threat of the dwindling food supply of the world and the increase in human infections motivated this study whereby two food borne pathogens were subjected to an array of extrinsic stresses. Furthermore the fact that most studies regarding pathogens have been performed on pure cultures, and not communal growth, this study attempted to address that by investigating communal growth. *Escherichia coli*, a Gram-negative, shiga toxin producing human enteric pathogen and *Pseudomonas aeruginosa*, an opportunistic, nosocomial spoilage and pathogenic bacterium were grown in duplicate under controlled conditions. Organisms were subjected to two sanitisers, two detergents and two household storage temperatures. Growth of the stressed samples was then compared with the unstressed samples for pure and communal growth. The working concentration taken from the 50% lethal dose (LD_{50}), was found to be 3.2×10^4 PPM for sanitiser 1 and sanitiser 2 and 1.6×10^4 PPM for the detergents. The response was generally the same throughout for the same stress. The finding for communal growth did not mimic the growth of the pure culture, nor was it an accumulative response of the two. Growth at the storage temperatures was not visibly inhibited, and once again

communal growth showed character and behaviour of its own. The possibility of the unique biofilm communication and supportive community could have been a strong possibility in communal growth.

2.2 INTRODUCTION

The basic human necessity for the intake of food puts every human being at risk of becoming infected by food-borne pathogens. This fact is a reality, not only in developing countries, but in many developed countries as well. Therefore specialists in food safety and public health professionals continuously emphasise the control of food-borne pathogens in industries during processing, transport and storage. Furthermore, data from a number of analyses performed in different countries have indicated that a large number of outbreaks originate in the home (Beumer and Kusumaningrum, 2003; Mead and Griffin, 1998). Interestingly, the data collected on the risk factors for food-borne diseases or infection, denotes that the prevalence of these outbreaks is the consequence of faulty food handling practices (Mead *et al.*, 1999).

The spate of human infections related to consumption of raw fruits and vegetables have amplified in recent years, owing to some factors such as changes in agronomic and processing practices, an escalation in *per capita* consumption of fresh produce and intensified international trade and dissemination. Probable sources of contamination in fresh produce include unprocessed produce, handling workforce and food processing plants (Sengun and Karapinar, 2004). Regardless of progress in hygiene, consumer consciousness and food treatment and processing, food-borne diseases initiated by pathogenic micro-organisms still represent a significant threat to public health worldwide (Mead *et al*., 1999).

2.2.1 Sanitisers and detergents

A sanitiser is a product used to treat food-contact surfaces to destroy most of the contaminant bacteria, but at the same time does not adversely affect the product or the safety of the consumer. Although an assortment of antimicrobials including chlorine, chlorine dioxide, calcined water and organic acids has been scrutinised for their ability to lower the levels of food-borne pathogens on fresh produce, several inquiries have

reported their inability to completely remove of inactivate pathogens on fresh produce (Kim *et al*., 2000).

Detergents on the other hand are synthetic chemicals, usually alkaline in nature, which acts by reducing the surface tension of water. This in turn increases the interaction of water with surface debris and which will subsequently be flushed way. Cleaning by detergent is defined as the elimination of adherent detectable soil, blood, protein substances and other waste from surfaces or apparatus by a manual or mechanical procedure. During the course of this process the detergent agent is a cleaning agent, which is not assumed to have antimicrobial effects. The entity is composed of hydrophilic and lipophilic constituents and can be apportioned into four classes: anionic, cationic, amphoteric and non-ionic (Exner *et al*., 2004). In contrast to this, the term “kills pathogenic bacteria”, mean that the product or substance kills bacteria which cause disease. This is more specific but similar to the term antibacterial or antimicrobial, which describes a substance or product that kills or inhibits the growth of bacteria.

On the other hand, surfactants are amphiphilic compounds, containing both hydrophobic (non-polar) and hydrophilic (polar) moieties that bestow on them the ability to aggregate between fluid phases such as oil/water. They then lower the surface and interfacial tensions and subsequently form emulsions (Desai and Banat, 1997). The surface activity properties make surfactants one of the most vital and versatile division of chemical products, used on a multiplicity of applications in household, Industry and agriculture (Deleu and Paquot, 2004).

2.2.2 *Escherichia coli*

Gram- negative bacteria, has been identified as food borne pathogens presenting a significant public health problem. Among these are the shiga toxin producing *Escherichia coli* (STEC) strains which have emerged as important human enteric pathogens. Strains which, for instance, express 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, and

may be transmitted in a variety of ways, including through food and water and through person- to- person and animal-to–person contact (Armstrong *et al.,* 1996). *E. coli* strains are typical inhabitants of the gut of warm blooded animals including homosapiens, which enable them to cause intestinal disease such as diarrhoea of hemorrhagic colitis. They may also cause extra-intestinal disease such as neonatal meningitis, nosocomial septicaemia, heamolytic uremic syndrome, urinary tract and surgical location infections (Falagas and Gorbach, 1995). Diarrhoeal diseases have for an extended period of time been recognised as a major cause of morbidity and mortality in children under the age of five. The incidence of diarrhoea cases is normally high in developing countries. In particular in the rural and farming communities where standards of public hygiene, sanitation and water supplies are low and cleanliness practices are below standard. Foods with pathogenic micro-organisms may be a major source of infectious diarrhoea in developing countries with low standards of personal hygiene (Armstrong *et. al.*, 1996).

2.2.3 *Pseudomonas aeruginosa*

In addition to *Escherichia coli, Pseudomonas aeruginosa,* a spoilage bacteria, 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). Recent studies have suggested that biofilm formation is a key factor in chronic *Pseudomonas* airway infection in cystic fibrosis, bronchi stasis, chronic urinary tract and medical device infections (Fux *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). This resultant toxicity may also lead to food spoilage.

Raw poultry has an exceedingly high perishability, and its spoilage may represent a critical economic loss for the poultry industry. Previous studies have demonstrated that the organoleptic changes which characterise a meat product as spoiled, i.e. off-odours, off flavours and slime, are the result of microbial growth (Jackson *et al.*, 1997). Psychotropic bacteria, commonly *Pseudomonas spp.,* have been identified as the

principal micro-organism responsible for spoilage of aerobically-stored meat products, and temperature as the most central environmental factor affecting its growth rate (Pooni and Mead, 1984). Even short temperature deviations may result in significant product spoilage in industry and households alike (Moore and Sheldon, 2003).

2.2.4 Household perspective

Cross contamination of food-borne pathogens in the household kitchen environment possibly contribute to the estimated 76 million cases of food-borne illness in the US each year (Mead *et al.*, 1999). A study done on 342 household refrigerators in Ireland revealed total viable and total coliform counts with values as high as 8,8 and 6 log CFU/cm ^{2}. Many food borne pathogens may linger on all kitchen utensils and be spread around continuously (Sharma et al., 2009). The focus on hygiene and proper sanitation in the food industry has resulted in an increasing use of chemical disinfection. The aim of disinfection in the home is to eliminate pathogens and spoilage micro-organisms present on food-contact surfaces, thereby avoiding contamination of raw materials and products, especially during refrigeration (Langsrud *et al.*, 2003).

2.3 AIMS

This study focused on the food borne pathogens *Escherichia coli* and *Pseudomonas aeruginosa* when subjected to common environmental stresses represented by the kitchen environment. The questions which now arise are: how sensitive a typical food spoilage organism (*P. aeruginosa*) and a food-borne pathogen (*E. coli*), to commercial sanitisers, detergents and storage temperature is and whether there is a change in the sensitivity of the organisms to the sanitisers, detergents and storage temperatures when it occurs in community autochthonous. Finally, the study looked at changes in growth patterns in communal growth.

2.4 MATERIALS AND METHODS

2.4.1 Strains, growth medium and growth conditions

Escherichia coli 0111 ATCC (ATCC25093) and *Pseudomonas aerigunosa* ATCC (ATCC27853) were obtained from culture collection of the UAFSB, CUT, Bloemfontein, SA. Organisms were grown on PCA (Plate count Agar) overnight and then used to inoculate media in growth flask. All sample 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, Bollinger, 2008), and this media is chemically defined which rules out the possibility of any interfering variables. Growth was allowed at 28°C, shaking for 30 hrs. Growth was estimated or monitored by optical density (OD) at 620nm. Samples were drawn at 0, 15 and 30 hours. Organisms were grown separately and in combination. All glassware and equipment used throughout the study was depyrogenated to rule out any unnecessary contamination.

2.4.2 Sanitiser and detergent concentrations

Stock solutions of two commonly used household sanitisers and two detergents were prepared. The selection and choice of sanitisers and detergents was based on the popularity throughout the commercialisation process in the media. That would include regular sales, the shelf availability and variety (data not shown). 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 doing 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 grown at all concentrations. By plotting the percentage difference between the samples with sanitiser and detergent and those without against the concentration, the LD_{100} and the LD_{50} was determined.

2.4.3 Temperature as variable

Growth conditions were as in 2.4.1 for this part of the study with the exception of temperature which was varied to allow samples to grow at 4°C and 10°C respectively. These temperatures were selected as they represent the most common household storage temperatures. Experiments were performed in duplicate and also samples at 0, 15 and 30 hours.

2.4.4 Calculations

All absorbance readings were taken, for bacteria stressed and unstressed, meaning grown with or without sanitiser and detergent, and then converted to a percentage difference.

This percentage difference in growth was determined as follows:

$$\frac{[OD_{620}\,CONTROL] - [OD_{620}TEST]}{OD_{620}TOTAL} \times \frac{100}{1}$$

2.5 RESULTS AND DISCUSSION

Similar growth profiles could be observed for both *E. coli* and *P. aeruginosa* under identical growth conditions (Fig. 2.1). Since the effect that the added sanitisers and detergents would have on the growth is largely unknown, 15 hours and 30 hours were selected as sampling intervals for subsequent experiments.

E. coli and *P. aeruginosa* were then grown in pure culture and in combination in the presence of two sanitisers and two detergents. The percentage difference in OD_{620nm} was calculated for samples taken at 0, 15, and 30 hours intervals. The working (LD_{50})

concentration for the sanitisers was determined as 3.2 x 10^4 PPM and that of the detergents as 1.6 x 10^4 PPM (Fig 2.2).

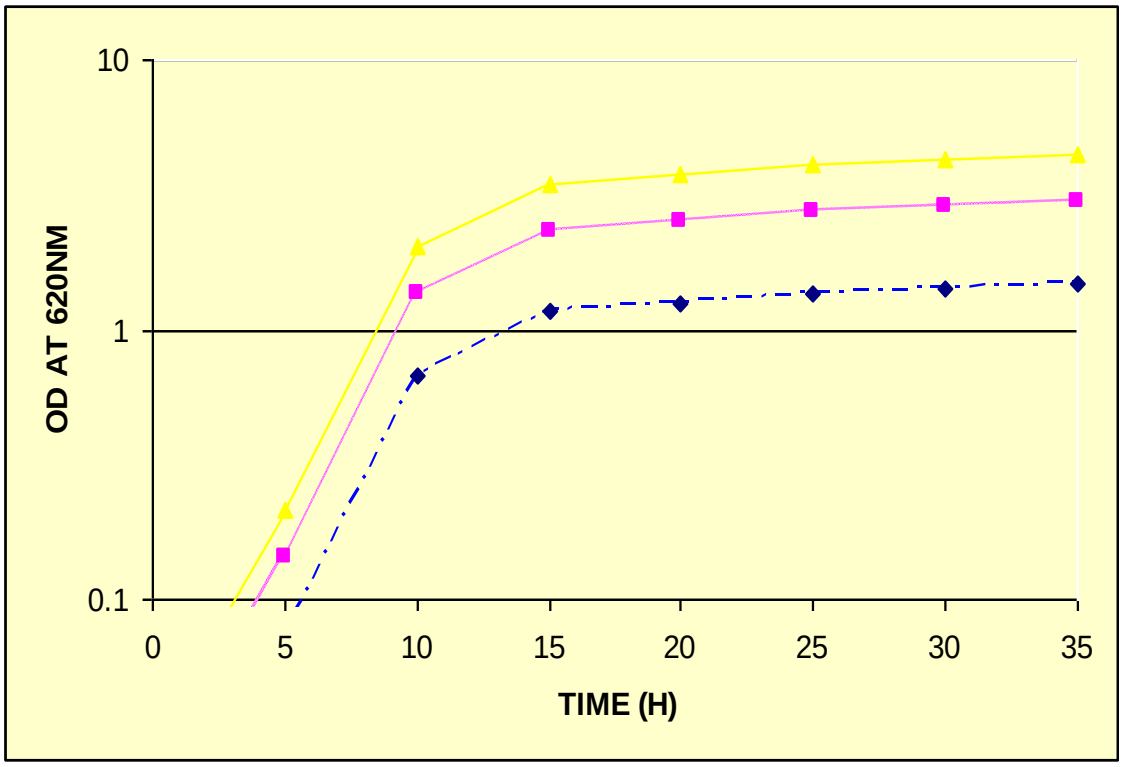

Figure 2.1: The growth of organisms in pure culture and in community to establish the time of growth of organisms for experiment.

------: *Escherichia coli*
------: *Pseudomonas aeruginosa*
------: Communal growth

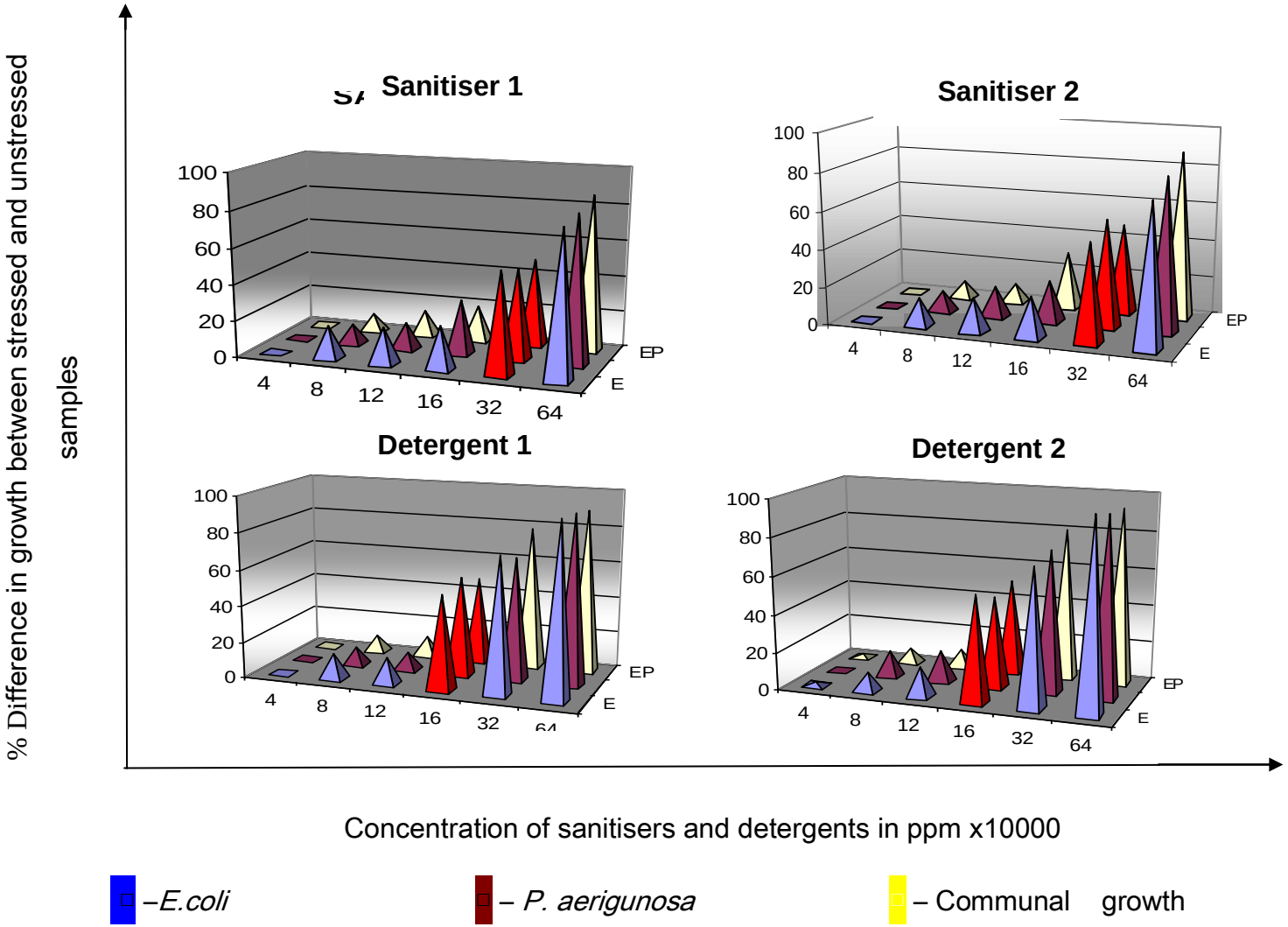

Figure 2.2: A graphical representation of the determination of a working concentration for the sanitisers and detergents. % difference in growth versus concentration in PPM. A LD_{50} is indicated for each by

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 (Fig 2.3A). For *P. aeruginosa* on the other hand, there was an 80% difference in growth when the control was compared with the stressed cells (Fig 2.3B). This difference decreases as the growth time increases, indicating that *P. aeruginosa* started to grow in the presence of sanitiser 1 between 15 and 30 hours. When these two organisms were grown in community the profile mimicked that of *E. coli* (Fig 2.3C). This would be expected in this instance, since this organism appeared least influenced by sanitiser 1.

Sanitiser 2: In pure culture there is no marked result of any influence introduced by the sanitiser on the growth of *E. coli*. *P. aeruginosa* on the other hand grew better when subjected to sanitiser 2 for 15 hours (Fig 2.3A, B). This could be indicative of a response to compensate for the stress to ensure survival of the culture. In communal growth after 30 hours of exposure, the percentage difference was 100% indicating, in all probability,

cell death and cell lyses of both organisms (Fig 2.3C). The competition variables could possibly have been too extensive for the survival of the organisms.

Detergent 1: A large percentage difference in growth is observed for *E. coli* over 15 – 30 hrs of exposure (Fig. 2.3A). It can thus be stated that detergent 1 had a dismal effect on the growth of this organism; in fact almost no growth was observed. This detergent also had a severe influence on the growth of *P. aeruginosa* during the first 15 hours, as is indicated by a 80% difference in growth (Fig 2.3B). Between 15 – 30 hours the organism seemingly adapted to the presence of the stress, and this can be seen in the decrease in percentage difference in growth at this stage. When the organisms were grown in community, the results at 15 – 30 hrs show a significantly large percentage difference in growth, which could also be indicative of cell death of cell lyses (Fig 2.3C). The communal growth represents the only other variable and therefore it is not unwarranted to suggest that the large percentage difference in growth may be attributed to lack of nutrients and/or other limiting factors. Also, the increase in the pH caused by the detergent may have hampered growth and flourishing of organisms.

Detergent 2: For *E. coli* the effect of detergent 1 and detergent 2 is not dissimilar. It is safe to say that 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 (Fig 2.3B). When the organisms were grown in community, detergent 2 affected the growth severely and over time an 80% difference in growth between the control and test samples was observed. Both the organisms are neutrophiles and the increase in pH may have affected the cell membrane, interfered with enzyme action and membrane proteins. This possibly caused the reduction in growth.

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* (Fig 2.4). This finding could possibly be attributed to the expected adaptation to the new environment and the sub-optimum growth temperature (Chapter 1, Table 1.3). *Pseudomonas* can usually thrive at this sub-optimum temperature due to its opportunistic nature, but in this instance it was

unexpected since literature indicates that it is able to survive better at refrigeration temperatures. *E. coli* has an optimum temperature of 37ºC (Table 1. 3, Chapter 1) and the reduction in growth is therefore the expected scenario. The organism did however show better growth in pure culture at 10ºC after 30 hrs. The communal growth showed the biggest difference between stressed samples and their control. This is most probably due to cell death at 4ºC after 30 hours. The thriving of *E. coli* at 10ºC is not observed in the community.

The temperature in combination with communal growth may now possibly represent two or a combined hurdle to growth of the organisms.

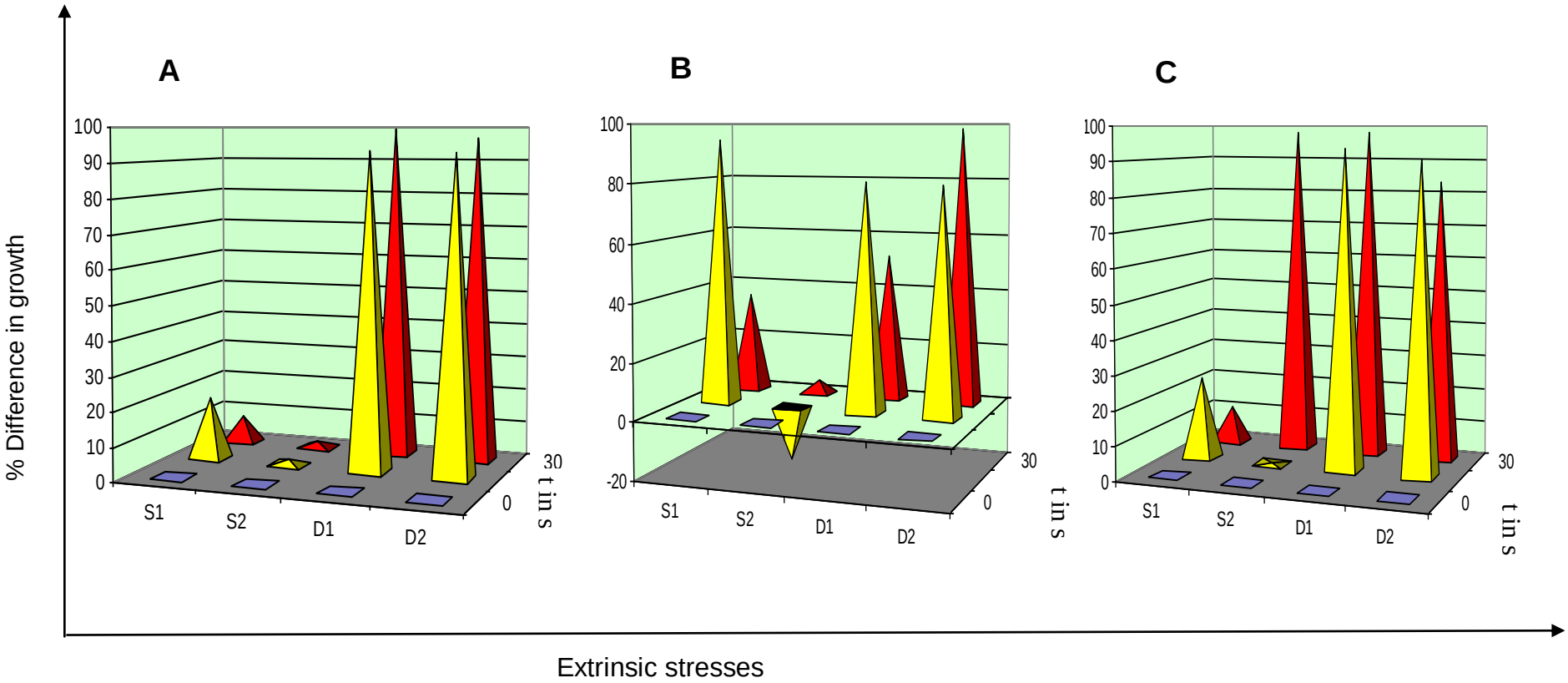

Figure 2.3: The percentage difference in growth between stressed and unstressed samples at 0, 15 and 30 hrs.

A *Escherichia coli*

B *Pseudomonas aeruginosa*

C Communal growth

S1 –sanitiser 1; S2 – sanitiser 2; D1 – detergent 1; D2 – detergent 2

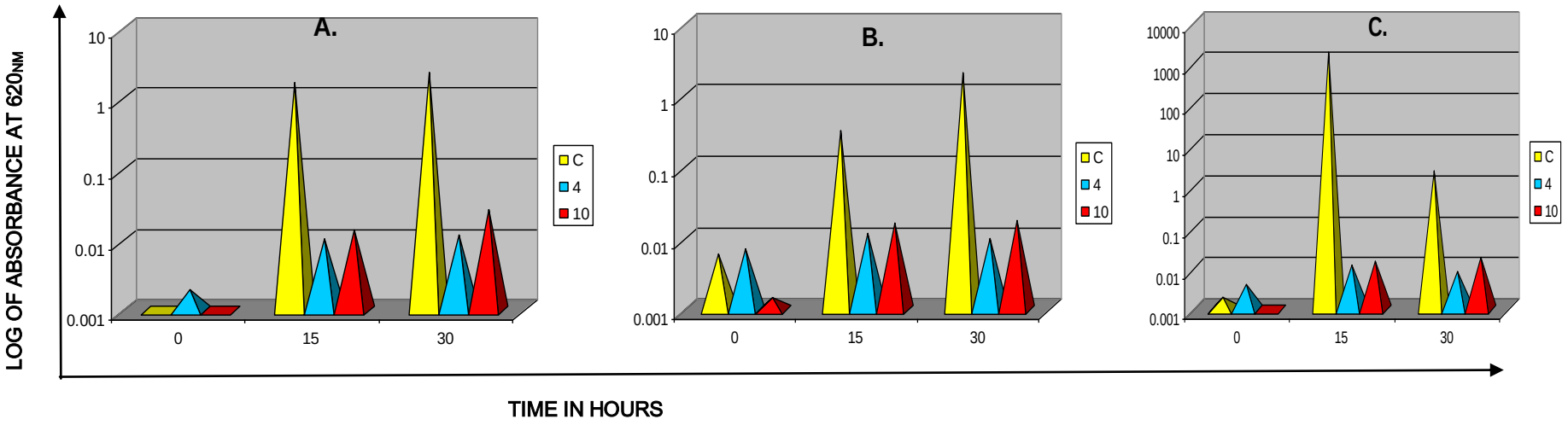

Figure 2.4: Growth at storage temperature as represented by the log of absorbance at 620_{nm}

A. *ESCHERICHIA COLI*; B. *PSEUDOMONAS AERUGINOSA*; C. COMMUNAL GROWTH

CONTROL

4°C

10°C

2.6 CONCLUSION

Addressing the questions as stated in the AIMS of this study:

The sensitivity of *E. coli*, a typical food borne pathogen and *P. aeruginosa*, a typical spoilage organism is varied to the sanitisers and detergents. What has been 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. *E. coli*, *P. aeruginosa* and the community exhibited a LD_{50} of 3.2 $x10^4$ PPM for sanitiser 1 and sanitiser 2, and a LD_{50} of 1.6 $x10^4$ PPM for detergent 1 and detergent 2.

The growth response delivered a myriad of patterns. For sanitizer 1 communal growth showed a response to the stress that was not an arithmetic sum of the response of the individual growth patterns. Sanitiser 2 showed no notable influence on *E. coli* and *P. aeruginosa* in fact grew much better and in community after 30 hours, a 100% difference in growth may have implied cell death and cell lyses of all cells. In the community, once again possible cell death is observed. It may be reasonable to assume that detergent 1

and 2 exhibit the same mode of action with the effect of detergent 2 was most severe. The household storage temperatures inhibited the growth of the organisms, but 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.

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CHAPTER 3

THE INFLUENCE OF EXTRINSIC STRESSES ON THE TOXICITY OF *ESCHERICHIA COLI* AND *PSEUDOMONAS AERUGINOSA* AS QUANTIFIED BY CHROMOGENIC *LIMULUS* AMOEBOCYTE LYSATE

3.1 ABSTRACT

The aim of this study was to identify the influence of extrinsic stresses i.e. sanitisers, detergents and storage temperature on the possible change in toxicity of common food borne pathogens. *Escherichia coli 0111*, (ATCC 25093) and *Pseudomonas aeruginosa* (ATCC 27853), were selected as test organisms. Both were grown in pure culture and in community to investigate the toxicity induced by the mentioned stresses. The change in toxicity was measured using the *Limulus* Amoebocyte Lysate (LAL) chromogenic test. The toxicity difference induced by the stresses varied between -2633 to 800 endotoxin units $cell^{-1}$ in pure culture. Two sanitisers, both of them chlorine based, were added at 32 x 10^4 PPM. For the sanitisers the average toxicity for *E. coli* varied from 250 EU/cell to 2, 5 EU/cell and for *Pseudomonas* from 41, 5 EU/cell to 30 EU/cell and for the communal growth from -71 to -4, 7 EU/cell. This could most likely be attributed to more or less LPS molecules per cell or the same LPS concentration but with more or less Hydroxy fatty acids in the Lipid A part. The 2 detergents, both ammonium based were added at 16 x 10^4 PPM. The average toxicity induced by this stress on pure cultures

varied from-1287 EU/cell (Endotoxin Units per cell) to 98 EU/cell for *E. coli* , from -1385 to 99 EU/cell for *Pseudomonas* and from -826,5 EU/cell to 98 EU/cell for communal growth. Two storage temperatures of 4°C and 10°C induced a much lower average change in toxicity. Alkaline and ammonium based compounds increased and decreased toxicity depending on the growth time and the sub-optimum storage temperature induced an almost constant increase in toxicity.

3.2 INTRODUCTION

3.2.1 Relevance of bacteria in households and industry.

Bacteria are able to inhabit or occupy a variety of food preparation surfaces, utensils, domestic dishcloths, sponges and other cleaning objects. They can then subsequently be transferred into food (Jackson *et al.*, 2005). In support of this, reported statistics indicate that the incidence of foodborne illness in Europe is undesirably high, even though it is a striking underestimation of the true vastness of the problem. This fact would imply that the refrigeration practices will continue to be major determinant in domestic food safety. In the event of failure to follow correct practices in the adjustment,

maintenance, use or cleaning of domestic refrigerators a number of risks could arise for consumers. This is significant because refrigerators form an important linkage in the extensive chain of cross-contamination, and causes 28% of outbreaks of domestic foodborne disease (Ryan *et al.*, 1996). This situation is worsened by bacteria contaminating unwashed raw foods, leaking packages and hands in refrigerators which may directly or indirectly contaminate other stored foods. Alternatively, biofilms may form, attach to and persist on the internal surface of the refrigerator posing risks of indirect longer term contamination during ensuing food preparation activities (Michaels *et al.*, 2001). Countless domestic refrigerator temperatures are incorrectly adjusted, operating above the advised temperature and are therefore capable of supporting sub-optimum but significant growth of mesophilic organisms (Jackson *et al.*, 2005). There is nonetheless very little information on the spread to, and persistence on the interior surfaces of domestic refrigerators, making it difficult to quantify the affliction of such pathogens in these environments, and at the same time to assess the threat they pose to consumers (Jackson *et al.*, 2005).

Cross contamination of food-borne pathogens in the household kitchen environment possibly contribute to the estimated 76 million cases of food-borne illness in the US, each year (Mead *et al.*, 1999). The focus on hygiene and proper sanitation in the food industry has resulted in an increasing use of chemical disinfection. The aim of disinfection in the home is to eliminate micro-organisms present on food-contact surfaces thereby avoiding contamination of raw materials and products, especially during refrigeration (Langsrud *et al*., 2003). A combination of microbial knowledge, experimental data and mathematical techniques, predictive microbiology design models can describe, understand and predict microbial behaviour in food products. The execution of these predictive models may improve control of food safety and food spoilage (Derlinden *et al*., 2008).

Looking at the two organisms representing the focus of the study: Illness caused by *Escherichia coli*, a Gram-negative bacterium, occurs sporadically (see Chapter 2). Psychotropic bacteria, commonly *Pseudomonas spp.,* have been identified as the principal micro-organism responsible for spoilage of aerobically-stored meat products

(see Chapter 2), and temperature as the most central environmental factor affecting its growth rate (Pooni and Mead, 1984).

3.2.2 Adaptation of *E. coli* and *P. aeruginosa* to stress

In an attempt to control bacterial presence and cross contamination in food processing plants, sanitation of the working surfaces and equipment usually includes the stepwise application of a detergent, an acid/alkali rinse, a disinfectant treatment followed by a final rinsing (Musgrove *et al*, 2004). Injury to cells *in vitro* as well as *in vivo* causes increased expression of a variety of (early stress response) proteins (van de Water *et al*., 2006). A parallel between the resistance of micro-organisms and the efficacy of the sanitation techniques in the food manufacturing industry is known to exist and is a direct result of the inborn ability of bacteria to adapt to a new environment and form increasingly resistant survival structures such as biofilms (Kumar and Anand, 1998). This adaptation is ascribed to several phenotypic changes that commonly occur in, amongst others, the outer membrane (first line of defense) and include changes in the

macromolecular structure of the lipopolysaccharides (LPS) of Gram-negative bacteria (Frank, 2001).

Apart from being an endotoxin, the LPS is the major constituent of the cell wall of the Gram-negative bacteria (see Chapter 1). 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). Little mention has, however, been made of the influence of sanitisers and detergents applied in the food industry on the LPS structure. Therefore the aim of this study was to address this change in toxicity.

3.2.3 Toxicity

In the research path of this century, bacterial endotoxins have been classed as one of the most interesting and exciting molecules found in nature. Their uncharacteristic structure, their miscellany chemically and physically and their expansive spectrum of biological activities has effected worldwide research in this field. Regardless of the fact that the knowledge about chemical composition and structure of endotoxins is well

developed, many questions remain about the role of endotoxins in human health, especially pathophysiology (Petch *et al.,* 2000). Endotoxins, more accurately known as lipopolysaccharides (LPS), are conceded as the most potent microbial mediators implicated in the pathogenesis of sepsis and septic shock. Notwithstanding its discovery over a century ago, the primary role of the endotoxin in most patients with septic shock remains a mystery and its value as a target for therapeutic intervention continues to be a contentious clinical issue. LPS is viewed by the host as a dread molecule indicating microbial invasion by Gram-negative bacterial pathogens (Opal, 2007). Although endotoxins are firmly anchored within the bacterial wall (see Chapter 1), they are continuously liberated into the surrounding medium. Endotoxin release unmistakably does not happen only with cell death, but also during growth and division (Petch *et al.*, 2000).

3.2.3.1 Toxicity of *E. coli* and *P. aeruginosa*

Shiga toxin producing *Escherichia coli* (STEC) were initially distinguished as human pathogens in 1982 when *E. coli* 0157:H7 was accountable for two outbreaks of

hemorrhagic diarrhea (Doyle and Padhye, 1989). Since this realisation, more than 100 serotypes of STEC have been isolated from animals, foodstuff and other sources. In the USA and Europe most outbreaks of STEC have been related to serotype 0157:H7 (Griffin, 1995), but in concert, several were caused by STEC of other serotypes. Outbreaks caused by *E. coli* 0111:NM, in particular, has been reported in Italy (Caprioli *et al.,* 1994). The study of Shiga toxins is widespread and they are viewed as well established virulence factors (Meng *et al*., 1998). *Pseudomonas sp,* the second organism of interest is an ever-present Gram-negative organism which can be found in water, soil and organic waste; *Pseudomonas aeruginosa* is customary found in the nasopharynx, oropharynx and lower digestive tract of numerous vertebrates (Baker, 1998). It is a noteworthy opportunistic pathogen, which is likely to abound in hospitals together with the ability to form biofilms, and cause critical infections in both humans and animals (Gales *et a.l*, 2001). This psychotrophic bacterium is routinely found in milk and dairy products (Cousin, 1982). *E. coli* and *P. aeruginosa* could in pure culture and in community be the source of the resulting toxicity.

3.2.3.2 Measurement of toxicity

For an expanded calculation of toxicity and risk assessment an improved understanding of the mode of action of toxicants is required. Since toxicity is commonly related to biological perturbation at the cellular level, improving our knowledge of the cellular perturbations and the outcomes for biological function is indispensable. Contemporary technologies allow genome wide analysis of stress responses as a result of the exposure of biological systems to an assortment of stresses. Gene expression is not adequate to appreciate toxicity; since the possibility exists that there may not be a direct relationship between gene expression and protein expression (van de Water *et al.*, 2006). Endotoxins released by Gram-negative bacteria, may trigger a response in humans ranging from mild fever to shock or even death. A steadfast method of detecting bacterial endotoxins in pharmaceuticals is, therefore, of fundamental importance for *in vivo* use in humans (Zijlstra *et. al.*, 1997).

3.2.3.3 Endotoxin versus pyrogenicity

The name endotoxin was established to differentiate heat stable toxin release during bacterial lyses from secreted, heat labile bacterial exotoxins. When endotoxins are in pure chemical form it is known as lipopolysaccharides (LPS) (Hodgson, 2006). Variations in LPS structure can play a role in the avoidance of immune detection through molecular mimicry of host structures (Moran and Prendergast, 2001). LPS plays a major role in pathogen-associated molecular patterns that not only alert the host to infection but also prevents unsuitable autoimmune reactions. What can also happen is the overreaction of the host immune system to LPS (Krug *et al.*, 2001). The LAL test is commonly used to detect or confirm the absence of these endotoxins.

3.2.3.4 *Limulus* Amoebocyte Lysate

The technique of *Limulus* Amoebocyte Lysate (LAL) has been extensively used for ±25 years for the detection of endotoxin in quality control of injectable drugs and medical devices. The LAL tests detect the presence and concentrations of intact LPS, and by enlarge (in the absence of other straightforward tests), is taken as a comprehensive

gauge signaling the presence (or assuring the absence) of pyrogens and/or pro-inflammatory species (Schindler, 2009). As far back as 1942, the rabbit pyrogen test was approved by the U.S Pharmacopoeia (USP). In principle during this test, the increase in temperature (a pyrogenic response) in rabbits is monitored after injection of a test substance. About thirty years later, in 1971, an alternative for the *in vivo* rabbit test was introduced by (Cooper *et al.,* 1971). This *Limulus* Amoebocyte Lysate (LAL) test is an *in vitro* test utilising lysate blood cells of the horseshoe crab, which enzymatically interacts with endotoxins. There are several techniques or principles known such as the gel-clot test, the chromogenic test and the turbidimetric test (Zijlstra *et al.,* 1997). The gel-clot test is a semi-quantitative test where, the incidence of occurrence of an endotoxin is observed by the formation of a solid gel; whereas the chromogenic and turbidimetric are fully quantitative tests and they measure the colour or turbidity developed by the reagent in the presence of endotoxins (Bohrer *et al*., 2001). In this report, we have made use of the chromogenic test to quantify the toxicity of stressed foodborne pathogens. In addition an attempt was made at a correlation between pure culture organisms as compared to communal growth.

3.2.3.5 Test in pure culture

Scrutinisation of pure cultures has for an extensive period of time been significant in the advancement of the development of new growth media, establishing optimum growth conditions, the response to antibiotics and extrinsic and intrinsic stresses. The characterisation of the pure culture growth approach has been favoured over communal growth. In contrast, it should be noted that many micro-organisms exist in microbial assemblages. These cultures often have surface associated commensal partners, which often lead to biofilm formation. A biofilm, therefore, is a population of microbes, aggregated on surfaces, embedded in a matrix of extracellular polymers (Chapter 1). For the purposes of this study a comparison of change in toxicity was investigated between the responses of pathogens to extrinsic stresses in a pure culture, as compared to communal growth.

3.3 AIMS

The aim of the study was to investigate the influence of extrinsic stresses such as household sanitisers, detergents and storage temperature on the change in toxicity of *Escherichia coli* and *Pseudomonas aeruginosa* grown in pure and communal cultures.

3.4 MATERIALS AND METHODS

Escherichia coli and Pseudomonas aeruginosa were grown in pure culture and in combination as is described in Chapter 2. Determination of the working concentration is outlined in Chapter 2. LAL Kit 50-648U (Cambrex) was used for the chromogenic toxicity testing. LAL reagents, endotoxin-free water, endotoxin-free pipette tips, endotoxin free test tubes, stop reagent, sodium hydroxide (0.1 N) were used. For this study all samples, stressed and unstressed were standardised in terms of their concentrations. A method of dilution, using 0,8 % saline, re-examining the OD_{620nm} (Chapter 2) for the purpose of presenting samples with the same cell density, was performed. The endotoxin concentration or the resulting toxicity was determined using the quantitative,

chromogenic LAL assay (end-point method). 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 OD_{410} versus EU per absorbance unit. Sample preparations (with exact same cell density) were also assayed in duplicate and the concentration plotted against $OD_{410.}$ 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 prepared for the graphical representation according to the equation 3.1.

Equation 3.1: $$\left(1-\frac{OD_{test}}{OD_{control}}\right)X\,\frac{100}{1}$$

A positive reading indicates better growth by the control or unstressed sample; whereas a negative reading shows that the stressed samples exhibited better growth when stressed.

The toxicity analysis of the EU values obtained from the LAL assay were analysed according to the equation 3.2.

Equation 3.2: $$\left(\frac{LAL_{TEST}}{OD_{TEST}}\right)-\left(\frac{LAL_{CONTROL}}{OD_{CONTROL}}\right)$$

A positive reading on the graphs in Fig. 3.2 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.

3.5 RESULTS AND DISCUSSION

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; Table 3.1). The results from the LAL assay were read against a standard calibration curve to express the results in endotoxin units (EU) (Hansen *et al.*, 1999). It is evident that endotoxins amass in lamellar, cubic and hexagonal inverted arrangements, such as micelles and vesicles, with diameters up to 0, 1 µm. In addition, endotoxin micelles and vesicles are much more stable than those of simple detergents and sanitisers. Thus, vesicles are even found in ultra pure water. Monomers have to unambiguously be created by using detergents, bile acids and chelators (Petch *et al.*., 2000).

The stresses varied in their influence on the monocultures. What can be observed when growth occurred in combination or community is a shock response 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 increase in toxicity is simultaneously observed which is as a result of the favourable growth (Fig.

3.2A; Fig. 3.1A). *P. aeruginosa* exhibited weaker growth when stressed with a concomitant decrease in toxicity (EU's), at 15 hours of growth (Fig. 3.2A; Fig. 3.1A). This margin of reduction becomes minimized at 30 hours of growth. This reduction in toxicity may possibly be ascribed to an increase in lipid synthesis with a decrease in endotoxin production. In colonial 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 3.1: Common endotoxin concentrations in everyday life.
(Adapted from Petch *et al.,* 2000)

Source	**Solution**	**Concentration: EU/ml**
Tap and mineral water		1 – 20
Water: swimming pool		25 600
Marjoram tea		533
Protein from cell density cultures of *E. coli*	Supernatant after homogenization	>2 000 000
Proteins from shaking flask cultures *E. coli*	Culture filtrate	70 000 – 500 000
Murine IgG_1 from cell culture	Culture filtrate	$\leq$ 100
Whey processed from milk	Supernatant after acid precipitation of milk	≈10 000
Commercial preparations of BSA	1 mg ml^{-1} of lyophilized protein	0.5 – 50

Sanitiser 2 caused a small difference in percentage growth of *E. coli* with a more marked decrease in toxicity when stressed. A possible decrease in lipid A concentration, which is associated with an decrease in 3-hydroxy fatty acid concentration implies a greater presence of LPS. (Fig. 3.1B; Fig. 3.2B). 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 slight noticeable 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 (Fig. 3.1B; Fig. 3.2B). No observable growth correlated with no noteworthy change in toxicity at 30 hours of growth. A most likely reason could be cell death and no endotoxin activity.

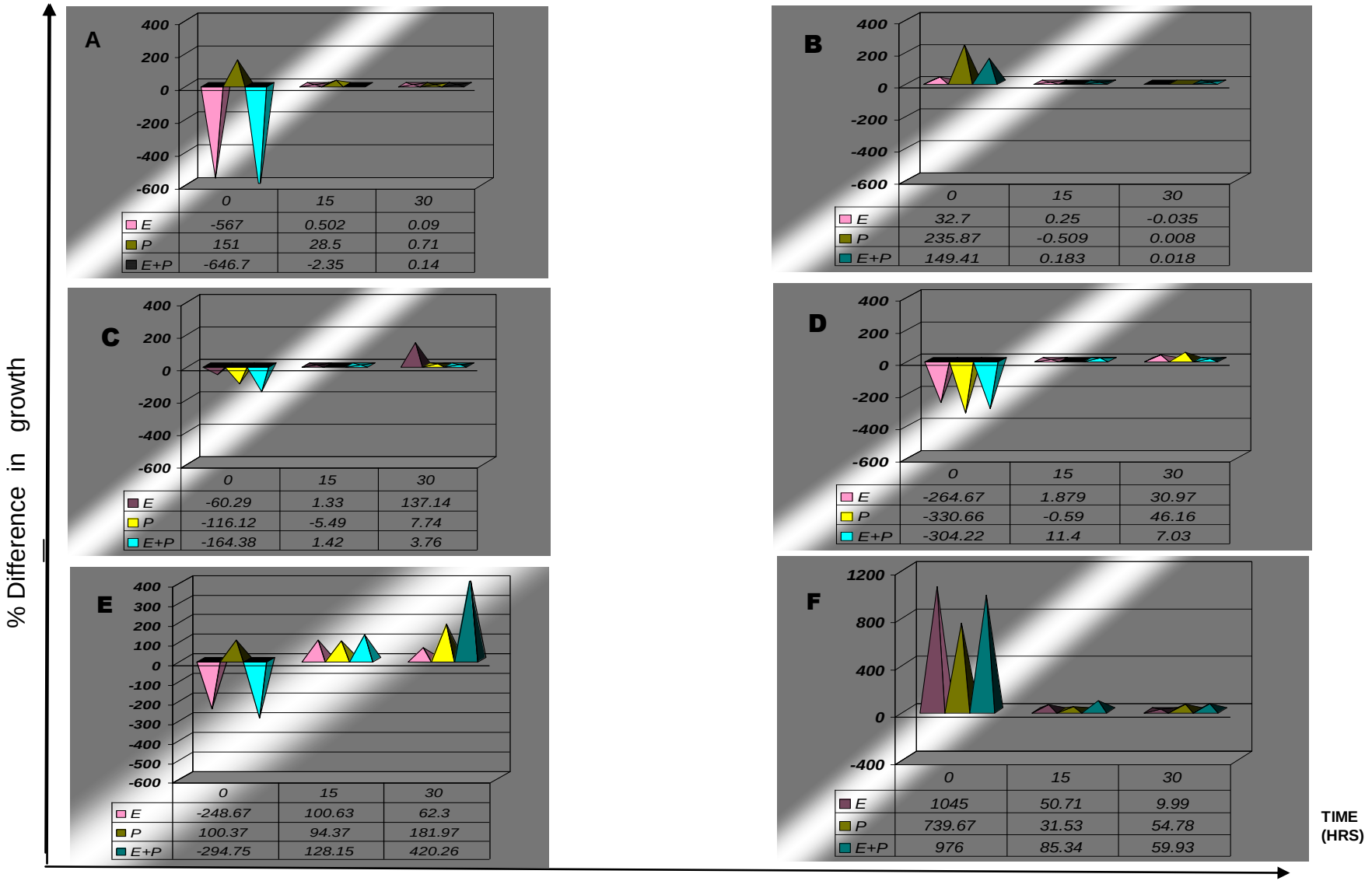

Figure 3.1: The percentage difference in growth ability between test/stressed samples and their control counterparts versus time of growth. A –sanitiser 1; B-Sanitiser 2; C- Detergent 1; Detergent 2; E-4ºC; F-10ºC

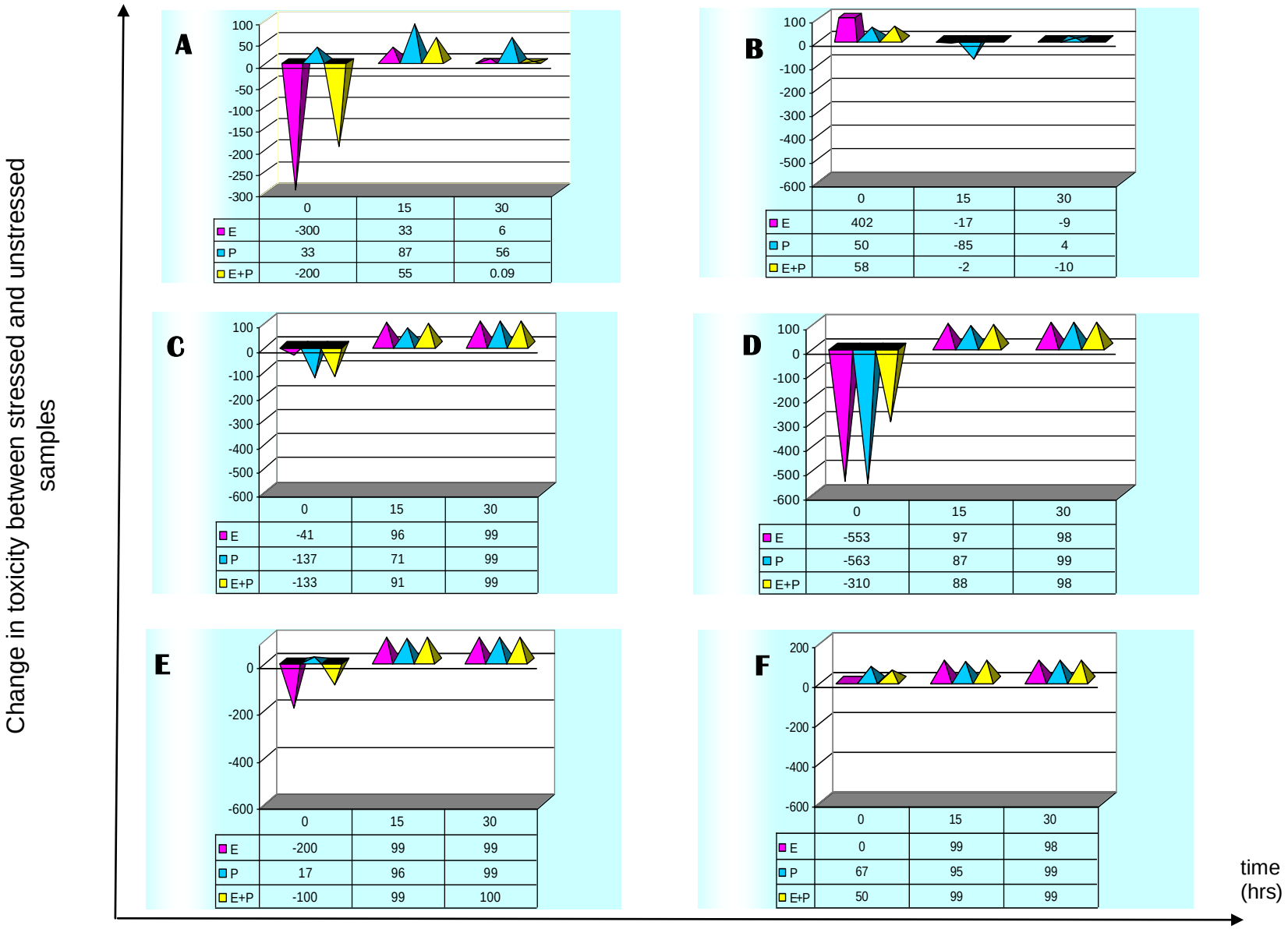

Figure 3.2: The change in toxicity measured by LAL assay due to extrinsic stresses versus time of growth

A=Sanitiser 1; B=Sanitiser 2; C=Detergent 1; D=Detergent 2; E=4ºC; F=10ºC

E= *Escherichia coli*; P= *Pseudomonas aeruginosa*; E+P= community

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 hardly any noticeable difference in growth percentage can be observed except for *E. coli* at 30 hours. At the same time, all the samples show a similar reduction in toxicity, with the change at 30 hours exactly the same (Fig. 3.1C; Fig. 3.2C). When compared to detergent 1, detergent 2 initially caused a feast scenario with the cultures possibly engaged in lipid biosynthesis. (Fig. 3.1D; Fig. 3.2D). It would then be reasonable to assume that there would be an increase in the concentration of 3 –Hydroxy fatty acids (Fig 2D; Fig 3D).

Growth difference at 4^{o} C initially was characterised by better growth by *E. coli* and the community, which most likely represented *E. coli* rather than *P. aeruginosa*. The initial difference in toxicity also showed an increase, except for *P. aeruginosa* (Fig. 3.1E; Fig. 3.2E). At 15 hours of growth the percentage difference in growth matches the reduction in toxicity, whereas at 30 hours of growth the large reduction in growth of the community correlates with a much more conservative reduction in toxicity (Fig. 3.1E; Fig. 3.2E). The

question thus aroused; was there a change in the amount of the LPS or was there just an alteration in structure because of the stress?

At 10 ºC samples surprisingly did not respond well to the stress and a very large difference in growth percentage was initially observed (Fig. 3.1F; Fig. 3.2F). 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 (Fig. 3.1F; Fig. 3.2F).

3.6 CONCLUSION

In conclusion, results obtained in this study 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 colonial fashion. The subsequent toxicity from growth varied between a

feast and famine response. 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.

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CHAPTER 4

THE INFLUENCE OF EXTRINSIC STRESSES ON THE TOXICITY OF *ESCHERICHIA COLI* AND *PSEUDOMONAS AERUGINOSA* AS QUANTIFIED BY PORCINE-IL-6 PRODUCTION

4.1 ABSTRACT

The study attempted to establish whether extrinsic stresses could have an effect on the toxicity as represented by Interleukin-6 production of foodborne pathogens grown in community. *Escherichia coli*, a typical inhabitant of the gastro-intestinal tract of warm blooded animals causing intestinal disease and *Pseudomonas aeruginosa*, an opportunistic pathogen responsible for causing severe infections in both humans and animals was subjected to common household sanitisers, detergents and storage temperatures during growth. These extrinsic stresses have effects which range from being bactericidal, lowering the surface tension of the water medium and removal of adherent debris. A method for whole porcine blood testing as proposed by Andrade *et al.,* (2003) was used in an IL-6 bioassay to determine the amount of IL-6 production in response to the stresses, as compares to the unstressed samples. The comparative concentrations of IL-6 were varied. Growth with sanitiser 1 and 2 yielded much lower IL-6 concentration for the community, suggesting symbiotic growth rather than competition.

Higher values for the control samples could have been due to pre-stimulation of the porcine blood. Overall it could be concluded that IL-6 concentration might not be a reliable marker for pyrogenicity or an assessment tool for the change in LPS structure of samples grown in community.

4.2 INTRODUCTION

4.2.1 Relevance of *Escherichia coli* and *Pseudomonas aeruginosa* in households and industry.

For the dilemma regarding the occurrence of food-borne disease, the safety and security of food products, management of food safety and inconsistencies in the manufacturing industry, refer to Chapter 1 and 2.

4.2.2 Control of micro-organisms by sanitisers, detergents and storage temperature.

Sanitisers are chemical products used administer to food-contact surfaces to destroy most of the disease causing bacteria, but at the same time does not adversely affect the product or the safety of the consumer. An assortment of sanitisers including chlorine,

chlorine dioxide, calcined water and organic acids has been scrutinised for their capability to lower the levels of food borne pathogens on fresh produce. Several explorations have reported their inability to completely eliminate or inactivate pathogens on fresh produce (Kim *et al.*, 2008). Household detergents on the other hand are synthetic chemicals, usually alkaline in nature, which act by lowering the surface tension of water. This in turn increases the interaction of water with surface debris and these will subsequently be flushed way (see Chapter 2).

4.2.3 Toxicity of micro-organisms

Endotoxins are lipopolysaccharides (LPS) present in the outer wall of Gram-negative bacteria, and these endotoxins may be shed during growth and death of bacteria. The toxic effect of endotoxins is embedded in their lipid A component. The polysaccharide chain on the other hand can determine the virulence of the bacterium (Rietschel *et al.*, 1993). LPS effectively obstructs a number of host antimicrobial defenses during infection and has accordingly gained acknowledgment as a virulence determinant for Gram-negative bacteria (Piotrowski, 1998). The endotoxic or LPS component of the cell wall

plays a pivotal role in the pathogenesis of shock and tissue damage associated with Gram-negative infection septicaemia. Sepsis is characterised by fever, chills, leucopoenia or leukocytosis, and hypertension and systemic inflammation that may result in spread of intravascular coagulation, haemorrhage and pulmonary oedema. This may advance into acute respiratory distress syndrome (Opal and Cross, 1999). The LPS signaling cascade involves the elaborate collaboration of a throng of receptors, cofactors and messenger proteins. As seen from Fig 4.1, the processing of LPS for signal transduction commences in the extracellular space with the ligation of LPS by LPS-binding protein. The direct result of this signal is fever; the body's most manifested sign of infectious illness, the only one of a concatenation of complex, non-specific host defense responses to infectious pathogens characterised as the acute-phase response (Fig 4.1). It arises in most endotherms by means of the activation of a combination of various autonomic and behavioural mechanisms (Blatteis, 2003).

4.2.4 IL-6

Once LPS is bound to the binding protein (LBP), the complex LPS–LBP activates different populations of cells by binding to its receptor. Interleukin 6 (IL-6) is a 26 kDa multifunctional cytokine which performs an important role in the host defense, acute phase reactions, immune responses, nerve cell function, hematopoiesis and bone remodeling. IL-6 is generated by an array of normal and transformed lymphoid and nonlymphoid cell types (Hirano, 1998). This IL-6 production is up regulated by a number of signals such as mitogenic or antigenic stimulation, lipopolysaccharides, calcium ionophores, cytokines and viruses. The expression of this cytokine is impeded in monocytes by cytokines such as IL-4, IL-10 and IL-13. Raised serum IL-6 levels have been observed in a number of pathological conditions, including bacterial and viral infections, trauma, autoimmune disease, inflammation and malignancy (Hibi *et al.*, 1996). IL-6 is a prototypic constituent of the IL-6 super family of cytokines that make use of a gp130 as their signal transducing receptor subunit (Taga and Kishimoto, 1997). Porcine-il-6 cDNA encodes a 212 amino acid precursor polypeptide that contains a 29 amino acid signal sequence with a 183 amino acid mature fragment. Mature porcine-il-6

possesses 58% and 39% amino acid uniqueness with human and mouse IL-6, respectively (Richards and Saklatvala, 1991).It is, therefore, apparent that the ability to test for the presence of LPS or in this case IL-6 would elucidate the degree of endotoxin infection.

4.2.5 Toxicity testing

Two pharmaceutical investigations for pyrogenic contamination exist: the rabbit pyrogen test and the *Limulus* amoebocyte Lysate (LAL) test. The rabbit pyrogen test necessitates the determination of the rise in body temperature induced in rabbits by the intravenous injection of the solution being tested. This test uses live experimental animals, is expensive and is non quantitative. The tenet of the LAL test is the extracellular coagulation of the blood of *Lymulus polymephus*. The LAL test is more sensitive but detects only the presence of LPS from Gram-negative bacteria and may give false negative and false positive results (Poole *et a*l., 1988). In view of the shortcomings of the rabbit pyrogen test and the LAL test, *in vitro* pyrogen tests that employ the exacting sensitivity of monocytes to exogenous pyrogen was then proposed

(Hansen and Christensen, 1990). Endotoxin (LPS) inducible cytokine release by human whole blood is increasingly used to model inflammatory responses *in vitro*, to detect the presence of pyrogenic contaminations, as well as to monitor disease states or immunomodulatory treatments ex vivo (Hermann *et al*., 2002). For the purpose of this study the porcine whole blood IL-6 detection test was applied to communal growth of the two Gram-negative food borne pathogens *Escherichia coli* and *Pseudomonas aeruginosa.*

4.3 AIM

This study attempted to quantify the influence of extrinsic stresses such as sanitisers, detergents and household storage temperatures on the toxicity of food borne pathogens *Escherichia coli* and *Pseudomonas aeruginosa* by using the Porcine-il-6 production.

4.4 MATERIALS AND METHODS

4.4.1 Growth of pathogens

Escherichia coli and *Pseudomonas aeruginosa* were grown in duplicate in 200 ml chemically defined Tryptone soy broth (Merck, SA) at 28°C log phase (30 hrs) and was estimated by optical density (OD) at 620 nm (all growth conditions and methods as outlined in Chapter 2 and 3). For the purpose of this study, as in the case for the LAL analysis (Chapter 3), all collected samples, stressed and unstressed were standardised in terms of their concentrations. A method of dilution, using 0,8% saline was performed after re-examining the OD_{620nm} (Chapter 2) for the purpose of presenting samples with the same cell density for the possible stimulation of IL-6.

4.4.2 Porcine whole blood cell culture

The method as proposed by Andrade *et al*. (2003) for human whole blood was used. Pooled porcine blood samples from 10 pigs were processed within 2 hours of collection to minimize the undesired activation of cytokine expression. Porcine heparinized blood was then diluted 1:3 with sterile saline. The respective samples (50 µl) and endotoxin

standard was transferred to 1 ml sterile Eppendorf tubes. Saline (50µl) and 150 µl of diluted blood was added per tube to give a final volume of 250 µl. The tubes were incubated at 37°C for 24 hours in 5% CO_2 humid atmosphere. The cell supernatants were drawn and assayed for the presence of IL-6 by porcine specific immunoassay.

4.4.3 Porcine interleukin-6 bioassay

The serum concentration of interleukin-6 was determined using the Quantikine® Porcine-il-6 enzyme kit (R & D Systems, Whitehead Scientific, SA). The methodology of the test employs a quantitative sandwich enzyme immunoassay technique and the analysis of samples was conducted according to the proposed method of the manufacturers. Standards, controls and samples were pipetted into the microtitre plate wells and any porcine-il-6 present was bound by the immobilized antibody. After washing away any unbound reactants, an enzyme-linked polyclonal antibody specific for porcine-il-6 was added to the wells. A wash to remove any unbound antibody-enzyme followed, before a substrate was added. The enzyme reaction yielded a blue product that turned yellow on the addition of the stop solution. The Optical density and subsequently the IL-6

concentration were determined at 540 nm by the Elysses ELISA machine (Human diagnostics, SA). The intensity of the colour was measured indicating the amount of porcine-il-6 bound during the initial step. Sample concentration values were calculated by using the standard curve which gave a $r^2 = 0.98801$.

4.4.4 Analysis of readings.

The collected results reflected optical density and the sample concentration of IL-6. Sample IL-6 concentrations were set against the growth density of stressed and unstressed samples. To determine a comparison between the amount of growth of the pathogens and the possible expression of cytokine IL-6, the following equation was used:

$$\frac{IL\text{-}6_{TEST}}{OD_{TEST}} \quad \frac{IL\text{-}6_{CONTROL}}{OD_{CONTROL}}$$

This arrangement was then represented graphically to illustrate the degree of IL-6 stimulation in contrast or relation to cell density (Fig 4.1). A positive result would therefore signify an increase in IL-6 production in the stressed samples, and therefore an increase in toxicity.

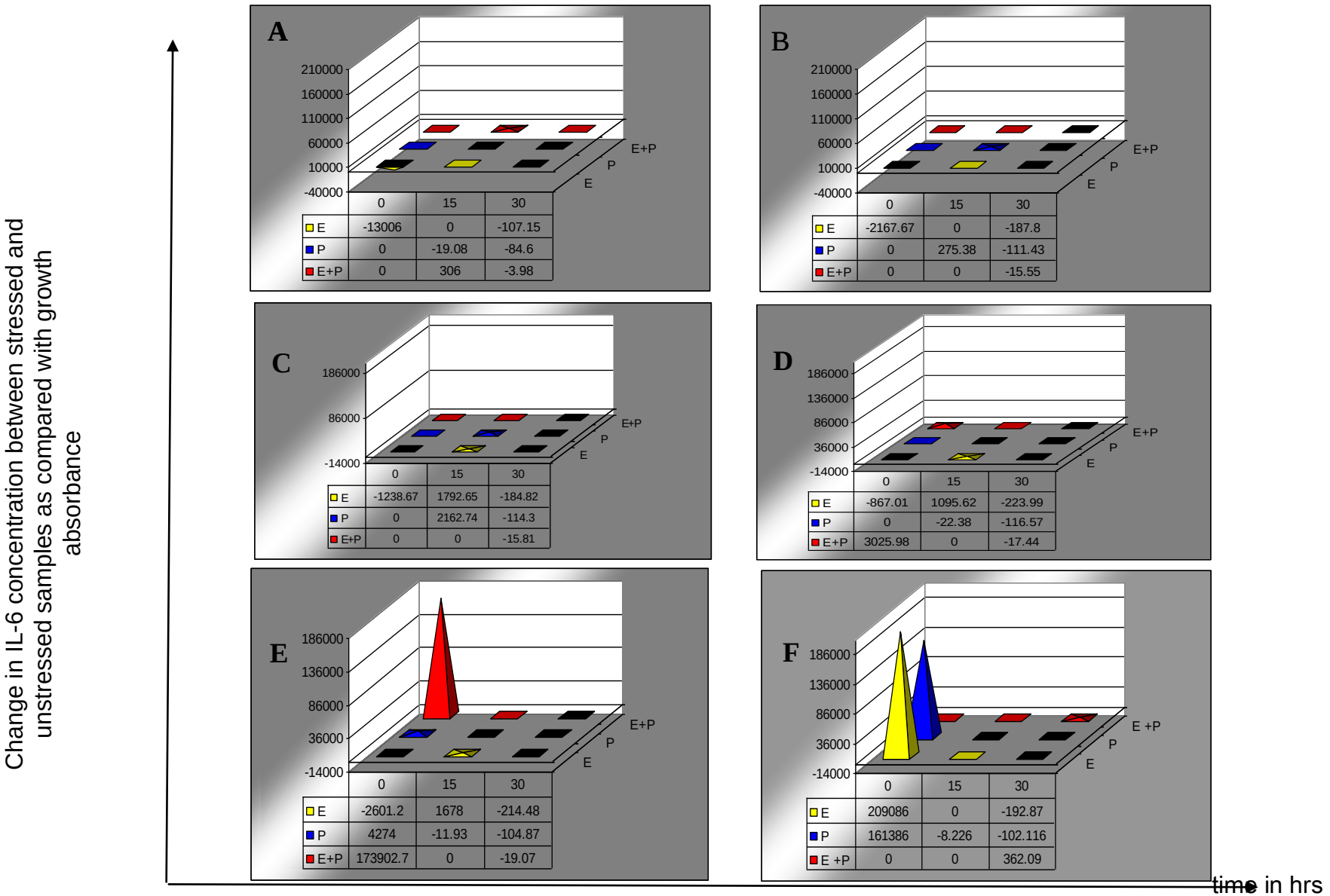

Figure 4.1: the change in IL-6 concentration between stressed and unstressed samples vs. time

A =Sanitiser 1; B = Sanitiser 2; C = Detergent 1; D = Detergent 2; E = 4°c; F = 10°c

4.5 RESULTS AND DISCUSSION

4.5.1 Values for IL-6

In 1996, Hartung and Wendel, performed incubations of human whole blood in the presence of bacterial stimuli with LPS, and after 24 h incubation revealed cytokine concentrations as follows: 1.2 x 10^3 pg/ml IL-6 for 0,001 µg/ml LPS, 16 x 10^3 pg/ml for 0,1 µg/ml LPS and 20 x 10^3 pg/ml IL-6 for 10 µg/ml LPS (Hermann *et al.*, 2003). In addition to this, IL-6 produced by Murine LPS in the hepatocytes is represented in Fig. 4.2.

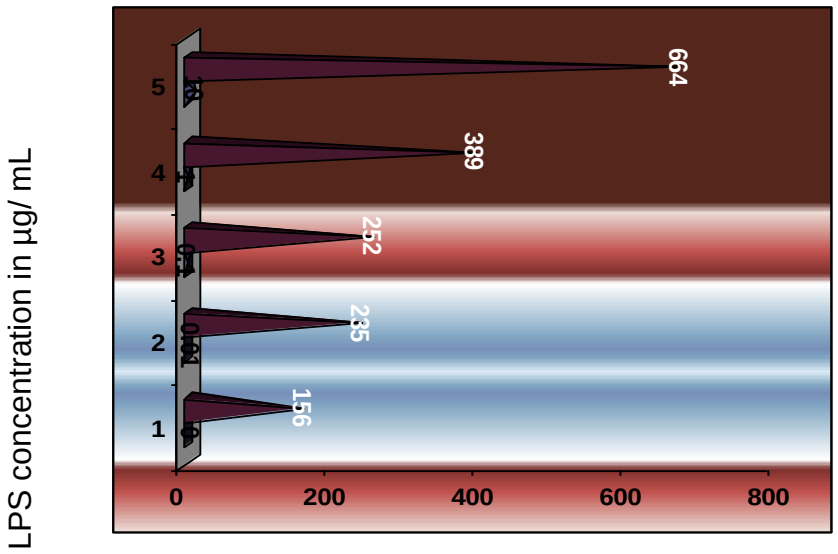

Figure 4.2: IL-6 concentration produced in Murine hepatocytes versus LPS concentration. Values as adapted from Panesar *et al.*, 1999

Through initial inspection there are certain samples which exhibited a notable or observable difference in IL-6 concentration when the challenged samples were compared with the controls. The communal growth samples' IL-6 concentration also appeared different. If the notable readings from Fig. 4.3 are tabulated, the comparison can be made with the values in Fig. 4.2, as represented in Table 4.1

The results for sanitiser 1 reflects a much lower IL-6 concentration for *E. coli* than for *Pseudomonas aeruginosa* and the community, suggesting symbiotic growth rather than competition. The derived LPS concentration that falls below the detectable concentration denotes a low IL-6 synthesis in the test sample (Panesar *et al*., 1999). The insignificant small or no difference on IL-6 concentration observed in *Pseudomonas aeruginosa* and the community could be because of no physical effect of the sanitiser allowing for a defense mechanism in the organisms membranes. The effects of sanitiser 2, in some way follow that of sanitiser 1. *E. coli* shows a lower IL-6 concentration in the control as compared to the test sample (Fig. 4.1).

Table 4.1: Derived values for LPS concentration using the values from listed values and Figure 4.2

Sample	Value from graph (from listed equation)	IL-6 concentration in pg/mL	Derived possible LPS concentration
1. A *E. coll* (0hrs)	-13006	0,00	0
2. B *E. coll* (0hrs)	-2167,67	0,00	0
3. B *P. aeruginosa* (15hrs)	275,38	500,361	8 µg/mL
4. C *E. coli* (0hrs)	-1238,67	0,00	0
5. C *E. coli* + *Pseudomonas* (15hrs)	1792,65	112,937	0 0
6. D Communal growth (0hrs)	3025,98	242,079	0,01 µg/mL
7. E Communal growth (0hrs)	173902,70	695,611	>10 µg/mL
8. F *E. coli* (0hrs)	209086	222,092	0,01 µg/mL
9. F *Pseudomonas* (0hrs)	161386	242,079	0,01 µg/mL

It would suggest that the unstressed sample may have contained stimulated porcine blood. *P. aeruginosa* at the same time shows a noticeable elevated IL-6 concentration during the exponential phase which would suggest stimulation in the porcine blood in response to the pathogen adapting. This adaptation subsequently produced a higher LPS concentration resulting in a higher IL-6 concentration (Sample 3, Table 4.1). It would thus seem that measure of toxicity is in response to a stress. Sanitisers in general would have a physical effect on the bacterial cell membrane causing the peripheral

extremes to fold back and in so doing closing the pores to any influence by the sanitisers. The cell membranes are therefore not relaxed/open and therefore not susceptible to LPS stimulation. This is a defense mechanism and therefore leaves its influence on IL-6 production debatable or unanswered at this stage. Another question arises and that is whether an alteration in the LPS structure may have been caused by the sanitisers and how, if at all, this had an effect on the acute-phase response in the blood.

In comparison to this, the IL-6 concentration produced by *E. coli* and *P. aeruginosa* shows a noticeable elevation during the exponential phase (Samples 5, Table 4.1) and this would once again suggest an aggressive response to stress. An increase in LPS concentration on the other hand is not that noticeable as it falls below the measurable level. This is significant when the marked increase in cytokine is observed in community during the lag phase for detergent 2 (Sample 6, Table 4.1). This could be because of two reasons. Firstly, it may represent stimulation in the porcine blood before test, or secondly it could represent competition for nutrients between the pathogens, resulting in

an increase in LPS concentration with the concomitant rise in IL-6 concentration or toxicity.

The samples grown at 4°C exhibit a significantly high IL-6 concentration for the community at the lag phase. It would be expected that the LPS during the lag phase would be at its lowest. This would therefore be redolent of the fact the quantity of the LPS might not necessarily be indicative of toxicity, but rather the quality too. There is some response to each other in terms of IL-6 production. Samples 8, 9 in Table 4.1 show a clearly higher IL-6 concentration. The community shows no difference between the control and tested samples throughout the growth time. When the concentration of LPS, IL-6 and Optical density (From Chapter 2) are compared with each other it is noticed that is no correlation between cell density and IL-6 concentration. This would also mean that there is no correlation between the number of viable CFU's and the LPS concentration.

4.6 CONCLUSION:

From this study it may be deduced that IL-6 is not an accurate or relevant biomarker for pyrogenicity. In addition to that it may also be said that it should also not be considered for the assessment of the change in LPS concentration. The sensitivity of the test is limited although it would seem that the possibility of inflammation or acute-phase response may be detected. The cytokine IL-6 could therefore be a useful marker for LPS induced inflammation at higher concentrations. Further studies are necessary to corroborate this hypothesis, but as seen in this study, with success *in vitro* studies, an *in vivo* study could be promoted. This could then be useful for the elucidation of possibly challenged LPS, *in vivo*.

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CHAPTER 5

A COMPARISON OF THE *LIMULUS* AMOEBOCYTE LYSATE TEST AND THE PORCINE – IL-6 PRODUCTION FOR THE QUANTIFICATION OF CHANGE IN TOXICITY

5.1 ABSTRACT

The *Limulus* amoebocyte lysate test (LAL) and the Porcine-il-6 Immunoassay were compared for their effectiveness to quantify endotoxin concentration and activity. The two tests compared favourably in principle, but differed in the complexicity of the technique, the cost involved and the sensitivity to what was tested for. The difference in sensitivity made it challenging to compare exact findings. As a result of the dissimilarity in the application extensive and more sophisticated methods are needed to test for further changes brought about by the effect of the extrinsic stresses. The LAL test was found to be more sensitive than the porcine-il-6 immunoassay as the latter has a sensitivity not accommodating low concentrations.

5.2 INTRODUCTION

5.2.1 Endotoxins

Endotoxins are lipopolysaccharides (LPS) found in the outer wall of Gram–negative bacteria, and these endotoxins are cast off during growth and death of bacteria. The

toxic upshot of endotoxins lays in their lipid A component, and the polysaccharide chain can establish the virulence of the bacterium (Reitschel *et al*., 1994) (Fig. 1.3, Chapter 1). Primarily, the pyrogenicity of endotoxins was of particular interest and worry in pharmacology and clinical fields, and it is soundly documented that the pyrogenicity of endotoxins is directly dependent upon the strain of Gram-negative bacteria which produce it (Weary *et al*., 1980). The endotoxin molecules are frequently thought of naively as singular, static structures; however the chimeric LPS molecule is able to change form depending on intrinsic factors, but also on extrinsic factors. Extrinsic factors which may affect the heterogeneity of the LPS molecule include the growth environment of the bacterial culture, the method of extraction, the non-LPS cellular constituents, bacterial type and the mechanism of LPS release (Williams, 2001). The mechanism of LPS discharge may be because of natural cell death, membrane vesicle liberation, host phagocyte ingestion and antibiotic release (Williams, 2001). For the purposes of this study, the growth environment was altered by the addition of sanitisers, detergents and household storage temperature. The systemic effects of endotoxins have been investigated intensively for many years (Reitchel and Brade, 1992) and the

structure-activity relationship with respect to systemic effects have been evaluated (Reitschel *et al*., 1994).

5.2.2 *Limulus* Amoebocyte Lysate

During the last 10 years, stress induced release of LPS from pathogens has been the object of extensive studies *in vitro* as well as *in vivo* (Hurley, 1992). The original discovery in 1956, that Gram-negative bacterial endotoxin trigger *Limulus* blood to clot was followed by the formulation of the initial LAL. This then lead to the Food and Drug Administration's (FDA) approval and commercialization of LAL for testing endotoxin presence (Levin, 1970). The rabbit pyrogen assay served as the only official pyrogen test for more than 30 years. However, during the early 1960's, an unlikely replacement was discovered, characterised by a blood product (Lysate) derived from the horseshoe crab *Limulus polymephus* (Williams, 2001). The *Limulus* Amoebocyte Lysate (LAL) test, based on the enzymes from the blood of this horseshoe crab, is very sensitive in revealing the presence of endotoxins. In just about all epidemiological studies on health

effects of endotoxins in airborne organic dust the LAL assay has been used for quantitative evaluations of endotoxins.

However, it is documented that endotoxins from different bacterial strains display different potencies in the LAL assay (Olenchock *et al.*, 1989). It is suggested that the LAL assay quantifies the proportion of endotoxin that is available to the assay (Hansen *et al.*, 1999). The ability of heamocytes to coagulate in the presence of gram-negative bacteria or their endotoxins is not restricted exclusively to *Limulus* and has been demonstrated in lobster, oyster and insects (Williams, 2001). Upon the invasion of gram-negative bacteria of the hemolymph, the hemocytes distinguish LPS on their surface and discharge their granular contents. To date, the known biosensors consist of coagulation factor C and factor G. These serve as the activation for the coagulation cascade that alters soluble coagulogen to the insoluble coagulin gel (Williams, 2001). For the purposes of this study the chromogenic test was performed (Fig. 5. 1).

5.2.3 IL-6

The porcine-IL-6 assay uses a quantitative sandwich enzyme immunoassay technique. The polyclonal antibody which is specific for the porcine-il-6 had been used to coat a microplate. The pleiotropic cytokine interleukin-6 (IL-6) shows a broad spectrum of biological tasks among which stimulation of B cells and induction of acute phase protein synthesis in the liver are mostly noteworthy (Kalan *et al.*, 1997). IL-6 wields its activity on target cells by binding to an IL-6 specific surface receptor (IL-6R). This receptor/ligand complex aids in the homodimerization of gp130 the second subunit of the IL-6 receptor complex. Dimerization of gp130 gives rise to transduction of an IL-6 signal (Taga and Kishomoto, 1997).

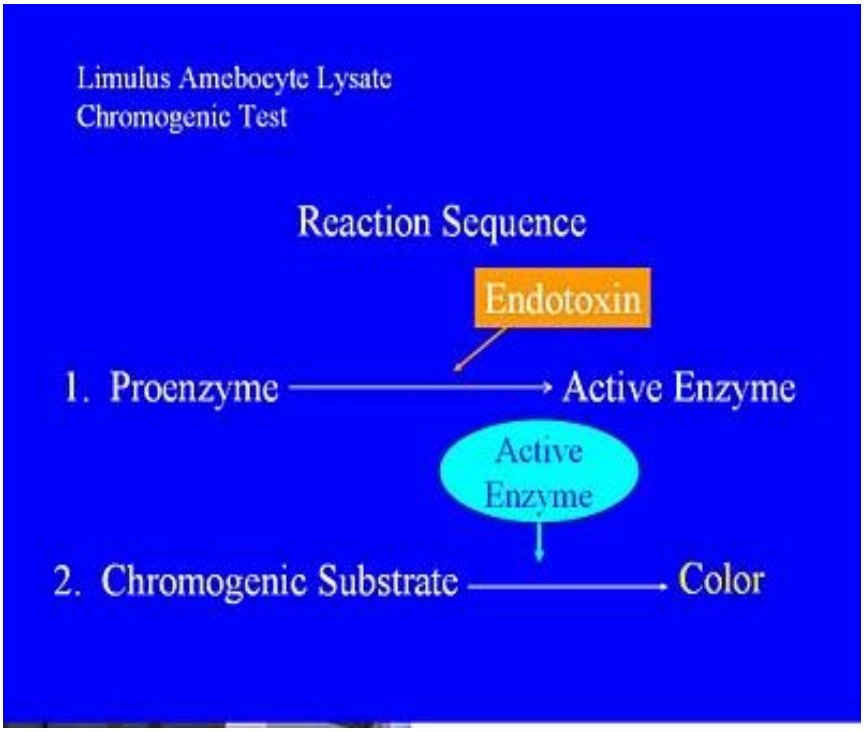

Figure 5.1: The mechanism of the *Limulus* Amoebocyte Lysate chromogenic test

5.3 AIMS

This study was conducted utilizing findings from previous chapters and existing findings from literature. The porcine-IL-6 and LAL methodologies are qualitatively validated.

5.4 MATERIALS AND METHODS

The qualitative validation compared the two techniques LAL (Chapter 3) and Porcine-il-6 production (Chapter 4). The techniques were compared according to their accuracy, sensitivity, cost and applicability for similar studies.

5.5 LIMITATIONS

5.5.1 LAL

The preference of the LAL technique may be prescribed by the pharmaceutical fraternity but may also be influenced by additional issues such as the inherent turbidity of the experimental sample as well as mandatory sensitivities. Although a positive LAL test designates the presence of pyrogens, a negative result does not necessarily imply the product is pyrogen free, as non-endotoxin pyrogens may exist but not picked up by the LAL test (Hartung, 2002). Endotoxin modification is a problem involving the amphiphilic properties of the LPS unit. This would imply that a strict lack of interference with structure would be required for a standard reading. When considering the different effects possible due to extrinsic stresses, this cannot be assured. The price of the LAL

kit is about 50% more than the porcine-il-6 Quantikine kit, making this investigation more expensive.

5.5.2 IL-6

The Quantikine Porcine-il-6 Kit from R&D Systems has to date, only been utilised for the purposes of research and thus has not displayed reliability diagnostically or *in vivo*. In addition to this, the design of the assay is to eradicate interference by soluble receptors, binding proteins and other factors existing in biological samples but, until all receptors have been put to the test in the Quantikine Immunoassay, the possibility of interference cannot be ruled out (Taga and Kishomoto, 1997). Furthermore, this assay recognises both recombinant and natural porcine-il-6, making the final result questionable in terms of whether it is as a result of experimental stimulation or not. The pooled blood taken from a number or pigs represents a blend of pre-sampling conditions and does not rule out the possibility of stimulation before testing or an inherent acute-phase condition in some of the sample candidates. What is more is the fact that although porcine blood has

an extensive similarity with human blood, it does not necessarily imply that the effect would be the same when performed *in vitro* or *vivo*, using human blood.

In terms or the technical aspects of the test, the minimum detectable dose of porcine-il-6 is typically 10pg/mL. The significance of this being that any readings falling below this concentration will not be reflected. This therefore reduces the degree of sensitivity of the test. Besides this, the test may be compromised by LPS concentrations ≥ 40 ng lps/mL (Pedersen *et al.*, 1995). The sensitivity is therefore at this point a questionable quality. Having said this, it now raises the question of the need for a more sensitive detector. Analysis through mass spectrophotometry could possibly rule out the low level of sensitivity and at the same time provide information regarding the alteration, if any in the structure of the LPS molecule.

5.6 CONCLUSION

The validation of the two tests should be approached on two different levels due to the different principles or mode of action. The LAL procedure would seem to be fit for the

purpose it was designed for. In contrast to this, the porcine-il-6 quantification gave a meaningful view of the presence of the cytokine IL-6, but its sensitivity is rather questionable and warrants a technique with greater or improved sensitivity.

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CHAPTER 6

GENERAL CONCLUSIONS

6.1 CONCLUDING REMARKS

The dangers facing the global food supply and the concern for human and animal health, as a result of food-borne diseases have been established as an on going problem. The safety of food, in particular, is of importance and unease to consumers and producers alike. Knowing this, it has become imperative that a mechanism which may combat the cause of disease needs critical attention.

The sensitivity of *Escherichia coli* and *Pseudomonas aeruginosa* to the respective sanitisers and detergents was similar for the same sanitiser and detergent. The sensitivity in communal growth was varied, but in some cases it generally followed that of the sensitivity of the individual organisms. For sanitiser 1 communal growth showed a response to the stress that was not an arithmetic sum of the response of the individual growth patterns, whereas sanitiser 2 showed no observable influence on *E. coli*, and it in fact facilitated better growth for *P. aeruginosa* and the community after 30 hours. Furthermore, the effect of detergent 1 and 2 was very similar indicating a possible

related mechanism or action on the organisms. The effect of detergent 2 was more acute. Household storage temperatures of 4ºC and 10ºC inhibited the growth of organisms but did not prevent it. The growth profile for communal growth at these temperatures did not resemble that of the pure cultures at all. For the quantification by LAL it became evident that the enumeration of the organisms isolated from households might not be because of any acclimatisation and underlying stress induced and thereby affecting their toxicity. The sanitisers and detergents affected growth and toxicity in a competitive colonial fashion. The stresses also had a more marked effect on the older "biofilm" formation as this is seen by the decrease in toxicity. For the porcine-il-6 analysis it was shown that this procedure is not an accurate or relevant biomarker for pyrogenicity testing at very low concentrations. In addition, it should not be considered for the assessment of the change in LPS concentration. Having said that, this procedure may be used instead to detect any LPS induced inflammation at higher concentrations.

It is therefore no surprise that the validation for the two tests when compared in terms of complexicity of technique, sensitivity, reliability and cost, favours the LAL procedure.

The LAL test also seems fit for the purpose it was designed for. The large room for pre-test stimulation in pigs' blood also tends to cast a shadow on the IL-6 findings. To corroborate these findings, further investigation and more sensitive techniques could be used.

6.2 FUTURE RESEARCH

As this study only evaluated the influence of extrinsic stresses on the communal growth of *Escherichia coli* and *Pseudomonas aeruginosa*, it is suggested that the following would require further investigation:

- The change in LPS structure and the way it influences toxicity

- How and where bacteria alter lipid A during the course of disease. This would provide clues necessary to identify host lipid A recognition factors.

- The question of free LPS, as compared to or in combination with intact LPS counterparts.

- The need for a biomarker to quantify the change in LPS induced growth in community.