THE INFLUENCE OF THERMAL AND NONTHERMAL FOOD PRESERVATION METHODOLOGIES ON THE LIBERATION AND ULTRASTRUCTURE OF BACTERIAL ENDOTOXINS

by

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DECLARATION OF INDEPENDENT WORK

I, Dedré Olivier, do hereby declare that this research project submitted to the Central University of Technology, Free State for the degree Doctor Technologiae: Biomedical Technology, is my own independent work. This research has not been submitted before to any institution by me or any other person in fulfilment of the requirements for the attainment of any qualification.

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SIGNATURE DATE

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GLORIA IN EXCELSIS DEO

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SUMMARY

Consumer demands for fresh, microbiologically safe foods with high organoleptical and nutritional quality has led to the development of novel food preservation technologies as alternatives or enhancements to traditional preservation techniques. An example of these novel preservation technologies is high hydrostatic pressure (HHP) processing. It involves the applications of static pressure of 50 to 1 000 mega Pascals (MPa) to solid or packaged liquid foods, with varying holding times. The combination of factors to enhance preservation is increasingly being used in industry, e.g. the use of different temperatures and additives (hurdles) can enhance the preservative effect of HHP. In this study the influence of HHP on organism viability and growth response was assessed. The organisms evaluated included *Escherichia coli* O111, *Listeria monocytogenes* (UAFSBCC) and *Staphylococcus aureus* (ATCC 25923), in peptone water, which was subjected to HHP of 200 MPa for 15 minutes at 8 and 50 ºC respectively. Subsequent to the mentioned pressurisation, sub-culturing was performed and growth responses were evaluated at 0, 6, 18, 24, 30, 42 and 48 hours. Bacterial survival and growth response was measured by means of intact cell count, colony forming units and optical density. From the results it was eminent that bacterial cells were only sublethally injured and were able to repair within 48 hours of enriched sub-culturing. *E. coli* O111 proved to be most sensitive to HHP with *Staphylococcus aureus* (ATCC 25923) most resistant. This study also proved that bacterial concentration and inactivation rate are inversely proportionate to each other. Subsequent to growth and cell repair assessments, *E. coli* O111 was selected

as a model to evaluate the effect of sublethal HHP on the liberation and toxicity of bacterial endotoxins (free and cell wall bound). It is also known that different extraction procedures extract different lipopolysaccharides (LPS) fractions and therefore LPS was extracted from the test broth by a combination method of Folch, Lees & Sloane-Stanley, and Venter and Ivanov. The extraction yielded a biphasic system, LPS with reduced lipid content in the upper phase (aqueous) and LPS with increased lipid content in the lower phase (organic). Following extraction the *Limulus* amebocyte lysate (LAL) test was performed to quantify the concentration (assumed) of LPS in the aqueous and organic phases. Free LPS was detected within six hours in the supernatant in the high and low bacterial loads, moreover the toxicity response of post HHP cell damage was more pronounced at 50 ºC (hurdle) than that observed for the treatments at 8 ºC (hurdle) and more so in the organic phases. The latter implied that HHP not only resulted in quantity LPS variation but also in structural change. However membrane repair was apparently complete after 48 hours, as differences in toxicity were no longer evident. Furthermore, the use of a porcine IL-6 ELISA assay was evaluated as an alternative for the customary LAL as a biomarker for pyrogenic substances in matrixes. Porcine whole blood was challenged for IL-6 production by LPS in the samples from the organic and aqueous systems. A porcine IL-6 enzyme-linked immunosorbent assay was used to assess IL-6 expression in whole blood after being challenged with LPS. From the results it emanated that HHP caused in a change in LPS structure which resulted in a decreased IL-6 expression in whole blood, indicating that structural adaptation of the cell membrane in response to HHP influenced the ability of LPS to

stimulate macrophages and monocytes. Therefore, further research and development would be required to evaluate the influence of post HHP LPS on human IL-6 expression. When comparing the porcine IL-6 with the LAL no correlation in toxicity could be established in any of the treatment parameters.

Finally it can be concluded that HHP had an influence in the structural morphology of LPS. These structural changes could result in LPS being more toxic, it could also have an effect on the accuracy of immunological assessments, the ability to form biofilm, and susceptibility to phages.

KEYWORDS*: E. coli* O111, *Staphylococcus aureus, Listeria monocytogenes*, LAL, IL-6, HHP, sublethal injury, endotoxin

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ACRONYMS

ACRONYM	
µl	micro litre
ATCC	American type culture collection
a_w	water activity
CFU ml^{-1}	colony-forming units per millilitre
CFUs	colony forming units
CO_2	carbon dioxide
ctrl	control
$_{D,D}$-Hep	$_{D}$-glycero-$_{D}$-manno-Heptose
DNA	deoxyribonucleic acid
E. coli	*Escherichia coli*
Eh	redox potential
EHEC	enterohaemorrhagic *E. coli*
ELISA	enzyme-linked immunosorbent assay
EU	endotoxin units
Gal	galactose
Glc	glucose
Hep	L-glycerol-D-manno-heptose;
HHP	high hydrostatic pressure
HIV	human immunodeficiency virus
HPLC	high-performance liquid chromatography
IL	Interleukin

INF	interferon
IRAK	interleukin-1 receptor-associated kinase
kDA	kilo Dalton
Kdo	2-keto-3-deoxyoctonic acid
Ko	2-keto-D-glycero-D-talo-octonic acid
LAL	limulus amebocyte lysate
LBP	LPS-binding protein
$_{L,D}$-Hep	L-glycero-$_{D}$-manno-heptose residues
LOS	lipo-oligosaccharide
LPS	lipopolysaccharide
LTA	lipoteichoic acid
ml	millilitre
mm	millimetre
MPa	mega Pascals
MyD88	Myeloid differentiation primary response gene (88)
NGa	N-acetyl-galactosamine
NGc	N-acetyl-glucosamine
nm	nanometre
NMR	nuclear magnetic resonance
PD	percentage difference
PEFs	pulsed electric fields
pH	acidity or alkalinity
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis

STEC	Shiga toxin-producing *E. coli*
Stx	Shiga toxins
T_H	T helper
TNF	tumour necrosis factor
TNFα	tumour necrosis factor alpha
TRAF6	tumor necrosis factor receptor-associated factor 6
UAFSBCC	Unit for Applied Food Science and Biotechnology culture collection
VTEC	verocytotoxin producing *E. coli*
vtx$_1$	verocytotoxin 1
vtx$_2$	verocytotoxin 2
WHO	World Health Organization

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CHAPTER 1

1. INTRODUCTION

Some of the most prevalent health problems encountered are the result of foodborne diseases, which influence the health of individuals and the development of societies (Schlundt, 2002). A broad spectrum of microbial pathogens can contaminate food and water supplies and cause illness following ingestion of them or their toxins. This causes a range of illnesses, where the invasive organism can produce clinical syndromes and toxins that can affect almost any system in the body. Therefore, the term “foodborne disease” relates to many pathogens and many diseases (Tauxe, 2002). The World Health Organization (WHO) defines food hazards as “biological, chemical, or physical agents in or property of food that may have an adverse health effect,” and food-related risks are traditionally defined as “a function of the probability of an adverse effect and the magnitude of that effect, consequential to a hazard in food” (WHO, 1995).

Toxigenesis is an underlying mechanism by which many bacterial pathogens cause disease. At a chemical level, there are two main types of toxins produced by bacteria. Exotoxins secreted by Gram-positive bacteria, acts enzymatically or through direct action with host cells and stimulate a variety of host responses. Lipopolysaccharides, also known as endotoxins, are associated with the cell wall of Gram-negative bacteria and are liberated from bacterial cells upon death, division and as blebs (Todar 2008). Lipopolysaccharides (LPS) are essential for the viability of Gram-negative bacteria. Despite the wide variety of LPS expressed in

nature, they all have a common structure (Erridge, Benner-Guerrero & Poxton, 2002). Nevertheless, their structural details strongly influence their activity (Caroff & Karibian, 2003). Differences between species and strains exist in LPS, and most variation occurs in the O-chain, followed by the core region and finally the lipid A moiety. The endotoxicity of LPS is determined by the quantity, nature and arrangement of acyl chains and phosphate groups on lipid A, with lipopolysaccharide (LPS) structures displaying an acylation pattern similar to that of *Escherichia coli* (*E. coli*), being the ones optimally recognised by human monocytes (Erridge *et al.,* 2002).

LPS are among the most potent bacterial stimuli of the human immune system and are of great significance in the pathophysiology of many disease processes (Erridge *et al.,* 2002; Proctor, Delinger & Bertics, 1995). LPS induce the production of large amounts of inflammatory cytokines and mediators and provide numerous antigenic sites (Proctor *et al.,* 1995). LPS do not act directly against cells or organs, but through activation of the immune system, especially through monocytes and macrophages. These cells release mediators such as tumour necrosis factor (TNF), interleukins and free radicals, having potent biological activity and being responsible for the adverse effects caused by LPS exposure (Martich, Boujoukos & Suffredini, 1993; Pabst & Johnston, 1989; Rietschel, Kirikae, Schade, Mamat *et al.,* 1994). In an infected host, small amounts of LPS can be protective by stimulating the immune system and have even been used to shrink

tumours. Large amounts, however, have an adverse effect (Caroff & Karibian, 2003).

1.1 ORIGIN OF BACTERIAL LIPOPOLYSACCHARIDES

Lipopolysaccharides were discovered in the eighteenth century during the search for a fever- and disease-producing substance that was associated with unclean conditions. This substance was referred to as a pyrogenic substance, putrid poison, or toxin. Richard Pfeiffer (1858-1945) identified the heat-stable substance produced by Gram-negative bacteria, and named it endotoxin (Rietschel & Westphal, 1999; Vaara & Nikaido, 1984). The development of techniques for the extraction and preparation of endotoxins pure enough for structural elucidation was slow. In the 1930s and 1940s it was reported that the structure consisted of a polysaccharide, a lipid part and a small amount of protein, hence the name lipopolysaccharide (Rietschel & Westphal, 1999). In the 1950s, numerous techniques were developed for the extraction, purification and characterization of LPS, with further adaption and application of sugar and lipid chemistry to define the structure (Caroff, Karibian, Cavaillon & Haeffner-Cavaillon, 2002).

LPS are an integral part of the outer membrane of Gram-negative bacteria and are responsible for the organisation and stability of the bacteria (Vaara & Nikaido, 1984). [See Figure 1.1 for the structure of the Gram-negative bacterial cell wall]. LPS are anchored within the bacterial cell wall and are incessantly liberated into

the environment when the cells die, during cell proliferation and division (Raetz, 1990), and in the form of vesicles (Kuehn & Kesty, 2005).

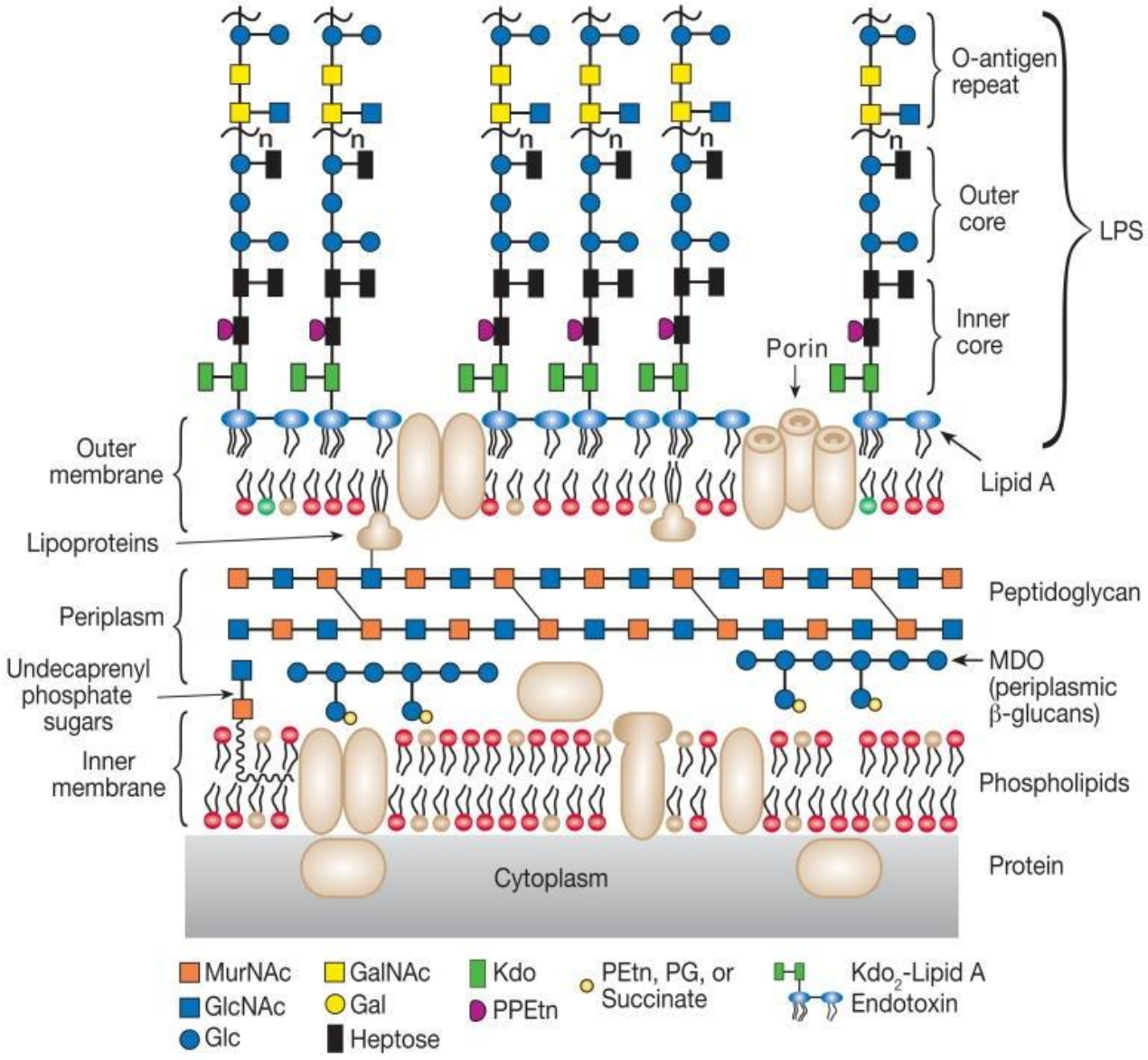

Figure 1.1 Structure of the Gram-negative bacterial cell wall (Varki, Cummings, Esko, Freeze, Stanley, Bertozzi, Hart & Etzler, 2009).

1.2 LIPOPOLYSACCHARIDE ULTRA STRUCTURE

With techniques such as high-performance liquid chromatography (HPLC), nuclear magnetic resonance (NMR) and epitope studies, the structure of the LPS of numerous Gram-negative bacteria have been elucidated and generally all conform to a common architecture. [See Figure 1.2 for a schematic structure of *E. coli* O111:B4 LPS].

The basic structure for the LPS of Gram-negative bacteria comprises three distinct regions: Lipid A, which is hydrophobic and the part responsible for the endotoxic activity; the core, which is covalently attached to lipid A and which is divided into an inner and an outer core; and a polymer of repeating subunits called the O-polysaccharide (O-chain), which is attached to the core (Erridge *et al.,* 2002).

The core region close to lipid A and lipid A itself are partially phosphorylated; therefore, at neutrality, LPS exhibit a net negative charge (pK_a=1.3) (Hou & Zaniewski, 1990). The molar mass of an LPS monomer varies between 10 and 20 kilo Dalton (kDA), owing to the variability of the oligosaccharide chain. In some instances, masses of 2.5- (O-antigen-deficient) and 70 kDa (very long O- antigen) were found (Kastowsky, Gutberlet & Bradaczek, 1990).

The three-dimensional structure of LPS, especially the long surface antigen, is more flexible than the globular structure of proteins (Kastowsky *et al.,* 1990).

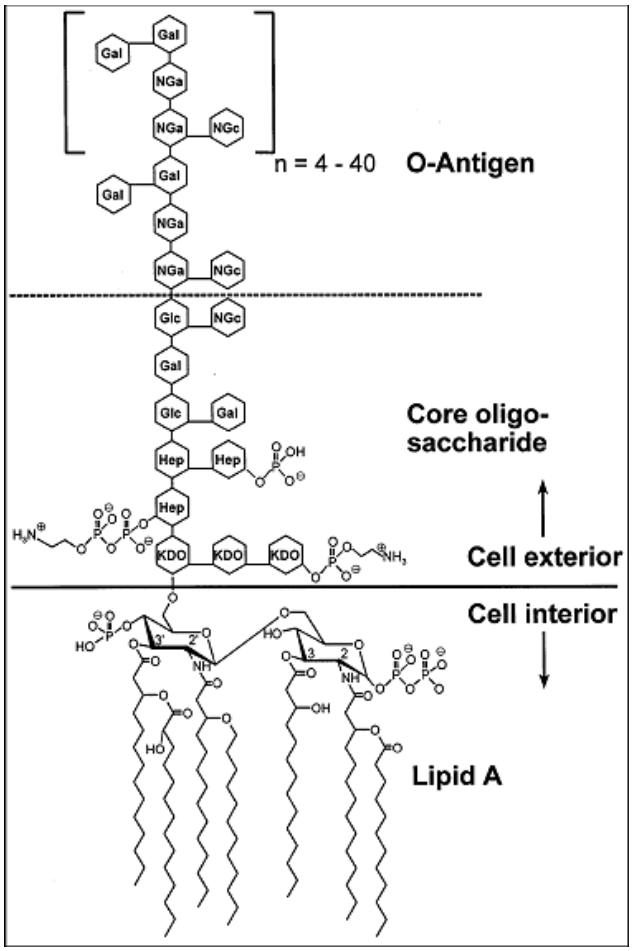

Figure 1.2 Schematic structure of *E. coli* O111:B4 LPS (Ohno & Morrison, 1989) (Hep = L-glycerol-D-manno-heptose; Gal = galactose; Glc = glucose; KDO = 2-keto-3-deoxyoctonic acid; NGa = N-acetyl-galactosamine; NGc = N-acetyl-glucosamine)

Another characteristic is the formation of supramolecular structures, owing to non-polar interactions between lipid chains, as well as to bridges generated among phosphate groups by divalent cations. Hence, micellar structures and vesicles are produced that have much higher molar masses and diameters of up to 0.1 μm, which, even in dilute aqueous solutions, show high stability levels (Anspach, 2001).

1.2.1 Lipid A

The most conserved part of the LPS is lipid A, showing a narrow structural relationship between different bacterial genera (Hou & Zaniewski, 1990). The lipid A of *E. coli* is composed of a β-D-GlcN-(1-6)-α-D-GlcN disaccharide carrying two phosphoryl groups at positions C-1 and C-4. These phosphates can be substituted with groups such as ethanolamine, phosphate, ethanolamine phosphate, ethanolamine diphosphate, D-arabino-furanose, GlcN and 4-amino-4-deoxy-L-arabino-pyranose (Moran, Lindner & Walsh, 1997). Four primary (R)-3-hydroxytetradecanoic acids are attached to this backbone by either ester (position 3 and 3') or amide (positions 2 and 2') linkages. In addition, two secondary acyl chains are found, tetradecanoic acid 14:0 ester linked to 14:0(3-OH) in position 3' and dodecanoic acid 12:0 linked to the hydroxyl group of the 14:0(3-OH) in amide linkage to position 2' (Gutsmann, Schromm & Brandenburg, 2007). The acyl chains can be substituted by fatty acids to provide LPS with up to seven acyl substituents, which vary noticeably between species in nature, number, length, order and saturation. The fatty acids can be attached to lipid A either asymmetrically (4+2, e.g. *E. coli*) or symmetrically (3+3, e.g. *Neisseria meningitidis*). The most common fatty acids in lipid A have 10-16 carbon atoms, although longer chains do exist (Moran *et al.,* 1997). The number of fatty acid groups present in the LPS has a direct effect on its toxicity. Fatty acid chain length is critical for lipid A toxicity since C_{12}, $C_{12}OH$, C_{14} and $C_{14}OH$ fatty acids are those found in the most toxic lipid A (Zähringer, Knirel, Lindner, Helbig *et al.,* 1995).

Removal of a particular group of lipid A leads to a dramatic decrease in endotoxicity. Cleavage of the acid-labile 1-phosphate (monophosphoryl lipid A) causes a reduction in cytokine production by one order of magnitude, and the removal of one acyl chain such as the secondary one in position 3' (pentaacyl lipid A) leads to a reduction by more than three orders of magnitude. Upon removal of both secondary acyl chains (tetraacyl lipid A), there is a complete loss of activity. Changes in chemical primary structures lead to corresponding changes in the aggregate structures, i.e. to the molecular shape and to the intra-molecular conformation, which has a major influence on the biological activity (Gutsmann *et al*., 2007). [See figure 1.3 for the structure of lipid A in *E. coli*].

The polysaccharide portion of the LPS is almost invariably linked to lipid A by means of a Kdo residue at position 6'. This portion of the molecule constitutes the most conserved region seen between LPS molecules: a phosphorylated diglucosamine backbone attached to at least one Kdo residue (Moran *et al.,* 1997). [See figure 1.4 for the function of LpxA in lipid A biosynthesis].

1.2.2 Core Polysaccharide

The O-polysaccharide region of the LPS can vary. The oligosaccharide structures in the core part of the LPS are limited, with some regions being highly conserved between different strains and species. The inner core is proximal to lipid A and

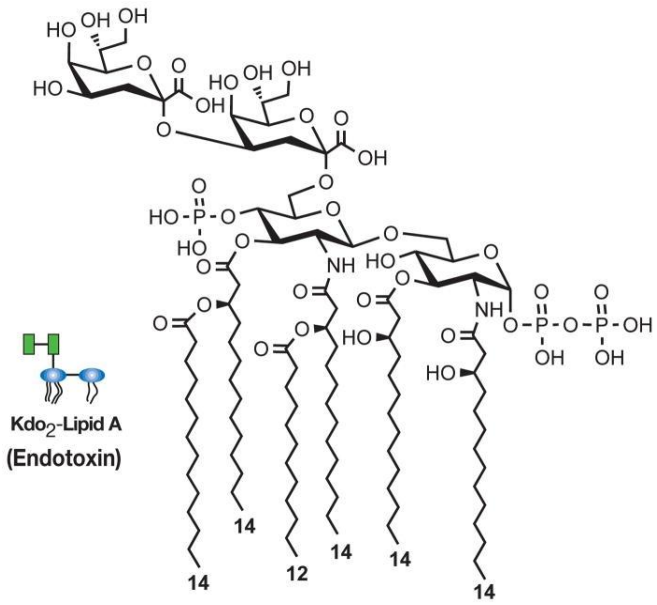

Figure 1.3 Structure of lipid A in *E. coli (*Raetz, Reynolds, Trent & Bishop, 2007)

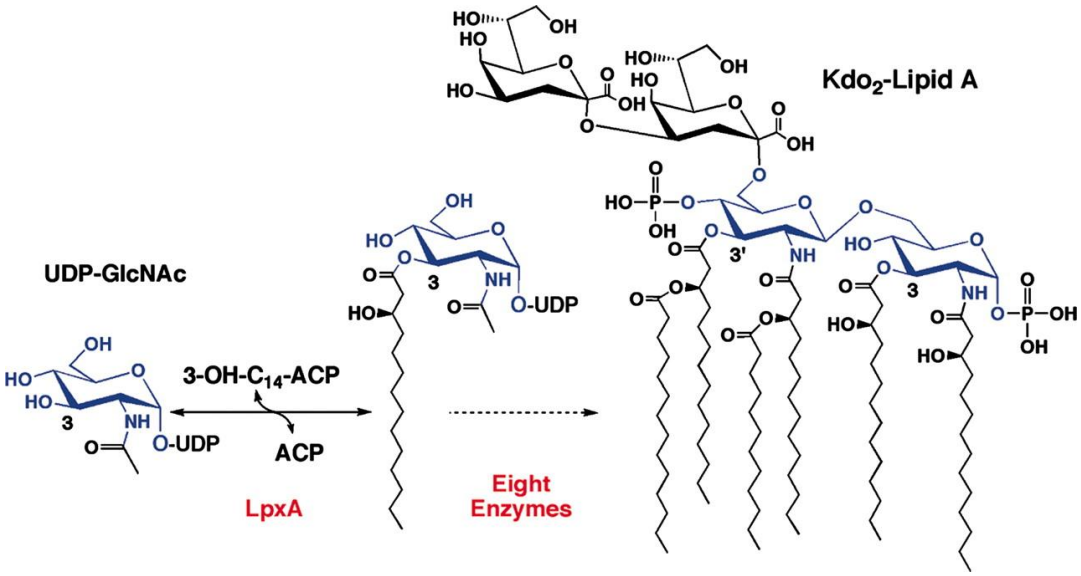

Figure 1.4 Function of LpxA in lipid A biosynthesis (Williams, Immormino, Gewirth & Raetz, 2006)

contains a large amount of unusual sugars such as L-glycerol-D-manno heptose (Hep) and 3-deoxy-D-manno-octulosonic acid (Kdo) (Erridge *et al.,* 2002). Kdo is seen in almost every LPS, and is α-bound to the carbohydrate backbone of lipid A. Exceptions to this have been seen with *Acinetobacter* and *Burkholderia cepacia* LPS, which employ the alternative 2-keto-D-glycero-D-talo-octonic acid (Ko) in its place. The bond between the first Kdo residue and lipid A is acid labile, and with moderate pH (<4.4), easily hydrolysing the bond to release the core from lipid A. The Kdo residue is required for bacterial viability. The outer core extends further from the surface of the bacteria and is more likely to consist of more common sugars such as glucose, galactose, N-acetyl galactosamine and N-acetyl glucosamine (hexose region). The outer core is generally more variable than the inner core (Erridge *et al.,* 2002).

The extent of heterogeneity in the core of bacterial strains depends somewhat on the number of non-stoichiometric components present. Non-stoichiometry is often attributed to incomplete biosynthesis, but could also be the result of partial degradation after complete biosynthesis (Caroff & Karibian, 2003). "Phase variation" is another kind of core heterogeneity, which has been studied in the O-chainless genera of *Haemophilus, Neisseria, Campylobacter* and other pathogens that invade mucosal tissues (Masoud, Martin, Thubault, Moxon & Richards, 2003; Moran & Prendergast, 2001). Structural variations may have any of several types of genetic origin, one of which is replication slippage (Gilbert, Karwaski,

Bernatchez, Young *et al.,* 2002). The term “phase variation” applies to heterogeneities in bacterial cell surface components other than LPS cores. The basis for this variation in *Haemophilus* and *Neisseria* is probably polymerase slippage during replication of short nucleotide repeats in genes encoding biosynthetic enzymes. The reading frame on the deoxyribonucleic acid (DNA) is shifted and results in a switch-like change in the synthesis and addition of certain groups of sugars to a core (Cox, Wood, Martin, Makepeace *et al.,* 2002). The result of the modifications in the core structure is either the masking of an antigenic group (epitope) on the core or the mimicking of a host structure. In both cases, the effect is a dulling of the potential reaction of the host (Preston & Maskell, 2002). Although the endotoxically active part of the molecule is lipid A, the nature and number of attached saccharide residues and substituents do have a considerable impact on modulating this activity (Erridge *et al.,* 2002).

1.2.3 Enteric Cores

LPS cores typically consist of 8-12 branched sugar units. The sugar at the reducing end is always α-3-deoxy-$_{D}$-manno-oct-2ulosonic acid (Kdo) (2$\rightarrow$6)-linked to GlcN^{II} of lipid A, and this bond is sensitive to mild-acid hydrolysis. One or two Kdo groupings may be found at the C-4 position of Kdo. Three L-glycero-$_{D}$-manno-heptose residues ($_{L,D}$-Hep) are also (1$\rightarrow$5) linked to the first Kdo. A phosphate, pyrophosphate or phosphorylethanolamine group, or another sugar, may substitute

a heptose to make up the inner core, as is found in *Shigella, Salmonella* and *Escherichia*. These substituents are often present in non-stoichiometric amounts (Holst, 1999).

The outer LPS core of *Shigella, Escherichia, Salmonella, Hafnia, Citrobacter* and *Erwinia* generally consists of an oligosaccharide (up to six sugar units) (1→3)-linked to Hep^{II}. It is often branched with Gal, Glc or derivatives thereof. However, there are exceptions found in the outer core: *E. coli* K12 has a unit of $_{L,D}$-Hep and strains of *Klebsiella pneumoniae*, which has one or two units of $_{D}$-glycero-$_{D}$-manno-Heptose ($_{D,D}$-Hep) linked to a Kdo unit serving as the attachment site of an O-chain (Vinogradov, Frirdich, MacLean, Perry *et al.,* 2002; Vinogradov & Perry 2001).

In the genus *Proteus* (*vulgaris, penneri* and *mirabilis)* the inner cores of almost all strains have their Kdo residue substituted at the C-8 position by 4-amino-4-deoxy-$^{\beta}$-$_{L}$-arabinose. As for the rest, this part of the core resembles that of other enterics. The outer cores can, however, vary considerably. Some of the unusual constituents include: a Kdo linked to Hep III, a GalA amide linked to an aliphatic amine or to the α-amino group of $_{L}$-lysine, 2-glycylamino-2-deoxy-$_{D}$-glucose, the open-chain form of GalNAc glycosidically linked to GalN in the form of a cyclic acetal (Vinogradov, *et al.,* 2002; Vinogradov & Perry, 2000; Vinogradov & Sidorczyk, 2002).

1.2.4 Non-Enteric Cores

Hep, Kdo and hydroxytetradecanoic acid are considered to be identifying characteristics of LPS. They are present in the LPS of wild-type enteric bacteria; however, it has been shown in mutants with shorter cores that these bacteria are able to survive under laboratory conditions without the heptose (Mäkelä & Stocker, 1984). Further research revealed that several non-enterics are devoid of heptose, and some had replaced Kdo with Ko. A *Neisseria meningitidis* mutant lacking a core had been cultured and had been able to survive and grow slowly in a laboratory (Steeghs, Den Hartog, Den Boer, Zomer *et al.,* 1998). The core of the *Francisella tularensis* LPS has been reported to be entirely lacking heptose components. The one Kdo unit present is linked to a β-mannose-(1→4)-α-mannose disaccharide substituted at O-3 and O-2 by α-Glc and α-GalN-(1→2)-β-Glc, respectively (Vinogradov *et al.,* 2002).

The phosphate groups and charged sugar residues in the inner core and lipid A contribute to the stability of the outer membrane via cross-linking cations. There are, however, variations in the number of negatively-charged components: one to three units of Kdo, the presence or absence of phosphate groups on lipid A, on Kdo and/or heptoses, and the presence or absence of an occasional hexuronic acid. Due to the variability in the density of these charges in the lipid A inner core regions of different bacteria, the specific environmental conditions and the nature of bacterial capsules must be considered in explaining the diversities. Bacterial

species are likely to have more O-chain types than core types (Amor, Heinrichs, Frirdich, Ziebell *et al.,* 2000). The inner cores of most LPS have Kdo and heptoses, but more diversified sugars are found in the outer cores of non-enteric bacteria. The majority of sugars encountered in LPS are hexoses in the pyranose form. There are, however, exceptions, e.g. rhamnose found in some *Pseudomonas aeruginosa* strains, fructofuranose and sedoheptulofuranose in *Vibrio cholerae*, and 3-deoxy-α-$_D$-manno-oct-2-ulofuranosonic acid in *Aeromonas salmonicida* (Holst, 1999).

1.2.5 O-Polysaccharide

The O-polysaccharide (also referred to as O-chain or O-antigen) is the outermost part of the LPS and is the major antigen targeted by host antibody responses (Joiner, Grossman, Schmetz & Leive, 1986). The repeating units of the O-polysaccharide region consist of between one and eight glycosyl residues and vary between bacterial strains by means of the sugars, sequence, chemical linkage, substitution, and ring forms utilised. There is a near limitless diversity of O-chain structures, which is verified in nature with the observation of hundreds of serotypes for particular Gram-negative species. The number of subunits used to complete the chain varies between 0 and ~50, and a single bacterium can produce a wide range of these lengths as a result of incomplete synthesis of the chain. This gives rise to the classical ladder pattern of molecular weights seen when smooth LPS is

visualised by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Erridge *et al.,* 2002). O-chain structures can be linear or branched and substituted by many different aglycones. The most frequent substituents are O- and N-acetyl, phosphate and phosphorylethanolamine groups (Moran, Rietschel, Kosunen & Zahringer*,* 1991). Amino acids in amide linkages, acetamidino groups and formyl groups, as well as glyceric acid, are often found as non-stoichiometric substituents (Janson, 1999). Serology of the O-chain is a valuable diagnostic tool in typing bacterial strains and LPS. The O-polysaccharide is recognised by the innate arm of the immune system, and plays a role in the activation and inhibition of complement activation (Joiner *et al.,* 1986). O-chains are typically composed of hexoses and are not ubiquitous, as they are truncated or lacking in a number of Gram-negative bacteria. Bacteria that colonise mucosal surfaces often express LPS with a truncated non-repeating O-chain known as lipo-oligosaccharide (LOS). Certain strains also carry mutations in the otherwise well-conserved '*rfb*' locus (which contains a selection of genes involved in O-chain synthesis and attachment) and are termed "rough mutants" to differentiate them from the wild-type "smooth" strains, which express O-chain-bearing LPS (Erridge *et al.,* 2002).

Rough-mutant bacteria are serum-sensitive and are lysed by complement, whereas smooth strains, protected by their O-LPS polysaccharide chains, can survive in the host. These smooth strains express a heterogeneous mixture of unsubstituted complete core (Ra) LPS and O-LPS with different chain lengths. Since only O-LPS and Ra-LPS occur in the host, these LPS are responsible for the

natural immunity detected when normal or clinical sera are studied with the various R-LPS and S-LPS as antigens in immunoassay. When the incomplete-core R-LPS are used as immunogens, they can elicit novel antibody responses to the "break points" in the core oligosaccharide chains normally occupied by the missing outer sugars. Such antibodies tend to be chemotype-specific, and may dominate following such vaccinations. Immunoblotting on more complete LPS can often reveal their lack of reactivity, especially for monoclonals (Barclay, 2010).

O-specific chains are present in smooth-type Gram-negative bacteria and pathogenic bacteria, and are in direct contact with the host during infection. These chains help the bacteria to escape the lytic action of complement by a "shielding" process (Joiner, Schmetz, Leive & Frank, 1984). A combination of monosaccharide diversity, the numerous possibilities of glycosidic linkage, the substitution and configuration of sugars, and the genetic capacities of the diverse bacteria all contribute to the uniqueness of the great majority of O-chain structures (Caroff & Karibian, 2003). Even though broad diversity seems to be the rule in bacteria, serological cross-reactivity sometimes indicates similarities between O-chain structures. *Brucella abortus* and *Yersinia enterocolitica* O9 both have a homopolymeric O-chain structure composed of 4-amino-4,6-dideoxy-L-mannose residues (Caroff, Bundle & Perry, 1984; Caroff, Bundle, Perry, Cherwonogrodzky & Duncan, 1984). Some O-chains consist of repetitive sugar units interlinked with other compounds such as hydroxybutyric acid or diamino-3-deoxy-nonulosonic acid (Vinogradov, MacLean, Crump *et al.,* 2003). Other O-chains are of the

teichoic-acid type, with glycerol phosphate or with a phosphorylated aminopentonic acid (Dabrowski, Dabrowski, Katzenellenbogen *et al.,* 1996). Deoxy sugars are also frequent components in O-chain structures, but sugars that are more characteristic of the inner core region, like heptoses, are seldom present (Janson, 1999).

1.3 CYTOKINES

During infection, the release of exogenous agents (microbial products) and endogenous mediators (cytokines and chemokines) contributes to the recruitment of circulating leukocytes to the inflamed tissue. These microbial products trigger multiple cell types to release cytokines, which are potent inducers of chemokines. The primary cytokines act as endogenous activators of the immune response, whereas the inducible chemokines act as secondary mediators to attract leukocytes (Gouwy, Struyf, Proost & Van Damme*,* 2005). Cytokines are protein molecules, and their name was derived from early observations that these molecules influences cell ("cyto") movement ("kinesis"). Cytokines play a pivotal role in regulating nearly every aspect of the immune response (Abbas & Lichtman, 2003). They are large molecules (≥15 kDa) and cannot cross the blood-brain barrier under normal circumstances (Maier & Watkins, 1998). Cytokines are categorised into three functional groups based on their roles in: innate immunity (interferon-(INF) alpha, Interleukin (IL)-1, IL-6, IL-10, IL-12, tumour necrosis factor

alpha (TNFα) and the chemokines); adaptive immunity (IL-2, IL-4, IL-5, INF-gamma and transforming growth factor-beta); and haematopoiesis (colony-stimulating factors, IL-3 and IL-7) (Abbas & Lichtman, 2003).

Innate immunity (inflammatory response) is a relatively non-specific frontline defense that evolves over a period of hours to days against pathogens and in response to the disruption of tissue integrity (Miller, Pierce & Pariante, 2000). The two principal types of immune cells, T helper (T_H) cells and macrophages, are potent producers of cytokines. Exposure to minute amounts of LPS provokes the innate immune responses *in vitro* and *in vivo* (Imrich, Ning, Koziel *et al.,* 1999). The nature of the immune response may be dependent on the developmental stage of the subject (adult or infant) and on the sequence and mode of exposure to LPS and immune stimuli (Beutler & Poltorak, 2000). The major pro-inflammatory cytokines TNF-α and IL-1ß are mainly produced by macrophages. The T_H1 subset (which also activates macrophages) mainly secretes IFN-γ, TNF-ß and IL-2. The T_H2 subset participates primarily in allergic and humoral responses and produces IL-4, IL-5 and IL-13. T_H1 and T_H2 are defined by their functions and distinct cytokine production profiles (Elenkov & Chrousos, 2002; Romagnani, 1998). IL-6 is a pleiotropic cytokine that can simultaneously generate functionally distinct and sometimes contradictory signals through its receptor complex, IL-6R alpha and gp130 (Kamimura, Ishihara & Hirano, 2003). [See Figure 1.5 for a ribbon representation of the IL-6 crystal structure.] Elevated levels of pro-inflammatory

cytokines in the brain have been associated with the development of neurological disorders such as Alzheimer's disease (Krabbe, Pedersen & Bruunsgaard, 2004; Gupta & Pansari, 2003), vascular dementia (Krabbe, Reichenberg, Yirmira, Smed *et al.,* 2005), Parkinson's disease (Gonzalez & Ezquerro, 2004), and human immunodeficiency virus (HIV)-associated dementia (Rafalowska, 1998).

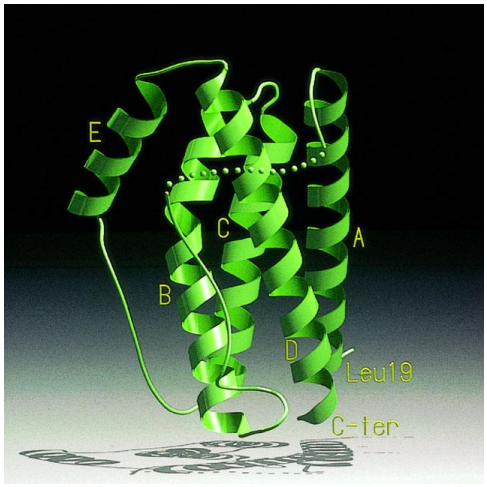

Figure 1.5 Ribbon representation of the IL-6 crystal structure. "The four main helices are labeled A, B, C and D. The extra helix in the final long loop is labeled E" (Somers, Stahl & Seehra, 1997).

Adaptive immunity is a slower appearing specific immune response, and is mediated by B-lymphocytes and T-lymphocytes, which target specific recognition molecules on invading pathogens, or virally infected or neoplastically transformed cells (Miller *et al.,* 2000). Exposure to LPS may influence subsequent adaptive

immune responses to other immune stimuli (Abraham, Finn, Milton, Ryan *et al.,* 2005).

1.4 RESPONSES TO ENDOTOXINS AND GRAM-NEGATIVE BACTERIA

The signaling pathways alerting the host to the presence of LPS are similar in vertebrates, insects and plants (Hoffmann, Kafatos, Janeway & Ezekowitz*,* 1999). An important function of the innate immune system is to detect and recognise invaders. The innate immune system initiates host defenses against invasive microbial pathogens using specific recognition mechanisms. The pattern-recognition systems of the innate immunity are highly specific, being capable of differentiating between “self” and pathogens. Although LPS are the major virulence factor of Gram-negative bacteria, other components have been identified that are also able to induce inflammation [See table 1.1 for other components that can induce inflammation] (Heumann & Roger, 2002).

Many extracellular and cellular elements in the complex host response systems to LPS have been elucidated (Elsbach, 2000). Once bacterial components have been shed into the host, they activate host cells such as monocytes/macrophages, neutrophils and endothelial cells, to generate an inflammatory response. This response can take place locally or systemically (Heumann & Roger, 2002).

Table 1.1 Components of Gram-negative bacteria known to induce inflammation (Heumann & Roger, 2002)

Lipopolysaccharides Porins Lipopeptides Pili DNA (CpG motifs)	Peptidoglycan Lipoproteins Lipid A-associated proteins Flagellin

Upon entry of LPS in the host environment, plasma levels of LPS-binding protein (LBP) increase (Ulevitch & Tobias, 1999). LBP is essential for the rapid induction of an inflammatory response by small amounts of LPS or Gram-negative bacteria (Jack, Fan, Bernheiden, Rune *et al.,* 1997). LPS aggregates are dissociated by LBP to form LPS/LBP complexes (Hailman, Lichenstein, Wurfel, Miller *et al.*, 1994). The LPS/LBP complexes are transferred to a plasma membrane-bound LPS glycoprotein receptor, CD14 (mCD14), present prominently on mononuclear phagocytes and in smaller quantities on polymorphonuclear leukocytes (Jack *et al.,* 1997). Granulocytes, monocytes and macrophages are not the only cells to possess the LPS-membrane receptors CD14 and Toll-like receptor 4 (TLR4); human corneal epithelial cells are also reported to express CD14 and TLR4 (Song, Abraham, Park, Zivony *et al.,* 2001). [See Figure 1.6 for an illustration of immune system activation]. Multiple mammalian receptors for LPS have been identified, i.e. β_2-integrins CD11/CD18, the macrophage scavenger receptor for acetylated LDL, $_L$-selectin and CD14 (Wright, Ramos, Tobias *et al.,* 1990).

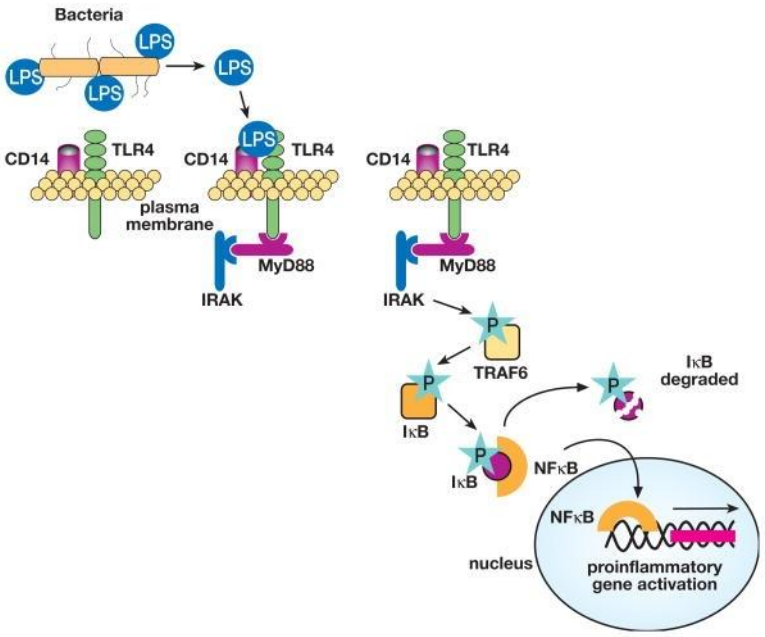

Figure 1.6 Activation of the human immune system by LPS (Nizet & Esko, 2009)

1.5 DISPOSAL OF LIPOPOLYSACCHARIDES

Removal of intact and presumably partially degraded LPS from the host is mainly through the liver and the biliary tract. Various hepatic cellular elements are sequentially involved in the translocation of LPS and, after initial uptake by the Kupffer cells, LPS are present in the parenchymal cells (Feingold, Funk, Moser, Shigenaga *et al.,* 1995). Bacterial-associated LPS and cell-free LPS can enter extracellular and cellular disposal pathways. Complex formations of LPS with a broad range of extracellular host proteins, including opsonins, antibodies and lipoproteins with, respectively, bacteria and shed LPS, lead to uptake by host cells¸

primarily phagocytes that contain at least one LPS-degrading enzyme (acyloxyacyl hydrolase) (Elsbach, 2000).

1.6 FOOD PRESERVATION

"Then Moses said to them, 'No one is to keep any of it until morning'. However, some of them paid no attention to Moses; they kept part of it until morning, but it was full of maggots and began to smell." Exodus 16:19-20 (NIV). Since Biblical times there has been evidence of Man's struggle to preserve food for later use. Food preservation implies subjecting micro-organisms to hostile conditions, thereby causing death, inhibiting growth, or curbing their survival (Leistner, 1995a, 1995b). Most methods for extending the shelf-life of food rely on one or more physical factors, such as conventional thermal processing, which involves the effect of temperature on micro-organisms and food constituents followed by appropriate packaging and storage conditions (Lund, 2003). [See table 1.2 for a summary of the environmental factors that affect changes in food constituents.]

[See table 1.3 for the most important traditional and more recent methods for extending the shelf-life of food]. Within a food matrix, microbial growth kinetics depends on the effects and interactions of intrinsic and extrinsic conditions [See table 1.4 for factors affecting microbial growth] (McDonald & Sun, 1999). During food preservation, one or more of these conditions are manipulated to a level

outside the range of growth of most foodborne micro-organisms (McDonald & Sun, 1999).

Table 1.2 Environmental factors affecting food constituents (Lund, 2003)

PHYSICAL	Temperature
	Pressure
	Oxidation / reduction potential
	pH
	Enzymes
	Metals
	Leaching
	Light
	Processing / packaging chemicals
	Water activity
TISSUE	Maturity
	Cultivar
	State of the tissue
	Composition

Table 1.3 Processes for extending the shelf-life of foods (Lund, 2003)

TRADITIONAL	Thermal processing
	Freezing
	Cooling
	Drying
	Fermentation
	Packaging
RECENT	High hydrostatic pressure
	Ultrasonic treatment
	Pulsed electric field
	Oscillating magnetic fields
	High-intensity light
	Irradiation

Table 1.4 Intrinsic and extrinsic factors affecting microbial growth (McDonald & Sun, 1999)

INTRINSIC FACTORS	pH, acidity, acidulant identity
	% buffering power
	Water activity and content
	Humectant identity
	Redox potential
	Presence of antimicrobials
	Identity and distribution of natural microbial flora
	Presence of physical structures
	Presence of biological structures
	Availability of nutrients
	Colloidal form
	Substrate surface-to-volume ratio
EXTRINSIC FACTORS	Temperature
	Relative humidity
	Light intensity and wavelength
	Atmospheric gas composition and ratio
	Packaging characteristics and interactions
	Processing characteristics and interactions
	Storage, distribution and display considerations

1.6.1 Hurdle Technology

The development of alternative technologies for use as food preservation treatments has received considerable attention in response to consumer demands for more “fresh” and “natural” food products (Jeyamkondan, Jayas & Holley, 1999). In the case of most foods, the microbial safety and stability, as well as the sensory and nutritional quality, are based on the application of combined preservative factors (hurdles) (Leistner, 1995a). Hurdles are used in industrialised and in developing countries for the gentle but effective preservation of foods (Leistner, 2000). Nonthermal processing techniques (such as HHP) are used in combination with various hurdles to eliminate the use of elevated temperatures during food processing, avoiding the adverse effect of heat on the nutritive value, flavour and

appearance of foods (Barbosa-Canovas, Gongora-Nieto, Pothakamury & Swanson, 1999). There are, however, potential benefits to food safety when combining high temperature conditions with nonthermal processes, but the use of high heat negates the original goals of nonthermal processing (Ohlsson, 1994).

1.6.2 The Hurdle Concept

"Minimal processing" is a concept describing approaches to food safety and preservation that are directed at retaining the natural and as-fresh properties of foods (Manvell, 1997). Nonthermal processes such as pulsed electric fields (PEFs), high hydrostatic pressure (HHP), ionising radiation and ultrasonication, are able to inactivate micro-organisms at ambient or sublethal temperatures. Many of these processes do, however, require very high treatment intensities to achieve sufficient microbial inactivation in low-acid foods. Combining two or more processes can enhance microbial inactivation and allow the use of lower individual treatment intensities. Optimal microbial control can be achieved through the hurdle concept, with a synergistic effect resulting from different components of the microbial cell being targeted simultaneously, while their impact on the sensory and nutritive properties of the food is minimised (Leistner, 1992; Ross, Griffiths, Mittal & Deeth, 2003).

Some of the hurdles applied to food preservation are temperature (low or high), water activity (a_w), acidity (pH), redox potential (Eh), preservatives (e.g. sulphite,

nitrite, sorbate), and competitive micro-organisms (e.g. lactic acid bacteria). More than 60 potential hurdles that improve the stability and/or quality of the products have been described (Leistner, 1999). Some hurdles will influence the safety and the quality of foods, because they have antimicrobial properties and at the same time improve the flavour of the product (Leistner, 1994). Therefore, the intelligent application of hurdle technology requires knowledge of the mechanisms of each hurdle applied (Leistner, 2000).

1.6.3 High Hydrostatic Pressure

Several authors have evaluated the effect of high hydrostatic pressure (HHP) processing on food chemistry and/or microbiology (Chawla & Chander, 2004; Erkmen & Doğan, 2004; Smiddy, O'Gorman, Sleator, Kerry *et al.,* 2005; Van Opstal, Banmuysen, Wuytack *et al.,* 2005). HHP processing involves the applications of static pressure of 50 to 1 000 mega Pascals (MPa) to solid or packaged liquid foods, with holding times varying from seconds to minutes (Mertens & Deplace, 1993; Williams, 1994). Loss of cell viability begins at approximately 180 MPa, and the rate of inactivation increases exponentially as the pressure increases (Hauben, Wuytack, Soontjes & Michiels, 1996). Substantial count reductions (>4$\log_{10}$ units) of most vegetative bacterial cells are obtained when a pressure treatment of 400-600 MPa at room temperature is applied (Hoover, 1993).

At low or moderate temperatures, HHP causes destruction of microbial vegetative cells and enzyme inactivation, leaving the vitamins intact, without changing the organoleptic characteristics of the product. The efficacy of the treatment is dependent on the achieved pressure, the treatment temperature, the exposure time, the food matrix, and micro-organism resistance (Hugas, Garriga & Monfort, 2002). [See Figure 1.7 for a diagram of a typical HHP apparatus].

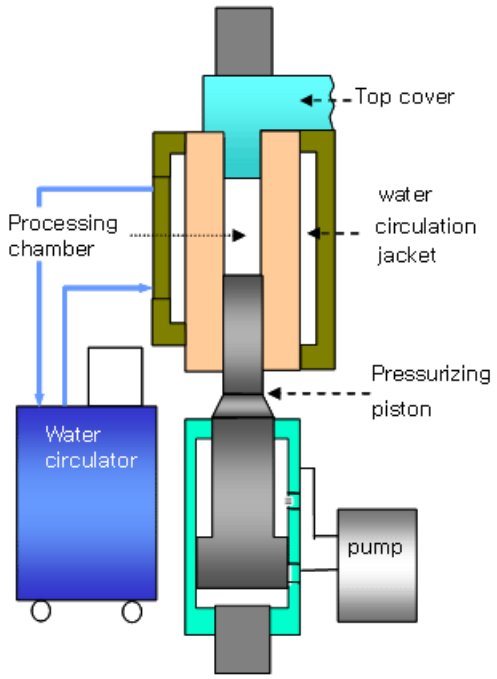

Figure 1.7 Schematic representation of a typical high hydrostatic pressure apparatus (Biomaterial Process Engineering Lab, 2010).

Inactivation of the micro-organisms is probably the result of a combination of factors, so cell death is due to multiple or accumulated damage inside the cell

(Simpson & Gilmour, 1997). The primary target of pressure damage is the cell membrane, mainly by altering its permeability as a consequence of phospholipid crystallisation. The differences in membrane properties are determining factors in the pressure or stress sensitivity of micro-organisms (Vogel, Molina-Guiterrez, Ulmer *et al.,* 2001). Membrane permeability is affected by compression of the membrane's bilayer and a reduction in the cross-sectional area per phospholipid molecule (Datta & Deeth, 1999). HHP damages the membranes, affecting the transport phenomena involved in nutrient uptake and disposal of cell waste. Intracellular fluid compounds have been found in the cell-suspending fluid after pressure treatment, demonstrating that leaks occur while cells are held under pressure (Shimada, Andou, Naito, Yamada *et al.,* 1993). The high pressure affects noncovalent bonds, leaving covalent bonds intact (Knorr, 1993) and, as a result, induces alterations in the structure of secondary- and tertiary-bonded molecules (Datta & Deeth, 1999). Other cellular functions that are sensitive to pressure are cell morphology, protein denaturation, inhibition of enzyme activity, and destabilisation of DNA replicative complex (Vogel *et al.,* 2001).

Spores and Gram-positive bacteria are on average more resistant to HHP than Gram-negative bacteria. The higher resistance of Gram-positive bacteria to HHP may be linked to the rigidity of the teichoic acids in the peptidoglycan layer of the Gram-positive cell wall (Arroyo, Sanz & Prestamo, 1999). The higher resistance of bacterial spores to physical stress, solvation and ionisation is linked to the protective effect of the membranes and coat surrounding the core, the presence of

dipicolinic acid, and the low water activity in the spore core (Timson & Short, 1965). Cocci are also more resistant than bacilli, which appears to be because the reduced cell surface (cocci) area in contact with the environment may limit cell leakage at a given treatment intensity, thus minimising the effect of the treatment (Arroyo *et al.,* 1999). Cells in the exponential growth phase are more sensitive to pressure than cells in the stationary phase (Archer, 1996).

Variation in HHP resistance in different strains must be taken into consideration when setting up processing regimes (Hugas *et al.,* 2002). Inactivation of micro-organisms is improved with the application of temperatures that are higher or lower than the optimal growth temperature of the organism (Smelt, 1998). [See table 1.5 for a summary of the opportunities and drawbacks of HHP].

Table 1.5 Opportunities and drawbacks of HHP (Devlieghere, Vermeiren & Debevere, 2004)

OPPORTUNITIES	High retention of nutrients and vitamins High fresh-like organoleptical quality Applicable for acid foods (as spores will not germinate in acidic foods) Spores can be inactivated when combined with heat or lactoperoxidase system or lysozyme
DRAWBACKS	Discontinuous for solid, viscous and particulate foods Semi-continuous for liquid foods High investment costs Spores are not sensitive Bacteria have been shown to become resistant

1.7 RATIONALE

Foodborne diseases are a worldwide public concern, in both developed and developing countries. In order to manage food safety effectively, it is essential to acquire knowledge of the occurrence and control of these foodborne pathogens. The changing lifestyles of the modern population have resulted in an increased demand for ready-to-eat food. This, however, has also led to the increased risk of food poisoning. It is estimated that in the United States of America, more than 75 million cases of food poisoning occur annually, resulting in approximately 5 000 deaths per year (Acheson, Calderwood, Moynihan & Baron, 2009).

Various effective preservation methodologies are used to control these pathogens in food. However, consumer demand for safe food with a fresh taste has resulted in the development of alternative technologies to the conventional heat preservation methods. HHP is a method of food processing that involves the application of pressure to achieve microbial inactivation. This methodology can be used in conjunction with other preservation techniques, such as heat or cold and antimicrobials, to enhance the efficacy of the treatment. HHP does not change the organoleptic characteristics of food and is especially beneficial for heat-sensitive products. Numerous studies have evaluated the efficacy of this preservation methodology on the viability of organisms. However, as far as is known, none of these studies have focused on the toxicity of post-HHP organisms. By means of this study, the influence of high hydrostatic pressure on organism viability and

liberation and the toxicity of LPS produced by foodborne pathogens will be assessed.

The specific aims of this study were:

1. To quantify bacterial cell viability after the application of HHP (*E. coli, Staphylococcus aureus and Listeria monocytogenes*).
2. To evaluate the influence of HHP on the quantity of LPS of *E. coli* O111 liberated into the extrinsic environment.
3. To determine the influence/effect of HHP treatments on the LPS toxicity of *E. coli* O111.
4. To evaluate a porcine cytokine (IL-6) expression system as a possible alternative to the customarily applied Limulus Amoebocyte Lysate (LAL) endotoxin quantification kit (*E. coli* O111).

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CHAPTER 2

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2. GROWTH RESPONSE OF *ESCHERICHIA COLI* O111 SUBJECTED TO SUBLETHAL HIGH HYDROSTATIC PRESSURE

2.1 HIGH HYDROSTATIC PRESSURE

Modern food preservation techniques are progressively relying on processes that have negligible effects on food systems but will result in microbiological safety by an intelligent synergistic combination of mild stress factors. The use of high hydrostatic pressure (HHP) for food processing is finding increased application within the food industry although the technology has been available for over a century (Hite, 1899). Conversely, high-pressure bioscience has reached maturity with a sphere of application that is unlimited. Due to these technological developments, the use of HHP is no longer restricted only to food processing; it is gradually being applied in the medical and pharmaceutical sciences. For example, high-pressure inactivation is being successfully used to sterilise fragile biopharmaceuticals and medical substances. Moreover, the enhanced immunogenicity of pressure-inactivated micro-organisms could be applied for the production of new vaccines and animal tissue, while blood and cells and organs for transplants could be stored at subzero temperatures without freezing (Masson, Tonello & Balny, 2001).

Advances in the food industry have shown that the application of HHP, which does not necessarily make use of heat, enhances the appearance, texture, flavour and

nutritional qualities of food, thus yielding a product with better quality than those processed by conventional methods (Farkas & Hoover, 2000; San Martin, Barbosa-Cánovas & Swanson, 2002). High pressure, in contrast to heat, does not disrupt covalent bonds of the primary protein structures and renders them relatively unchanged and for the most part suitable for food with ingredients that are undesirably altered by heat (Cheftel & Culioli, 1997; Murchie, Cruz-Romero, Kerry, Linton, Patterson, Smiddy *et al.,* 2005). The most commendable characteristic of HHP processing is that pressure acts instantaneously and uniformly through the treated matrix and consequently independently of matrix composition, size or shape and equipment size (Butz & Tauscher, 2002). Because transmission of pressure to the core is not mass or time dependent, the process is minimised (Aymarich, Picouet & Monfort, 2008). HHP has the ability to inactivate micro-organisms, as well as enzymes, thereby extending the shelf-life of the treated matrix (San Martin *et al*., 2002). Several extrinsic/intrinsic factors do, however, govern successful microbial inactivation: the type and strain of organism, the growth phase, temperature, time, pressure, pH and water activity, and the composition of the surrounding medium (Hugas, Garriga & Monfort, 2002).

2.2 INACTIVATION OF PATHOGENIC MICRO-ORGANISMS

Consumer demand for high-quality food with a “fresh” taste and a stable shelf-life has led to the concept of minimal processing and has prompted research into non-thermal food preservation techniques as alternative to the traditional techniques.

HHP is increasingly being employed for the commercial processing of oysters (*Crassostrea*), and high-pressure technology is proving particularly useful to the industry. Oysters, which are bivalve shellfish, are typically consumed raw or slightly cooked and can pose a severe threat to public health if contaminated with food-borne pathogenic micro-organisms (Altekruse, Yang, Timbo & Angulo, 1999; Kural & Chen, 2008). Oysters are filter feeders, passing copious volumes of water through the gills for nutritional and gaseous requirements, and thereby bio-accumulating micro-organisms from the surrounding waters (Cliver, 1995; Murchie *et al*., 2005). The presence of pathogenic bacteria, noroviruses and enteric viruses such as hepatitis A are not only a threat to the safety of shellfish worldwide (Beuret, Baumgartner & Schluep, 2003; Chironna, Germinario, De Medici, Fiore, Di Paquale, Quarto *et al.*, 2002; Kingsley, Meade & Richards, 2002) but could also have an impact on international trade (Ristori, Iaria, Gelli & Rivera, 2007). In recent years members of the Vibrionaceae family, in particular *Vibrio vulnificus* and *Vibrio parahaemolyticus,* have received attention from researchers due to their prevalence in outbreaks of food-borne diseases related to the consumption of contaminated shellfish (Cook, 2003; Kural & Chen, 2008; Kural, Shearer, Kingsley & Chen, 2008; Mahmoud, 2009; Prapaiwong, Wallace & Arias, 2009). [Table 2.1 highlights some of the effects of HHP on various oyster pathogens].

In addition to extending the shelf-life of oysters, HHP treatment has the additional advantage of shucking (opening) shellfish, making this food-processing technology

Table 2.1. Inactivation profiles of some of the pathogenic bacteria and viruses responsible for food-borne diseases resulting from the consumption of contaminated shellfish

Reference	Organism	Suspending medium	Treatment parameters			Log reduction
			Pressure (MPa)	Time (minutes)	Temp (ºC)	
Li *et al*. (2009)	Murine norovirus-1	Oysters	400	5	0	Undetectable
Kural *et al*. (2008)	*V. parahaemolyticus*	Oysters	≥350	2	1-35	5
		Oysters	≥300	2	40	5
Kural and Chen (2008)	*V. vulnificus*	Oysters	150	4	-2	4.7
		Oysters	150	4	1ºC	2.8
		Oysters	150	4	20	0.5
Grove, Forsyth, Wan, Coventry, Cole, Stewart *et al.* (2008)	Polio virus	Cell culture medium	600	5	ambient	no reduction
Calci, Meade, Tezloff and Kingsley (2005)	Hepatitis A	Oysters	350	1	8.7–10.3	>1
		Oysters	375	1	8.7–10.3	>2
		Oysters	400	1	8.7–10.3	>3
Smiddy, O'Gorman, Sleator, Kerry, Patterson, Kelly *et al.* (2005)	*E. coli*	PBS	200	5	20	no reduction
		Oysters	400	5	20	1
		PBS	400	5	20	3
Cook (2003)	*V. vulnificus*	Oysters	250	2	-	>5
	V. parahaemolyticus (O3:K6)	Oysters	300	3	-	>5
Kingsley, Hoover, Papafragkou and Richards (2002)	Feline calicivirus	Cell culture medium	275	5	ambient	>6

particularly advantageous to the shellfish industry and consumers (Murchie *et al*., 2005). Li, Tang, Wang, Wang, Zhao and Xue (2009) showed that HHP treatment applied at 200-400 MPa did not influence the visual appearance of the oysters and effectively facilitated shucking. He, Adams, Farkas and Morrissey (2002) also reported that HHP treatment at 242 MPa for two minutes caused detachment of the adductor muscle in 88% of oysters, while treatment at 310 MPa with instantaneous pressure release resulted in 100% shucking (He *et al*., 2002).

2.3 ESCHERICHIA COLI

E. coli is a Gram-negative, facultative anaerobic bacillus that forms part of the normal biota in the human intestinal tract. Some serotypes are nonetheless infamous for causing food poisoning. The inactivation of *E. coli* by HHP has received much attention, not only as far as the pressure treatment conditions (time, pressure, temperature) are concerned, but also with regard to the possible effect of HHP on the physiological status of the cell. A study conducted by Casedei, Mañas, Niven, Needs and Mackey (2002) proved that exponential- and stationary-phase cells showed surprising differences in response to pressure resistance. In exponential-phase cells, pressure resistance decreased as growth temperature increased, whereas in stationary-phase cells, pressure resistance increased to a maximum of 30 at 37 ºC and then dropped. The results showed that the difference in pressure resistance between exponential- and stationary-phase cells varies depending on growth temperature. At the optimum growth temperature of 37 ºC

for *E. coli* the differences were considerable, while the differences were less pronounced when cells were grown at 10 ºC. A possible explanation is that the protective stationary-phase responses were not induced to the same extent in cells grown at 10 ºC. The study confirmed that the membrane is a critical target in pressure inactivation, but also suggests that there are fundamental differences in the role of the cell membrane in determining the pressure resistance of exponential- and stationary-phase cells (Casadei *et al.,* 2002). Linton, McClements and Patterson (2001) showed that the pressure resistance of two *E. coli* strains (NCTC 11601 and NCTC 9706) also varied depending on the length of time the cells had been in the stationary phase. When cells were exposed to HHP in the early stationary phase, and after an extended time in the stationary phase, the number of cells that survived pressure treatment of 600 MPa for 10 minutes increased slightly, but not significantly, for up to five days. Cell viability then decreased considerably with time until, after seven days in the stationary phase, no surviving cells were detected after HHP treatment. This tendency was also comparable to viable counts in the cultures used for high hydrostatic pressure treatments, which started to decrease four to five days after the onset of the stationary phase. The increase in pressure sensitivity correlated with the onset of the death phase. This must be taken into consideration when foods are processed by high hydrostatic pressure, as micro-organisms may be present in foods in a wide range of physiological states and as a result their sensitivity to high pressure can differ extensively (Linton *et al*., 2001).

Enterohaemorrhagic *E. coli* (EHEC) is one of the leading microbiological challenges encountered in the food industry and poses a global threat. The presence thereof in fresh/organic products intended to be eaten raw can cause serious illness, leading to fatality in some cases and/or lifelong sequelae (kidney failure) in other cases. The minimum infective dose of these organisms compared to other food-borne pathogens is reported to be <100 colony forming units (CFUs) (Bell, 2002). Even though *E. coli* O157:H7 has received significant attention as a food-borne pathogen, other groups of verocytotoxin (VT) producing *E. coli* (VTEC) serogroups – also known as Shiga toxin-producing *E. coli* (STEC) – have emerged and have increasingly been implicated in food-borne illnesses, e.g. serotypes O26;H11, O103;H2, O111;H2 and O145;H28 (Bell, 2002). Various researchers have reported the presence of the virulence genes verocytotoxin 1 (vtx_1) and verocytotoxin 2 (vtx_2), which are associated with strains (O111) causing haemorrhagic colitis and haemolytic-uremic syndrome (Jothikumar & Griffiths, 2002; Nielsen & Andersen, 2003; Pearce, Evans, McKendrick, Smith, Knight, Mellor *et al.*, 2006). Within the STEC family certain strains appear to be of greater virulence for humans. *E. coli* O111 is responsible for enterohaemorrhagic and enteropathogenic diseases in humans (Nataro & Kaper, 1998) and is of special concern in developing countries where it is a classic cause of diarrhoea in children. Infections from EHEC may result from contact with infected animals (especially cattle) and their faeces, beef products, and fruits and vegetables such as cantaloupe, lettuce, apple cider and potatoes (Ackers, Mahon, Leahy, Goode, Damrow, Hayes *et al.*, 1998; Armstrong, Hollingswirth & Morris, 1996; Besser, Lett,

Weber, Doyle, Barrett, Wells *et al.*, 1993; Feng, 1995; Morgan, Newman, Palmer, Allen, Shepherd, Rampling *et al.*, 1988). STEC was also detected in retail shellfish, turkey, lamb, fish and pork (Samadpour, Ongerth, Liston, Tran, Nguyen, Whittam *et al.*, 1994). The infectious dose of STEC is low, as was observed with an STEC O111;H- outbreak in Australia where the implicated fermented sausages contained fewer than one *E. coli* O111 organism per 10 g. The diseases caused by *E. coli* have rendered EHEC a major threat to public health (Paton, Ratcliff, Doyle, Seymour Murray, Davos *et al.*, 1996). STEC is able to survive in slurries, farmyard manure and sludge, on pastureland and in aquatic environments. As the coast acts as a receptacle of agricultural and urban waste water effluents, STEC could therefore be present in coastal waters (Alam, Hasan, Ahsan, Pazhani, Tamura, Ramamurthy *et al.,* 2006; Garcia-Aljaro, Muniesa, Blanco, Blanco, Blanco, Jofre *et al.*, 2005; Gourmelon, Montet, Lozach, Le Mennec, Pommepuy, Beutin *et al.*, 2006; Pearce *et al.,* 2006). However, little is known about the prevalence of STEC non-O157 serotypes as contaminants in shellfish and the role they play in food-borne disease.

Considering that oysters are pressured in the region of 200 MPa, which is sufficient to shuck the oysters and inactivate various *Vibrio* species and some viruses, *E. coli* O111 was selected for high-pressure studies, as it is an emerging pathogenic contaminant in shellfish. This study aimed to investigate the response of *E. coli* O111 to 200 MPa, the influence of bacterial concentration on the inactivation of vegetative *E. coli* O111 cell suspensions, the use of different enumeration methods

to assess cell damage, and the evaluation of growth phases (surviving cells) in order to shed light on subsequent cellular repair after sublethal injury.

2.4 MATERIALS AND METHODS

2.4.1 Propagation Of Organisms

Stock cultures of *E. coli* O111 were kept in vials containing treated beads in a cryo-protective fluid (Microbank, Davies Diagnostics, SA) at -80 °C until needed. Bacterial growth was activated by placing a bead in 250 ml buffered peptone water (Biolab, Merck, SA), and incubated for 16 hours at 37 °C with agitation (75 revolutions per minute). The cells (exponential phase) where then subjected to HHP.

2.4.2 High-Pressure Equipment

Pressurisation experiments were performed in a prototype high-pressure instrument exclusively built for purposes of this study. The initial temperatures of the vessel jacket were adjusted to 8 °C and 50 °C respectively by circulating a water solution until the water temperature stabilised. This instrument had no heating/cooling jacket to control the temperature of the vessel; therefore the temperature was measured before the vessel was closed and again after high-pressure treatment. The average temperature of the pressurisation fluid after treatment was 18.5 ±1.5 ºC for the 8 ºC pressures and 26.5 ±1.5 ºC for the 50 ºC

pressures. The pressure transmission fluid used was distilled water. The diameter of the chamber was 40 millimetre (mm) and the depth 80 mm.

2.4.3 High-Pressure Treatment

Aliquots of 10 millilitre (ml) of the broth were transferred to sterile polyethylene bags (6 x 8 cm). The bags were double heat-sealed, taking care to expel most of the air from the bag. The samples (control and test) were kept on ice prior to pressurisation, which did not exceed one hour. The sealed bags were conditioned for five minutes prior to pressurisation in a water bath set to 8 °C for pressurisation at the same temperature and 50 °C for pressurisation at 50 °C. Test samples were pressurised at 200±5 MPa for 15 minutes at the starting temperature of the pressurisation fluid of 8 °C and 50 °C respectively. Come-up times varied from 36 to 42 seconds and decompression time was instantaneous. After pressurisation the sealed bags were removed from the chamber and kept on crushed ice prior to viability quantification (within one hour). Every test sample subjected to pressurisation (test) had a parallel control sample (held at 8 ºC and 50 ºC respectively) that was not subjected to pressurisation. Identically prepared samples treated for 15 minutes in an autoclave at 121 °C served as a dead cell control.

2.4.4 Enumeration Of Organisms

After high hydrostatic pressure treatment the samples were enumerated and subsequently subcultured by inoculating 100 micro litre (µl) of the sample in 150 ml buffered peptone water and incubating at 37 °C with agitation. Subsequently, enumeration of organisms and optical density determinations were performed at 6, 18, 24, 30, 42 and 48 hours.

2.4.4.1 Cell Viability

The number of viable culturable cells was quantified by the plate count method. One millilitre of each cell suspension was serially diluted in sterile physiological saline (9 ml) and 100 µl surface plated in duplicate on brain heart infusion agar. After 16 hours of aerobic incubation at 37 °C the colonies were enumerated using a semi-automated IUL colony counter (Optolabor, SA). Plates with colony counts lower than 50 and higher than 300 were deemed unsatisfactory. Results were calculated as colony-forming units per millilitre (CFU ml^{-1}).

2.4.4.2 Intact Cells

Intact cells in the suspensions were counted in a FAST-READ 102 counting chamber (Davies Diagnostics, SA) after mixing equal parts of the sample with Trypan blue stain. The number of cells within four squares (dimension 1 x 1 mm) was counted and the mean calculated and expressed as cells per µl.

2.4.4.3 Optical Density

Optical density for the various samples was determined at 620 nm on a Heλios ε spectrophotometer (Merck, SA).

2.4.5 Statistical Analysis

The experiments were performed in duplicate, and the data presented is the mean value ± standard deviation. The F-test was employed to qualify significance between different treatments and incubation times, and differences between the means were considered significant when $P \leq 0.05$.

2.5 RESULTS

Two sets of *E. coli* suspensions were subjected to pressure at 200 MPa for 15 minutes with starting temperatures of the samples at 8 ºC and 50 ºC. The first set of experiments[a] represented a lower bacterial load (OD < 0.2), while experiments[b] represented a higher bacterial load (OD >0.7). Percentage difference (PD) between the test organism (pressurised) and the corresponding control (ctrl) organism (not pressurised) was calculated at each time interval for colony-forming units, intact cell count and optical density. With the exception of 8[a] no significant difference ($P \geq 0.05$) in cell viability was observed when compared to the controls. Therefore, the difference at individual time intervals was considered and presented as percentage difference (PD), calculated as follows:

$$1-\left(\frac{T_t}{C_t}\right)\times\frac{100}{1}$$

T = Test value

t = Time

C = Control value

A PD of <25% was considered to be within experimental variability. A positive percentage value indicates that growth of the test organism (pressurised) was weaker than that of the control, while a negative value indicates the opposite (this is applicable to all the enumeration parameters). Table 2.2 shows an outline of the percentage differences.

2.5.1 Cell Viability

With the exception of 8^b and 50^b the test organisms and controls generally behaved the same. However, a notable PD (+34) was observed at zero hours for 50^b, and at six hours a high PD (>77) was observed for all the treatment parameters. Furthermore, an elevated PD was noted for both 8^a and 50^b during the time interval 18 – 42 hours. Within this timeframe 8^a presented a positive PD indicating that the control cells proliferated at a slightly increased rate compared to the test cells. In contrast, the PD at 50^a generally showed the opposite. The PD observed at 48 hours was insignificant.

Table 2.2 Percentage differences in the growth of *E. coli* O111 observed for each of the treatment procedures (200MPa 15min^{-1})

		Difference (%)							Comparison with control (P≤0.05)
	Treatment temp (ºC)	0 H	6 H	18 H	24 H	30 H	42 H	48 H	
CFU	8[a]	2	94	56	72	60	71	4	0.040*
	8[b]	-17	90	8	-2	-2	5	13	0.324
	50[a]	12	96	-66	-24	-413	40	-19	0.128
	50[b]	34	77	9	-22	1	11	8	0.367
ICC	8[a]	-13	40	-34	3	38	8	-25	0.417
	8[b]	8	69	5	7	22	20	6	0.486
	50[a]	90	25	-41	-103	24	-3	25	0.457
	50[b]	8	46	5	-4	8	14	6	0.486
OD	8[a]	5	67	18	12	4	0.2	-1	0.414
	8[b]	-2	72	7	6	5	6	5	0.410
	50[a]	11	97	16	29	21	2	2	0.489
	50[b]	2	48	2	-0.5	-3	-3	-2	0.394

CFU – colony-forming units; ICC – intact cell count; OD – optical density; H – time (hours)
([a]) – low bacterial load; ([b]) – high bacterial load
(*) – significant difference

2.5.2 Intact Cells

No significant difference (P≥0.05) between the test and control treatment parameters was observed. However, a notable PD (+90) was observed for 50^{a} at zero hours, which is the opposite of that observed with cell viability. A notable PD (>25) was observed after six hours of incubation for all the treatment parameters (similar to cell viability). At 18 hours a PD of -34 and - 41 was observed respectively for 8^{a} and 50^{a}, while the same tendency was also observed with cell viability. In general no elevated PD was observed with 8^{b} and 50^{b}, although a high PD was observed from 18 – 30 hours. The PD observed at 42 and 48 hours was insignificant.

2.5.3 Optical Density

As was observed with the intact cell count, no significant differences were observed between the test and control treatment parameters. In this instance the only significantly high PD (>48) was observed at six hours for all treatment parameters, with a slightly elevated PD at 24 hours for 50^{a}.

2.6 DISCUSSION

HHP involves the application of static pressure of 50 to 1 000 mega Pascal (MPa) to solid or liquid packed foods/matrixes, with holding times varying from a few seconds to several minutes (Mertens & Deplace, 1993; Williams, 1994). Loss of

bacterial cell viability begins at approximately 180 MPa, and the rate of inactivation increases exponentially as the pressure increases (Hauben, Wuytack, Soontjes & Michiels, 1996). Considerable count reductions (>4log$_{10}$ units) of most vegetative bacterial cells can be achieved when pressure treatments of 400-600 MPa are applied at room temperature (Hoover, 1993).

For this study, *E. coli* O111 was subjected to sublethal HHP treatment, and the recovery of surviving pressurised cells was facilitated by incubation in buffered peptone water. Pressurising organisms results in inactivation of or injury to the organisms, but this is nonetheless dependent on the species and strain of the bacteria, the pressure levels and subsequent storage conditions. Initial assessment showed that pressure-induced cell damage (PD – CFU/ICC) was not as pronounced as was observed for 8^{a} (CFU) and 50^{a} (ICC). Pressurised cells in the late exponential phase (after 14 – 16 hours of incubation) initiated growth with a short (six-hour) lag phase followed by exponential growth up to 48 hours and subsequent stationary-phase growth. A significant PD was observed (end of lag phase) at six hours with all assessments, indicating sublethal injury of the bacterial cells and denoting an increased amount of energy channelled towards metabolic adaptation rather than growth. In general, *E. coli* O111 (exponential cells) seemed resistant to HHP, especially at shucking pressures. These findings contradict some of the views expressed in the literature, since subsequent growth (after the lag phase) of the pressurised cells differed only slightly from that of the control

cells. According to Archer (1996) cells subjected to higher pressures (600MPa for 10 minutes) only recovered after exponential growth.

The conditioning temperatures of 8 ºC and 50 ºC respectively for five minutes and subsequent pressurisation at these temperatures did not seem to have a significant influence on PD. However, in this study the cells were incubated at 37 ºC prior to conditioning, and different results might be observed if the cells are incubated at a suboptimum temperature, inducing heat/cold shock (Aertsen, Vanoirbeek, De Siegeleer, Sermon, Hauben, Farewell *et al.,* 2004; Serrazanetti, Guerzoni, Corsetti & Vogel, 2009). Another contributing factor to the limited differences between the control/test cells could be that the treatment temperatures of 8 ºC and 50 ºC respectively were not maintained for the duration (15 minutes) of the pressurisation, and this had a negligible effect on bacterial inactivation. However, for 8^{a} (CFU), metabolic adaptation (prolonged lag phase) was more pronounced than for any other treatment conditions. This would indicate that a lower bacterial concentration and lower treatment temperature are more likely to induce cellular damage. This finding supports the results of Furukawa, Noma, Shimoda and Hayakawa (2002) who reported that bacterial concentration and inactivation rate are inversely proportionate to each other. Though HHP-induced damage to the cell membranes could result in cell death, substantial evidence exists that *E. coli* cells could survive or repair membrane damage in order to continue proliferation (Chilton, Isaacs, Mañas & Mackey, 2001). The variances observed in the PD of

CFUs and ICC could be attributed to cells aggregating and/or the presence of viable but non-culturable organisms, thus being reliant on the physiological status of the cell and cell age. From the results it would seem that cellular repair occurred within 30 to 42 hours (CFU and ICC). In view of the relatively small PD observed with optical density determinations, it would seem that differences in bacterial concentrations (^{a} vs. ^{b}) and the different treatment temperatures (8 ºC and 50 ºC) had a negligible effect on *E. coli* O111. The significant PD observed after six hours in all the treatment instances was most likely the result of the restoration of growth capabilities. Thereafter cellular repair as determined by OD seemed to have been completed after 18 hours. It should also be noted that sublethal HHP results in a more pronounced metabolic adaptation or structural correction rather than a morphological change as is seen for *E. coli* O111.

The intelligent application of HHP as a preservation process requires an understanding of microbial physiology and cell behaviour before, during and after treatment. From the results it could be concluded that sublethally injured micro-organisms could still present a potential threat in food safety given that they are able to repair themselves under suitable conditions. Care should thus be taken when culturing post-HHP micro-organisms on selective growth substrate, as the additional stress combined with the time to adapt metabolically might result in a false negative result. Therefore it is advisable to consider enrichment for at least 18 hours prior to selective cultivation. The concentration of microbes would also

dictate the preservation approach when applying HHP. Preservation methodologies ought to rely on a combination of factors such as depuration prior to HHP for more successful decontamination of fresh oysters and a reduction in microbial counts. It should be noted that high-protein environments such as oysters have a baro-protective effect (Narisawa, Furukawa, Kawarai, Ohishi, Kanda, Kimijima, *et al*., 2008) making it difficult to extrapolate the data obtained in this study directly to other matrixes. However, the results do show that an incorrect approach to the time of analysis for quality control purposes could yield false negative results. Moreover, when studying the post-HHP growth of *E. coli* O111, growth should be assessed at short intervals from the onset of growth to ensure that cell repair is observed. Furthermore, the application of hurdle technology (combined treatments) should be approached with care, as an increase in temperature combined with HHP could result in cells (present in low numbers) improving in condition (compared to control) after metabolic adaptation and structural repair. Finally, further studies should be conducted to assess the virulence of post-HHP *E. coli* O111, as metabolic adaptation and cell repair might also impact on lipopolysaccharides (LPS), toxin structure (Venter, Abraham, Lues & Ivanov, 2006), the ability to form biofilm, as well as the accuracy of serological assessment.

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CHAPTER 3

3. GROWTH RESPONSE OF *LISTERIA MONOCYTOGENES* (UAFSB CULTURE COLLECTION) AND *STAPHYLOCOCCUS AUREUS* (ATCC 25923) SUBJECTED TO SUBLETHAL HIGH HYDROSTATIC PRESSURE

3.1 FOOD-BORNE PATHOGENS AND PRESERVATION

Millions of episodes of illness annually can be traced back to contaminated food and water (Hayes, Elliot, Krales & Downer, 2003). The impact of food-borne pathogens on health is still an important issue, although the spectrum of food-borne infections has changed over time, as well-established pathogens have been controlled or eliminated and new ones have emerged. A vast number of pathogenic micro-organisms can contaminate food and water supplies and cause illness if they or their toxins are consumed. Included in this vast spectrum of food-borne pathogens are anaerobes and aerobes, parasites and viruses, enteric bacteria, marine dinoflagellates, bacteria that produce biotoxins in shellfish and fish, and the self-inducing prions of the transmissible encephalopathies. Regardless of the route of transmission, such pathogens cause an array of human illnesses, mostly gastrointestinal. The term 'food-borne disease' thus refers to numerous different pathogens and diseases (Tauxe, 2002). The pathogenesis of the food-borne bacteria that cause food poisoning is dependent on their capacity to produce toxins in the food matrix or to produce toxins after ingestion (Le Loir, Baron & Gautier, 2003).

Evaluation of any food preservation technology depends on a reliable assessment of its efficacy against pathogenic and spoilage food-borne micro-organisms. Research on alternative preservation technologies was originally focused on process design, product characteristics, and kinetics of microbial inactivation. Recently, however, there has been greater emphasis on understanding microbial physiology and behaviour of microbial cells before, during and after treatment (Lado & Yousef, 2002). Within a food matrix, microbial growth kinetics depends on the effects and interactions of intrinsic and extrinsic conditions. For that reason it is typical for one or more of these conditions to be manipulated to a level outside the range of growth of most food-borne and spoilage micro-organisms (McDonald & Sun, 1999). What is required, therefore, is a multidisciplinary research approach that combines microbiological, physical, biochemical and engineering principles to demonstrate the efficacy of these alternative food preservation methods (Balasubramaniam, Ting, Stewart & Robbins, 2004).

3.2 HIGH HYDROSTATIC PRESSURE

Consumer demand for fresher foods with an extended shelf-life and which are microbiologically safe and additive free has led to the development of different food preservation technologies as alternatives or enhancements to the traditional preservation techniques (Rastogi, Raghavarao, Balasubramaniam *et al*., 2007). Raso and Barbosa-Cánovas (2003) characterised the attributes of an ideal preservation method as: it must be acceptable to regulatory bodies and

consumers; the organoleptic and nutritional qualities of the food must remain intact; it must not leave any residues; it must be convenient and cheap; and it must inactivate micro-organisms.

For several years now, the practice has been to use high-pressure treatment in the production of plastics, ceramics, carbon graphite and composite material. Such technological developments have enhanced the feasibility of the use of high hydrostatic pressure (HHP) in food preservation (Smelt, 1998), and nowadays HHP is enjoying increased application in the medical and pharmaceutical sciences (Masson, Tonello & Balny, 2001). High-pressure technology has been in use since 1990 in the food industry as a food-preservation technique (Cléry-Barraud, Gaubert, Masson & Vidal, 2004). This technique has attracted considerable research attention as a means to inactivate spoilage and pathogenic micro-organisms in foods (Goodridge, Willford & Kalchayanand, 2006), and the technology is now commercially available for food preservation (Jofré, Aymerich, Grèbol & Garriga, 2009; Ross, Griffiths, Mittal & Deeth, 2003; Torres & Velazquez, 2005).

HHP is governed by Le Chatelier's principle, which predicts that pressure favours processes accompanied by negative volume changes and conversely inhibits processes accompanied by positive volume changes. As far as pressure is of biotechnological interest, it does not have any effect on covalent bonds, and as a result natural compounds such as aromas, dyes and flavours are not destroyed by

high pressure at room temperature (Masson *et al.*, 2001), resulting in minimally processed food with the organoleptic properties intact. HHP in many instances results in a better equilibrium between food safety on the one hand and food quality on the other (Van der Plancken, Grauwet, Oey *et al*., 2008). The key advantages of HHP can be summarised as follows: food processing is possible at ambient temperature or lower; pressure is transmitted instantaneously through the system, irrespective of geometry or size; the use of chemical preservatives or additives can enhance microbial inactivation; and food with novel functional properties can be created (Rastogi *et al*., 2007).

Inconsistencies seem to exist between the results produced by different researchers, and these were highlighted in a paper published by Balasubramaniam *et al*. (2004). The difficulty in comparing the effects of process treatments on micro-organisms can be attributed to: differences in reporting the data, making it difficult and/or impractical to compare results; differences in process equipment design and configuration; inadequate description of experimental methodologies; lack of definitions and understanding of the process parameters, resulting in a non-uniform process; and the influence of variable food matrixes on the inactivation and survival growth recovery of micro-organisms. [Table 3.1 illustrates some of the inactivation profiles of HHP on pathogenic food-borne micro-organisms].

Table 3.1 Inactivation profiles of some of the pathogenic bacteria associated with food-borne diseases

Reference	Organism	Suspending medium	Treatment parameters			Log reduction
			Pressure (MPa)	Time (minutes)	Temp (ºC)	
Koseki, Mizuno and Yamamoto (2008)	*Listeria monocytogenes*	Milk	550	5	25	Undetectable
Kural, Shearer, Kinglsey and Chen (2008)	*Vibrio parahaemolyticus*	Oysters	≥350	2	1-35	5
		Oysters	≥300	2	40	5
	V. vulnificus	Oysters	150	4	1ºC	2.8
Kural and Chen (2008)		Oysters	150	4	20	0.5
Viazis, Farkas and Jaykus (2008)	*Staphylococcus aureus* ATCC 6538	Milk	400	30	21-31	6
		Milk	400	30	21-31	8
	Staph. aureus ATCC 25923	Milk	400	30	21-31	6
		Milk	400	2	21-31	8
	E. coli ATCC 25922					
	L. monocytogenes ATCC 19115					
Chen (2007)	*L. monocytogenes*	Turkey meat	500	1	40	3.8
		Turkey meat	500	1	20	0.9
López-Pedemonte, Roig-Sagués, De Lamo *et al.* (2007)	*L. monocytogenes* NCTC 11994	Cheese	400	10	20	2.9±0.2
		Cheese	500	10	20	5
		Cheese	400	10	20	1.5±0.2
	L. monocytogenes Scott A	Cheese	500	10	20	5
Gao, Ju and Jiang (2006)	*Staph. aureus As 1.2465*	Milk	329.8	15.5	34.5	6
Smiddy, O'Gorman, Sleator *et al*. (2005)	*E. coli*	PBS	200	5	20	no reduction
		Oysters	400	5	20	1
		PBS	400	5	20	3

With regard to HHP treatment, *Listeria monocytogenes* and *Staphylococcus aureus* are two pathogenic non-spore-forming Gram-positive bacteria that have been subjected to thorough evaluation (Pilavtepe-Çelik, Buzrul, Alpas & Bozoğlu, 2008). The response of these bacteria to different combinations of treatment parameters (Wuytack, Diels & Michiels, 2002), food matrixes (Pilavtepe-Çelik *et al*., 2008), pH (Alpas, Kalchayanand, Bozoğlu & Ray, 2000), antibiotics (Black, Kelly & Fitzgerald, 2005) and bacteriocins (Alpas & Bozoğlu, 2000) are well documented. Some of these studies also documented the variation in pressure resistance among the different strains of *Listeria monocytogenes* and *Staphylococcus aureus* (Alpas, Kalchayanand, Bozoğlu *et al.,* 1999).

3.3 OYSTERS (*CRASSOSTREA)*

As the consumption of shellfish and seafood has increased worldwide, so has the incidence of associated outbreaks of bacterial and viral illnesses (Smiddy *et al*., 2005; Teplitski, Wright & Lorca, 2009). Oysters (*Crassostrea*) are bivalve filter-feeding molluscs, passing large amounts of water through their gills to obtain food and oxygen from the environment (Cliver, 1995). The quality of oysters is thus dependent on the environment (marine and estuarine water) in which they grow and feed. Raw oysters represent an important vector of pathogens and marine biotoxins due to their ability to bioconcentrate pathogens and toxins through filter-feeding (Rippey, 1994). Oysters are typically consumed raw or slightly cooked and are ingested whole (including the intestines), a fact that increases the risk of food-

borne diseases especially when the molluscs originate from a contaminated aquatic habitat or are handled under precarious hygienic conditions (Pereira, Nunes, Nuernberg *et al*., 2006), leading to post-harvest contamination. The use of post-harvest treatments is effective in inactivating shellfish-associated pathogens, but most of these treatments also kill the molluscs in the process.

HHP processing is increasingly being employed in the commercial processing of oysters due to its minimal effect on sensory and nutritional quality, as well as additional beneficial effects such as shucking and increased tissue yields. The effects of HHP on micro-organisms and seafood are dependent on processing parameters, and the intricate nature of HHP necessitates the careful design of processing regimes to exploit the goals of microbial and enzyme inactivation, whilst maintaining optimum product quality (Cruz-Romero, Smiddy, Kerry & Kelly, 2004, Murchie, Cruz-Romero, Kerry *et al*., 2005). Some of the bacterial genera associated with shellfish contamination are *Escherichia* (Brasher, DePaola, Jones & Bej, 1998), *Listeria* (Monfort, Minet, Rocourt *et al.*, 1998), *Vibrio* (Cook, 2003), *Salmonella* (Brands, Billington, Levine & Joens, 2005), *Staphylococcus* (Pereira *et al*., 2006), *Pseudomonas, Shewanella, Enterobacter* and *Serratia* (Cruz-Romero, Kelly & Kerry, 2008).

3.4 *LISTERIA MONOCYTOGENES*

Listeria monocytogenes are Gram-positive bacilli able to survive environmental conditions that are usually fatal to other micro-organisms (Cunningham, O'Byrne & Oliver, 2009). The organism is a facultative anaerobic bacillus capable of growth at temperatures ranging from 1 ºC to 45 ºC, tolerating high salt concentrations and acidic pH (Murray, Rosenthal & Pfaller, 2009). *Listeria monocytogenes* is a ubiquitous organism occurring in terrestrial and aquatic habitats (Karunasagar & Karunasagar, 2000) and this has led to its presence in the food-processing environment and the food chain. The ability of this organism to proliferate at refrigerator temperature (Hain, Chatterjee, Ghai *et al.*, 2007) enables this pathogen to remain on contaminated foods and establish infection after ingestion (Cunningham *et al*., 2009). It is a food-borne bacterial pathogen and the causative agent of listeriosis, a severe disease with high hospitalisation and fatality rates (Gandhi & Chikindas, 2007) and it is notorious for affecting high-risk subgroups of the population (Tauxe, 2002). The overt form of listeriosis has a mortality rate of circa 20 percent (Churchill, Lee & Hall, 2006), while the mortality rate of symptomatic listeriosis is higher than that of any other food-borne disease (Murray *et al*., 2009). Several studies have focused on the presence of *Listeria monocytogenes* or *Listeria* spp. in shellfish and seafood (Elliot & Kvenberg, 2000; Karunasagar & Karunasagar, 2000; Parihar, Barbuddhe, Danielsson-Tham & Tham, 2008; Wan Norhana, Poole, Deeth & Dykes, 2009), although data also suggests that *Listeria* spp. are not detectable in shellfish (Minami, Chaicumpa, Chongsa-Nguan *et al.,* 2009). The incidence of *Listeria* spp. in live shellfish must

therefore be known if one is to evaluate the actual role of secondary cross-contamination and bacterial multiplication in the microbiological quality of marketed shellfish (Laciar & De Centorbi, 2002).

3.5 *STAPHYLOCOCCUS AUREUS*

The Gram-positive *Staphylococcus aureus* are catalase- and coagulase-positive, non-sporing bacteria able to grow in media with a high salt concentration (up to 10 percent sodium chloride) and at temperatures ranging from 18 ºC to 40 ºC (Murray *et al*., 2009). *Staphylococcus aureus* is ubiquitous in humans and animals, and it is estimated that circa 20 percent of healthy individuals are persistent carriers and 60 percent are intermittent carriers (Bamberger & Boyd, 2005).

Food-borne diseases are a major concern across the world. Approximately 250 different food-borne diseases have been described, with bacteria being the causative agents of two thirds of these food-borne disease outbreaks (Le Loir *et al.,* 2003). *Staphylococcus aureus* is one of the most prevalent food-borne bacterial pathogens (Chokesajjawatee, Pornaem, Zo *et al.*, 2009) and a leading cause of gastroenteritis resulting from the consumption of contaminated food (Le Loir *et al.,* 2003). Staphylococcal food poisoning is caused by the ingestion of staphylococcal enterotoxins that were pre-formed in the implicated food (Chokesajjawatee *et al*., 2009), causing intoxication rather than infection (Murray *et al*., 2009). Staphylococcal enterotoxins are produced during all phases of growth,

but mostly during the middle and at the end of the exponential phase (Soriano, Font, Molto & Mañes, 2002). Various food sources have been implicated as the culprit in food-poisoning outbreaks, including meat, poultry, Minas cheese and raw milk (Do Carmo, Dias, Linardi *et al*., 2002), “Bgalla” (fried potato balls) (Nema, Agrawal, Kamboj *et al.,* 2007), dairy products (Veras, Do Carmo, Tong *et al*., 2008) and fish and shellfish (Wieneke, Roberts & Gilbert, 1993; Ayulo, Machado & Scussel, 1994; Cao, R., Xue, C-h. & Liu, Q. 2009).

The food matrix, the microbial species, and the ability of psychrotrophic micro-organisms to recover in food products are critical factors when evaluating the efficacy of HHP treatment (Jofré *et al*., 2009). Bearing in mind that oysters are pressured in the region of 200 MPa, which is sufficient to shuck the oysters and inactivate various *Vibrio* species and some viruses, *Listeria monocytogenes* and *Staphylococcus aureus* were selected as possible pathogenic contaminants in shellfish for purposes of this study on high-pressure treatment. With this study the authors explored the response of *Listeria monocytogenes* (obtained from the Unit for Applied Food Science and Biotechnology culture collection (UAFSBCC), Central University of Technology, Free State, South Africa) and *Staphylococcus aureus* (ATCC 25923) to 200 MPa, by employing different enumeration methods to assess cell damage and evaluate the growth phases (surviving cells) so as to shed light on subsequent cellular repair after sub-lethal injury.

3.6 MATERIALS AND METHODS

Refer to chapter 2 (sections 2.4.1-2.4.5; p63-66) for materials and methods description on the propagation and enumeration of the organisms, high pressure equipment and treatment, and statistical analysis.

3.7 RESULTS

Listeria monocytogenes (UAFSBCC) and *Staphylococcus aureus* (ATCC25923) suspensions were subjected to pressure of 200±5 MPa for 15 minutes, with the starting temperatures of the samples at 8 ºC and 50 ºC. No significant differences ($P \geq 0.05$) with any of the treatment parameters were observed when compared to the controls and as a result values were expressed as percentage difference. Refer to chapter 2 (p65-66) for calculation of percentage difference.

A PD of <25 percent was considered to be within experimental variability. A positive percentage value indicates that growth of the test organism (pressurised) was weaker than that of the control, with a negative value indicating the opposite (this is applicable to all the enumeration parameters). [Table 3.2 gives an outline of the percentage differences].

3.7.1 Colony-Forming Units

For *Listeria*, notable PDs (82 and 67) were observed at six hours at both treatment

Table 3. 2 Results of the percentage differences observed for each of the treatment procedures (200 MPa 15 min^{-1})

		Difference (%)							Comparison with control ($P \leq 0.05$)
	Treatment temp (ºC)	0 H	6 H	18 H	24 H	30 H	42 H	48 H	
CFUs	8^{L}	13	82	21	33	16	10	21	0.354
	50^{L}	-28	67	42	10	15	8	6	0.408
	8^{S}	28	18	9	18	29	-348	-35	0.209
	50^{S}	-360	-277	8	13	7	43	24	0.193
ICC	8^{L}	19	44	1	-16	-19	6	10	0.416
	50^{L}	19	11	4	-7	2	-10	7	0.471
	8^{S}	9	37	20	27	15	7	2	0.353
	50^{S}	-10	-31	-76	-38	15	6	-22	0.35
OD	8^{L}	5	70	-8	-8	-5	-1	2	0.401
	50^{L}	4	56	-6	-5	-4	-0.9	3	0.434
	8^{S}	1	21	0.3	2	0.2	0.02	0.4	0.479
	50^{S}	1	-146	-4	2	-3	-0.3	0.2	0.483

CFUs – colony-forming units; ICC – intact cell count; OD – optical density; H – Time (hours);
L – *Listeria monocytogenes*; S – *Staphylococcus aureus*

temperatures, becoming slightly elevated after 18 hours at 50 ºC and after 24 hours at 8 ºC. PDs observed after 30-48 hours at both treatment temperatures were not significant. Furthermore, *Staphylococcu*s showed notable PDs at zero (-360) and six (-277) hours at 50 ºC and a PD of -348 at 8 ºC after 42 hours, indicating that the test cells proliferated at a more rapid rate than the control cells. No significant PD for the 50 ºC treatment was observed in the case of *Staphylococcus*.

3.7.2 Intact Cell Count

For *Listeria* a notable difference (44) was only observed at six hours for the 8 ºC treatment. However, *Staphylococcus* had slightly elevated PDs after six (37) and 24 (27) hours at 8 ºC, while in the case of the 50 ºC treatments, elevated PDs were observed at six (-31), 18 (-76) and 24 (-38) hours, indicating a higher proliferation rate for the test cells.

3.7.3 Optical Density

As was observed with the CFU, a notable PD (70 and 56) was observed at six hours for *Listeria* at both treatment temperatures. *Staphylococcus* showed a notable difference at six (-146) hours at 50 ºC, while the same trend was observed in the case of CFU and ICC, indicating an increased proliferation of the test cells.

3.8 DISCUSSION

HHP is a preservation technology that has received much attention in recent years. It involves the application of pressures ranging from 100 to over 1 000 MPa at temperatures varying from 0-100 ºC and holding times varying from seconds to several minutes (Ramaswamy, Balasubramaniam & Kaletunç, 2006). Viability loss of pathogens is enhanced considerably as the pressure level and temperature increase (Alpas *et al*., 2000). When high pressure is applied to a food product, different reactions will occur, i.e. micro-organisms are inactivated, enzyme activity is enhanced or diminished, and chemical compounds are formed or destroyed (Van der Plancken *et al.*, 2008).

In this study *Listeria monocytogenes* and *Staphylococcus aureus* were subjected to HHP at 200 MPa, and recovery of surviving pressurised cells was facilitated by incubation in buffered peptone water. Normally, the pressurisation of organisms results in their injury or death; however, this is governed by the species and strain of the organism, as well as pressure levels, temperatures and subsequent storage conditions. Following pressurisation in the late exponential phase (after 16 hours of incubation), the cells initiated growth with a short (six-hour) lag phase, followed by exponential growth for up to 48 hours and subsequent stationary-phase growth.

Significant PDs (end of lag phase) at six hours were only observed for *Listeria monocytogenes* at both temperatures, indicating sub-lethal injury of the bacterial cells and denoting the channelling of an increased amount of energy towards

metabolic adaptation (recovery) rather than growth. It would seem that *Staphylococcus aureus* (exponential cells) was even more resistant to HHP, especially at shucking pressures, as the PDs observed at all treatment parameters were not significantly high. This supports the finding of Arroyo, Sanz and Préstamo (1999) that cocci are more pressure resistant than bacilli. The reduced cell surface area of cocci in contact with the environment minimises the effect of high pressure (Arroyo *et al.,* 1999).

The conditioning temperatures of 8 ºC and 50 ºC respectively for five minutes and subsequent pressurisation at these temperatures did not seem to have a significant influence on percentage differences. However, in this study, the cells were incubated at 37 ºC prior to conditioning; different results might be observed if incubated at a suboptimal temperature, inducing heat/cold shock (Serrazanetti, Guerzoni, Corsetti & Vogel, 2009). Another contributing factor to the limited differences between the control/test cells could be that the treatment temperatures of 8 ºC and 50 ºC respectively were not maintained for the duration (15 minutes) of the pressurisation, and this had a negative effect on bacterial inactivation. *Staphylococcus aureus* test cells proliferated much better than the control cells, as was observed with CFU, ICC and OD at six hours at the 50 ºC treatment temperature. This could indicate that the prolonged exposure (20 minutes) of the control cells to the elevated temperature resulted in pronounced stress in the controls cells, causing the test cells to proliferate better despite being subjected to HHP.

Although HHP is known to induce damage that could result in cell death, it would seem that *Listeria monocytogenes* was able to repair pressure damage and then continue to proliferate (extended lag phase). The slight variances observed in the PDs of the CFUs and ICC could be attributed to the physiological status and age of the cell. From the results it would seem that cellular repair occurred mostly within 18 hours of incubation.

In view of the insignificant PDs observed with optical density determinations, it would seem that different treatment temperatures (8 ºC and 50 ºC) had no effect on *Staphylococcus aureus*. The notable PDs observed after six hours for *Listeria monocytogenes* in all the treatment parameters are most likely to be the result of the restoration of growth capabilities. Thereafter cellular adaptation as determined by OD seems to have been completed after 18 hours. Sub-lethal HHP resulted in a more pronounced metabolic adaptation or structural correction rather than a morphological change as in the case of *Listeria monocytogenes*.

From the results it can be concluded that sub-lethally injured micro-organisms could still present a latent hazard in food safety given that they are able to repair themselves under favourable conditions. Preservation methodologies ought to rely on a combination of pre-harvest factors such as depuration and use of probiotics, and post-harvest factors such as preventing recontamination with pathogenic food

spoilage organisms prior to HHP for purposes of the successful decontamination of fresh oysters and the reduction of microbial counts.

Concerning the microbiological safety of food, quantifying the inactivation of pathogens and spoilage bacteria is imperative and critical in the establishment of an effective HHP treatment regime, and requires an understanding of microbial physiology and behaviour of the cells before, during and after HHP treatment. The target organisms in food preservation are for the most part vegetative organisms with various growth requirements. The storage conditions, selection of the recovery medium (selective vs. enriched) and subsequent culturing conditions will therefore determine the count of the surviving organisms and/or the recovery of the sub-lethally injured organisms. When assessing the efficacy of any HHP treatment regime, it is imperative that the most pressure-resistant organism is regarded as the target organism. Within the conditions of this study, this proved to be *Staphylococcus aureus*. Inactivation of the most resistant organism by HHP will thus result in inactivation of the less resistant organisms as well. In the instance where pressurisation of 200 MPa was applied, it is evident that *Listeria* and *Staphylococcus* were not optimally inactivated or injured to render a sufficiently safe product for human consumption. This treatment pressure can only be successfully applied to inactivate *Vibrio* species and shuck oysters. Several studies have proven that a combined use of pressure and elevated temperature is necessary to inactivate *Listeria* and *Staphylococcus* and that heat treatment or

pressure treatment alone is not sufficient to significantly reduce the number of organisms (Bozoğlu, Alpas & Kaletunc, 2004; Jofré *et al*., 2009).

Sub-lethal injury of *Listeria* could be adequate if combined with hurdles such as suboptimal growth conditions to extend the shelf-life of the product. However, since oysters are stored at refrigeration temperatures, psychrotrophic *Listeria* could have an opportunity to proliferate, thereby rendering the product unsafe and infectious to humans. Viable toxigenic micro-organisms such as *Staphylococcus aureus* can form toxins in food, but are harmless if inactivated by HHP. Sub-lethal injury of this organism by means of pressurisation at 200 MPa and subsequent refrigeration storage could result in this organism surviving at these lower temperatures and resuming growth when temperature conditions are once again favourable, resulting in the production of staphylococcal enterotoxins that render the product toxic. To ensure the microbiological safety of *Crassostrea* it is of the utmost importance to inactivate any pathogens, and as long as there is a market for fresh, live, raw oysters, researchers and food scientists can explore the effects of HHP as a preservation methodology.

It is a known fact that HHP modifies the structure of some proteins and the activity of some enzymes. Even though covalent bonds are not affected, hydrogen bonds as well as hydrophobic and intermolecular interactions may be modified or destroyed (Rastogi *et al*., 2007). However, little is known about the effects of HHP on the lipoteichoic acid (LTA) structure of Gram-positive organisms. Further

studies should be conducted to assess the virulence of post-HHP *Listeria monocytogenes* and *Staphylococcus aureus*, as metabolic adaptation and cell repair might also have an impact on the LTA structure, the ability to form biofilm, and pyrogenicity.

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CHAPTER 4

4. *IN VITRO* QUANTIFICATION OF *ESCHERICHIA COLI* O111 LIPOPOLYSACCHARIDE TOXICITY SUBSEQUENT TO SUBLETHAL HIGH HYDROSTATIC PRESSURE

4.1 *ESCHERICHIA COLI*

It is well known that micro-organisms damaged (injured) by food preservation methodologies could influence the accuracy of standard quality control assessments. During the latter, micro-organisms are frequently cultivated under stressful conditions (selective media) that select not only for specific organisms but also for "healthy" organisms within this group. Therefore, although organisms injured during food processing with high hydrostatic pressure (HHP) might yield a false negative result, within the processing line or final product they might regenerate viability (heal) to colonise or spoil the end product. Among these organisms are the ubiquitous *Escherichia coli* group of organisms, which include both pathogenic and non-pathogenic strains.

The pathogenic strain *E. coli* O111 is responsible for enterohaemorrhagic and enteropathogenic diseases in humans (Nataro & Kaper, 1998) and is of special concern in developing countries where it is a classic cause of diarrhoea in children. Infections from an enterohaemorrhagic *E. coli* (EHEC) strain may result from contact with infected animals (especially cattle), beef products and their faeces (Armstrong, Hollingsworth & Morris, 1996), and consumption of contaminated

foodstuffs, including fruits and vegetables such as cantaloupe (Feng 1995), lettuce (Ackers, Mahon, Leahy, Goode *et al.*, 1998), apple cider (Besser, Lett, Weber, Doyle *et al.*, 1993) and potatoes (Morgan, Newman, Palmer, Allen *et al.*, 1988). Furthermore, the infectious dose of shiga toxin-producing *E. coli* O111 is low, as was observed with an STEC O111;H- outbreak in Australia where the implicated fermented sausages contained fewer than one *E. coli* O111 organism per 10g (Paton, Ratcliff, Doyle, Seymour-Murray *et al*., 1996).

4.2 HIGH HYDROSTATIC PRESSURE

Nonthermal food-processing procedures were designed to eliminate the use of elevated temperatures during processing, avoiding the adverse effect of heat on the nutritive value, flavour and appearance of foods (Barbosa-Canovas, Gongora-Nieto, Pothakamury & Swanson, 1999). There are several additional benefits when combining high temperature conditions with nonthermal processes; however the use of high heat negates the innovative aims of nonthermal processing (Ohlsson, 1994). Pressure is an important thermodynamic variable and can disturb a wide range of biological structures and processes if it is significantly changed from the normal conditions established in the biosphere (Earnshaw, Appleyard & Hurst, 1995). At low or moderate temperature, HHP causes destruction of microbial vegetative cells and enzyme inactivation, without changing the organoleptic characteristics of the product and leaving the vitamins intact. The efficacy of the treatment also depends on the achieved pressure, treatment

temperature and exposure time. Food matrix and micro-organism resistance must also be taken into consideration. HHP treatment can induce special effects on the texture and structure of a given food and can be used for the development of new products or to improve the functionality of some ingredients (Hugas, Garriga & Monfort, 2002). Loss of bacterial cell viability begins at approximately 180 MPa, and the rate of inactivation increases exponentially as the pressure increases (Hauben, Wuytack, Soontjes & Michiels, 1996). Inactivation of micro-organisms by HHP is undoubtedly the result of a combination of factors; therefore cell death is attributed to accumulated damage inside the cell and not just a singular event (Simpson & Gilmour, 1997).

4.3 LIPOPOLYSACCHARIDES

Bacterial lipopolysaccharides, (endotoxins) are the main components of the outer membrane of *E. coli* O111, a Gram-negative bacterium (Caroff & Karibian, 2003; Wilkinson, 1996) and may exist as free and cell-wall bound (Mattsby-Baltzer, Lindgren, Lindholm & Edebo, 1991). LPS's are a unique class of macromolecules representing intrinsic cell-wall components of Gram-negative bacteria (Zubova, Melzer & Prokhorenko, 2005). Refer to chapter 1 for structural details on LPS.

It is well known that lipid A is the "endotoxic principle" of the molecule, and the biological activity of lipid is influenced by the fatty acid content, whereby a reduced amount of fatty acids in long-chain LPS may affect the biological activity (Mattsby-

Baltzer *et al.*, 1991). The reaction of the *Limulus* amebocyte lysate (LAL) assay is dependent on the chemical and physiochemical structure of the endotoxin (Rietschel, Brade, Brade, Brandenburg *et al*., 1987). Each species of bacteria has a specified lipid A structure, which is closely associated with endotoxin activities such as pyrogenicity and lethality (Ogikubo, Norimatsu, Noda, Takahashi *et al*., 2004). Brandenburg, Jürgens, Andrä, Lindner *et a*l. (2002) showed that the diglucosamine-4'-phosphate region in the lipid A moiety of LPS is the binding epitome in the LAL assay. However, inactive compounds such as tetra- and penta-acyl lipid A also exhibit high LAL activity (Schromm, Brandenburg, Loppnow, Moran *et al*., 2000).

Whereas LPS liberated from the outer membrane of Gram-negative bacteria remains the prototypic pyrogen, substances such as lipoteichoic acid (LTA) and peptidoglycan from Gram-positive bacteria (Daneshian, Guenther, Wendel, Hartung & Von Aulock*,* 2006), fungal spores (Tran, Ahmad, Xu, Ahmad & Menezes*,* 2003) and viral pathogens (Bowie & Haga, 2005) are now also recognised as powerful immune stimuli. As they are derived from the external environment of the body, they are called exogenous pyrogens (Zeisberger, 1999). Pyrogen is derived from the Greek word "pyros" meaning "fire" (Daneshian, Wendel, Hartung & Von Aulock, 2008), and pyrogens are fever-inducing substances usually derived from different pathogenic stimuli (Daneshian *et al.,* 2006). Moreover, LPS's are of interest in medicine, for they are strong immunomodulators in the infected host and elicit a variety of pathophysiological

effects such as endotoxic shock, tissue injury and lethality (Caroff. Karibian, Cavaillon & Haeffner-Cavaillon, 2002; Ogikubo *et al*., 2004). In addition to LPS's being amongst the most potent activators of the mammalian immune system, they are also essential for the viability of Gram-negative bacteria.

4.4 LIMULUS AMOEBOCYTE LYSATE

The LAL assay is widely used as an *in vitro* alternative to the labour-intensive *in vivo* rabbit pyrogen test for the detection and quantification of endotoxins in food and pharmaceutical products. There are, however, a few drawbacks: it does not exactly parallel *in vivo* pyrogenic activity but rather reflects the amount of LPS (Lee, Sung, Han, Lee *et al*., 2005), other pyrogenic stimuli are not detected, and numerous substances interfere with the assay, e.g. potassium chloride, sodium chloride, sodium bicarbonate and acetic acid (Bohrer, Hörner, Do Nascimento, Adaime *et al.,* 2001; Daneshian *et al*., 2008). The LAL utilises lysate of the blood cells of the arachnid, the horseshoe crab, which enzymatically interacts with endotoxins and is used to detect the presence of endotoxins in many different samples (Bohrer *et al*., 2001, Daneshian *et al*., 2006).

Variations in temperature, nutrient availability and exposure to stressors such as HHP and sanitizers (Venter, Abraham, Lues and Ivanov, 2006a) require an adaptive response for bacterial cells to survive. For *E. coli* O111, exposure to stressors can result in the activation of one or more stress responses that are

compartmentalised and managed by specialised systems (McBroom & Kuehn, 2007). Numerous studies have investigated the effect of different conditions (pH, temperature, time, antimicrobials, etc.) in combination with HHP on the viability of micro-organisms; however, as far as is known, no such studies have investigated the possible effect of HHP on LPS toxicity.

Gram-negative bacteria spontaneously release LPS's in the form of vesicles during normal cell growth (Horstman & Kuehn, 2000), and the rate of release varies among different bacterial species and strains (Russel, 1976). These vesicles may also contribute to pathogenicity by serving as vehicles for toxins to encounter host cells (Horstman & Kuehn, 2000). *E. coli* are part of the normal biota in human and animal intestinal tracts, and some strains of *E. coli* exhibit pathogenicity towards the human body. It is therefore important to monitor and determine the presence of this organism and its products (LPS) in food and clinical medicine and the general environment (Qu, Bao, Su & Wei, 1998).

Considering that oysters are pressured in the region of 200 MPa, which is sufficient to shuck the oysters and inactivate various *Vibrio* species and some viruses, *E. coli* O111 was selected for this high-pressure study because it is an emerging pathogenic contaminant in shellfish. It is further known that injured *E. coli* O111 cells are able to repair membrane damage and proliferate when cultured in enriched medium after sublethal HHP (Olivier & Venter, 2009). This study investigated the influence of sublethal HHP at 8 and 50 ºC respectively for 15

minutes at 200 MPa on the LPS toxicity of *E. coli* O111 as measured by a chromogenic LAL assay.

4.5 MATERIALS AND METHODS

Refer to chapter 2 (sections 2.4.1 - 2.4.3; p63-64) for materials and methods description on the propagation of the organisms, high pressure equipment and treatment.

4.5.1 Lipopolysaccharide Extraction

After high hydrostatic pressure treatment the samples were subcultured by inoculating 100 µl of the sample in 150 ml buffered peptone water and incubated at 37 °C with agitation. Subsequently LPS extraction was performed at 0 hours (immediately after pressurisation) and again at 6, 18, 24, 30, and 48 hours. LPS were extracted by means of a combination of the methods of Folch, Lees and Sloane-Stanley (1957) and Venter and Ivanov (personal communiqué). Five millilitres of each of the test and control culture broths were aliquoted in pyrogen-free tubes (Nunc, AEC Amersham, South Africa) and centrifuged for 30 minutes at 4 000 rpm (J.P. Selecta s.a., Mixtasel). *Circa* 4.5 ml of the supernatant (with free LPS not bound to cells) (about 4.5 ml) were aseptically removed and aliquoted in pyrogen-free tubes for LPS separation. Equal amounts of the supernatant (±4,5 ml) and chloroform-methanol (2:1; v/v) (Merck, SA) were mixed on a vortex and

stored overnight at 4 ºC to separate into a biphasic system with LPS with reduced lipid content in the upper phase (aqueous) and LPS with increased lipid content in the lower phase (organic).

The pellet (from the original centrifuged samples expected to contain the cell-bound LPS) was resuspended in sterile saline and washed three times with the saline. A similar extraction method to that mentioned was performed on the pellet. The two phases were separated and concentrated by rotary evaporation (IKA WERKE, PV06ML, GMBH & CO. KG) with a bath temperature of 46 ±2 ºC. The sides of the evaporation tubes were rinsed with either 400 µl methanol (aqueous phase) or 1 ml chloroform (organic phase) (depending on the phase) and the respective concentrates were then transferred to pyrogen-free sample vials. Samples were dried for four hours in a vacuum oven (Shinha JCS) at 40 ºC under maximum vacuum and reconstituted with 1 ml of pyrogen-free water before LAL analysis was performed.

4.5.2 Limulus Amoebocyte Lysate Assay

LPS toxicity on all the extracted LPS samples was determined using the chromogenic LAL, which is a quantitative test for Gram-negative bacterial endotoxin (CAMBREX, QCL-1000, *Limulus* Amoebocyte Lysate, WhiteSci, SA). The test was performed by the microplate method as prescribed by the manufacturer. The test kits included *E. coli* endotoxin standards with

approximately 15-40 endotoxin units (EU) lyophilised endotoxins. Endotoxin standards ranging from 0.1 to 1.0 EU ml^{-1} were prepared and a standard curve was constructed by plotting $OD_{410\ nm}$ against the EU per absorbance unit. The absorbance of the released p-nitroaniline (yellow colour) from the synthetic substrate was read at 410 nanometre (nm) with a microplate reader (Bio-Rad 680). Endotoxin concentration in the samples was determined by the graphic method proposed by the manufacturer. All equipment, reagents and consumables used were pyrogen free.

4.6 RESULTS AND DISCUSSION

LPS biosynthesis underlies quantitative differences in the bacterial growth phases, which results in changes in the endotoxin content of the cells, and therefore values were calculated as [toxicity]/OD_{620nm}, and not viability (Venter, Abraham, Lues & Ivanov, 2006b). Thereafter the differences at unit time intervals were considered and presented as the percentage difference (PD). Refer to chapter 2 (p66) for calculation of percentage difference.

A positive percentage value indicates that the toxicity of the test organism (pressurised) was less than that of the control (non-pressurised), while a negative value indicates the opposite (this is applicable to all the enumeration parameters). PDs lower than 200 were considered to be within experimental variation. [Table 4.1 shows an outline of the percentage differences].

Table 4.1 Percentage differences in toxicity of *E. coli* O111 LPS observed for each of the treatment procedures (HHP at 200MPa 15 min^{-1})

		Percentage difference					
	Treatment temp (ºC)	0 H	6 H	18 H	24 H	30 H	48 H
ST, OP	8[a]	475	-160	-17	27	-4	7
ST, AP	8[a]	-47	-160	-40	65	138	80
ST, OP	50[a]	-6	-3701	-25	-72	-65	-46
ST, AP	50[a]	-65	-3787	3	-43	11	36
PT, OP	8[a]	81	192	-21	-4	-24	118
PT, AP	8[a]	-52	-296	12	-85	1	-4
PT, OP	50[a]	-181	4658	157	-92	1306	103
PT, AP	50[a]	21	-1426	2	-31	33	33
ST, OP	8[b]	-3	-187	176	-222	203	469
ST, AP	8[b]	40	-179	-41	-10	-50	7
ST, OP	50[b]	-6	-96	-23	-105	137	223
ST, AP	50[b]	-4	-2438	22	0.8	1	55
PT, OP	8[b]	65	236	82	72	21	-142
PT, AP	8[b]	23	42	-39	-15	10	13
PT, OP	50[b]	-27	-35	-16	466	-621	-19
PT, AP	50[b]	7	-29	-0.5	0.0	-26	-69

ST - supernatant; PT – pellet; OP - organic phase; AP - aqueous phase; H - time (hours)
([a]) - low bacterial load; ([b]) - high bacterial load

In this study *E. coli* O111 was subjected to sublethal HHP treatment, and recovery of pressurised cells was facilitated by incubation in buffered peptone water. When micro-organisms are exposed to HHP it is to be expected that they will be injured or killed. This is, however, dependent on the species and the strain of bacteria, HHP conditions and subsequent storage. Subsequent to injury, bacteria might regenerate during storage, which may have an effect on the microbiological quality of foodstuffs (Bozoğlu, Alpas & Kaletunç, 2004), not only as an infectious agent but as a toxic product as well.

Cells that are only sublethally injured by HHP have the capacity to repair the site of injury (Russel, 2002) and return to a normal physiological state with initiation of growth and cell division under favourable conditions (Hurst, 1984), thus liberating LPS as a result of division, vesicle formation and death (Cadieux, Kuzio, Milazzo & Kropinski, 1983; Horstman & Kuehn, 2000). Bacteria from the late exponential phase (after 14-16 hours of incubation) were pressurised and immediately subcultured in fresh medium. The exponential growth phase commenced after six hours (all cells) of incubation followed by the stationary phase at 48 hours. The toxicity of cell-wall-bound (pellet) and free (supernatant) LPS from cultures of *E. coli* O111 subjected to HHP was studied *in vitro*. Repeated washings in physiological saline most probably removed the capsular polysaccharide (K antigens) prior to LPS extraction by chloroform:methanol, hence no interference from capsular material was expected in this study.

From the literature it is evident that free LPS can be detected within eight hours of growth in a culture medium (Bartková, Majtán & Ciznár, 1986). Within the parameters of this study, however, free LPS was detected within six hours in the supernatant of both the low and high bacterial loads. The biological activity of the free LPS in this instance seems to be higher for the 50 ºC treatment than that of the corresponding pellet LPS.

The PDs observed between the supernatant of the low and high bacterial loads differed notably from that observed in the pellet. Differences were observed after six hours of incubation in most cases; however, the differences seemed more pronounced within the 50 ºC treatment parameter with the exception for the pellet of the high bacterial load.

It could be that in response to HHP stress, *E. coli* O111 produced long-chain LPS (hexa-acylated lipid A), thereby increasing LAL activity. Chemical heterogeneity in the side-chain and the core regions could be influenced by HHP stress, resulting in the percentage differences observed. It is possible that metabolic adaptation of the cells, as observed at six hours, resulted in an increased synthesis of 3-deoxy-D-manno-octulosonic acid (Kdo), causing increased toxicity as observed at six hours (50 ºC) for the aqueous phases of the pellet and supernatant low bacterial loads and the supernatant high bacterial load. Long-chain LPS's, however, are not the only determinant for LAL activity – it is a possibility that HHP-induced stress resulted in R-mutant activities, thus increasing LAL activity as was observed.

Variations in toxicity as seen in the results are a function of the physiological status of the cell in response to sublethal HHP and cell death. Differences in the phosporyl group could also contribute to toxicity, where diphosphoryl lipid A (moderate toxicity) and complete R-LPS are the structures that display the highest level of toxicity. This could explain the high toxicity observed at six hours for the 50 ºC treatment – low bacterial load (both phases) and the 50 ºC high bacterial load. In the instances where toxicity was not as pronounced, monophosporyl lipid A (less potent) could be the contributing factor.

HHP damage to the diglucosamine-4'-phosphate region (binding epitopes for the LAL assay) could also inhibit LAL, as was observed at 0 hours (immediately after pressurisation) for the 8 ºC organic phase. LPS is well known for its dual biochemical characteristic in that the polysaccharide portion is hydrophilic and the lipid portion is hydrophobic. The supramolecular aggregate structure of lipid A could also have an influence on LAL expression, whereby conical molecules, which form cubic inverted aggregate structures, exhibit high endotoxin activity, as observed at six hours for the organic and aqueous phases of the 50 ºC treatments (supernatant high load), the 50 ºC pellet low bacterial load and the supernatant high bacterial load. It is also possible that the lower toxicity as observed at 24 hours (pellet high bacterial load) in the 50 ºC organic phase, at six hours (pellet low bacterial load) in the 50 ºC organic phase, and at 48 hours (supernatant high bacterial load) in the 8 ºC organic phase is the result of cylindrical molecules that

formed lamellar aggregate structures, resulting in lower toxicity expressed by the pressurised cells.

In bacteria the production of 3-hydroxy fatty acids (3-OH FAs) differs within species. The presence of 3-OH FAs is a result of Beta-oxidation (Venter, Kock, Kumar, Botha, Coetzee, Botes, Bhatt, Schewe & Nigam, 1997). Lipid A formed as a result of lipid synthesis could result in less 3-OH FAs, leading to lower toxicity as observed at six hours with the pellet low bacterial load. In the event of a stress condition (HHP), β-oxidation could become the principle supply of energy, mobilising the 3-OH FAs which then present as intermediates in the β-oxidation cycle. The presence of more 3-OH FAs will therefore constitute a higher toxicity, as observed at six hours for both the supernatant low and high bacterial loads (organic and aqueous phases) and the pellet low bacterial load aqueous phase.

From these results it can be concluded that the toxicity response of the pressurised cells to HHP damage at 50 ºC was more pronounced than that observed for the treatments at 8 ºC and more so in the organic phases. This could be explained by the higher amounts of lipids soluble in the organic phases rather than in the aqueous phases. In most cases, membrane repair seemed to be completed at 48 hours, as differences in toxicity were no longer that apparent.

A structural change in LPS could also influence the response of the immune system. It is therefore necessary to investigate the influence of HHP on the

immunogenicity of LPS on whole blood cell cytokine expression (IL-6). It is well known that the major target for bacterial inactivation by HHP is the membrane, and therefore the influence of HHP on the enzymes responsible for Kdo biosynthesis and membrane structure needs to be investigated further. In addition, the relationship between the LPS structure and toxicity of *E. coli* O111 deserves further investigation. GC-MS analysis of 3-hydroxy fatty acids could provide valuable information on the amounts of Gram-negative bacteria and the LPS in the extractions when performing a risk analysis of food preserved with HHP.

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CHAPTER 5

5. *IN VITRO* QUANTIFICATION OF PORCINE IL-6 INDUCED BY *ESCHERICHIA COLI* O111 LIPOPOLYSACCHARIDES EXPOSED TO SUBLETHAL HIGH HYDROSTATIC PRESSURE

5.1 LIPOPOLYSACCHARIDES

Lipopolysaccharides are the major outer surface membrane components present in Gram-negative bacteria and are potent immunostimulants to the innate or natural immunity in diverse eukaryotic species ranging from insects to humans (Alexander & Rietschel, 2001). They are also of great importance in the pathophysiology of numerous disease processes (Erridge, Bennet-Guerrero & Poxton, 2002; Proctor, Delinger & Bertics, 1995). Many cellular and extracellular elements that form part of a host response system of challenge by LPS have been identified and characterised (Elsbach, 2000). Picomolar concentrations of LPS can be detected by the innate immune system, which then triggers the cellular response (Refer to chapter 1 for a description of the immune response) (Gioannini, Teghanemt, Zhang, Coussens *et al*., 2004). LPS are an intoxicating stimulus for a variety of cells *in vivo* (epithelial cells, endothelial cells, leukocytes, platelets) and *in vitro*, which is comparable to bacterial infection and lethal shock (Day, Rubin, Vodovotz, Chow *et al*., 2006; Ulmer, Flad, Rietschel & Mattern, 2000; Wright, Ramos, Hermanowski-Vosatka, Rockwell, 1991). This response can take place locally or systemically (Heumann & Roger, 2002) and it induces the production of large quantities of inflammatory cytokines and mediators, providing numerous antigenic

sites (Proctor *et al.,* 1995). LPS do not act directly against cells or organs, but instead through the activation of the human immune system, especially through monocytes and macrophages. These cells release mediators such as tumour necrosis factor, interleukins and free radicals, which have a potent biological activity and are responsible for the adverse effects of LPS exposure (Martich, Boujoukos & Suffredini, 1993; Pabst & Johnston, 1989; Rietschel, Kirikae, Schade, Mamat *et al.,* 1994).

The signalling pathways alerting the host to the presence of LPS-bearing bacteria and free LPS are similar in vertebrates, insects and plants (Hoffmann, Kafatos, Janeway & Ezekowitz, 1999). The innate immune system initiates host defence against invasive microbial pathogens using specific recognition mechanisms. Pattern-recognition systems are the base of innate immunity that has considerable specificity, being able to distinguish between “self” and pathogens (Heumann & Roger, 2002). The pathogen-associated molecular pattern (PAMP) is a familiar molecular pattern, which is highly conserved and invariant amongst bacteria (Janeway, 1989), and the LPS is representative of a PAMP. The lipid A moiety of LPS represents the invariant pattern responsible for the proinflammatory effects of LPS. The innate immune cells then recognise the PAMP through the Toll-like receptors (Fujihara, Muroi, Tanamoto, Suzuki *et al.,* 2003).

Lipopolysaccharides are amphiphilic molecules that form large aggregates in solution. The supramolecular structure of the aggregates is defined by the

arrangement and pattern of the acyl chains, which define the “endotoxic” conformation (Seydel, Haekins, Schromm, Heine *et al*., 2003) that correlates with the biological activity (Jerala, 2007). The variability of LPS between different bacterial strains necessitates that the LPS receptors are targeted against the lipid A moiety, which is the minimal fragment that can trigger the cellular response, in order to give a response to a broad spectrum of Gram-negative organisms. The response, as well as the type of signalling pathway, can also be affected by variability in the LPS core and the O-antigen (Jiang, Georgel, Du, Shamel *et al*., 2005; Zughaier, Zimmer, Datta, Carlson *et al*., 2005).

LPS from the cell walls of Gram-negative bacteria are potent inducers of the inflammatory response. Studies using natural and synthetic analogues of lipid A have demonstrated a direct relationship between the structures of lipid A and their cytokine-inducing capacities (Akamatsu, Fujimoto, Kataoka, Suda *et al.*, 2006; Wang, Flad, Feist, Brade *et al*., 1991). LPS biosynthesis underlies quantitative differences in the bacterial growth phases, which results in changes to the endotoxin content of the cells (Venter, Abraham, Lues & Ivanov, 2006). It has been proven that for full expression of typical *in vivo* and *in vitro* endotoxic activity, a lipid A consisting of a ß-1,6-linked D-glucosamine disaccharide with two phosphate groups in position 1’ and 4’, substituted with 12 to 14 carbons in length, is required (Akamatsu *et al.*, 2006; Dehus, Hartung & Hermann, 2006). However, it must be noted that the LPS molecule itself is not toxic; it is the response of the host

cells to the LPS that renders it “poisonous” (Koyama, Sato, Nomura, Kubo *et al*. 2000).

5.2 CYTOKINES

Cytokines are protein molecules, and their name was derived from early observations that these molecules influences cell (“cyto”) movement (“kinesis”). Cytokines, which control almost every aspect of the immune system, have molecular weights ranging between 8 and 80 kDa (Gesualdo, Pertosa, Grandaliano & Schena, 1998; Strouse, 2007) and do not freely cross the blood-brain barrier under normal circumstances (Maier & Watkins, 1998). Interleukin-6 (IL-6) is a multifocal cytokine produced by lymphoid and non-lymphoid cells (Lee, Cho, Kang, Shin *et al*., 2004). IL-6, an endogenous pyrogen, has been proven to regulate T- and B-cell functions and is able to induce acute-phase response proteins and maturation of megakaryocytes (Van Snick, 1990). Pro-inflammatory cytokines, IL-6, IL-1β and tumour necrosis factor-alpha, synthesised by activated macrophages and monocytes in response to an LPS challenge, are considered important mediators of sickness behaviour and fever (Harden, Du Plessis, Poole & Laburn, 2006).

5.2.1 Methods To Assess Cytokines

Various methods exist whereby the presence of cytokines is determined in various bodily fluids. These methods are based on a sandwich enzyme-linked immunosorbent assays (ELISA) which was developed for the determination of human cytokines (Borg, Kristiansen, Christensen, Jepsen & Poulsen, 2002). However, swine presents with an important animal model for various diseases, and therefore porcine blood is increasingly being used for immunological investigations (Duvigneau, Sipos, Hartl, Bayer *et al*., 2007). With this study, an alternative methodology is tested in which IL-6 expression in porcine whole blood, as opposed to human blood, challenged with LPS, is used as a biomarker for possible food-borne pathogen toxicity.

5.3 MATERIALS AND METHODS

Refer to chapter 2 (sections 2.4.1 - 2.4.3; p63-64) for materials and methods description on the propagation of the organisms, high pressure equipment and treatment, and to chapter 4 (section 4.5.1; p124) for lipopolysaccharide extraction.

5.3.1 Porcine Whole Blood Cell Culture Assay

The method as proposed by Andrade, Silveira, Schmidt, Jùnior *et al.* (2003) for human whole blood was used. Pooled porcine blood samples were processed within two hours of collection to minimise the undesired activation of cytokine

expression. Porcine heparinised blood was diluted 1:3 with sterile saline. Samples (with LPS) and endotoxin standard were added at 50 µl per microtitre well respectively. Saline was added at 50 µl per well, and 150 µl per well of diluted whole blood were added to give a final volume of 250 µl. The plates were incubated at 37 ºC for 16-24 hours in 5 % carbon dioxide (CO_2) humid atmosphere. The cell supernatants were collected and assayed for IL-6 by porcine-specific immunoassay.

5.3.2 Porcine Interleukin-6 Enzyme-Linked Immunosorbent Assay

The serum concentration of interleukin-6 (IL-6) was determined using the Quantikine® Porcine IL-6 enzyme-linked immunosorbent assay (ELISA) (R & D Systems, White Sci, SA). The methodology employs a quantitative sandwich enzyme immunoassay technique, and analysis was conducted as proposed by the manufacturer. Standards, controls and samples were pipetted into the microtitre plate wells, and any porcine IL-6 present was bound by the immobilised antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific to porcine IL-6 was added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate was added to the wells. The enzyme reaction yielded a blue product that turned yellow when the stop solution was added. Readings were performed at 540 nm on a plate reader (Bio-Rad 680$^{TM)}$. The intensity of the colour was measured in proportion to the amount of porcine IL-6 bound in the initial step. Sample values were calculated

using the standard curve. The correlation coefficient for the different kits varied between 0.723 and 0.998.

5.4 RESULTS AND DISCUSSION

The differences at unit time intervals between the test and control values were considered and presented as percentage difference (PD). Refer to chapter 2 (p65-66) for calculation of percentage difference. A positive percentage value indicates that the IL-6 expression of the test organism (pressurised) was less than that of the control (non-pressurised), while a negative value indicates the opposite. PDs lower than 200 were considered to be within experimental variability. [Table 5.1 shows an outline of the percentage differences].

The ability to induce IL-6 in porcine whole blood, from cell-wall-bound (pellet) and free (supernatant) LPS from cultures of *E. coli* O111 subjected to HHP, was studied *in vitro*. Porcine whole blood was challenged with LPS, and subsequent IL-6 production by the cells was determined by a commercially available ELISA. *E. coli* O111 was subjected to sublethal HHP treatment, and recovery of pressurised cells was facilitated by incubation in buffered peptone water. After exposure to HHP, it is to be expected that bacteria will be injured or killed, although this depends on the species and strain of the bacteria, the HHP treatment variables

Table 5.1 Percentage differences in IL-6 production induced by *E. coli* O111 LPS observed for each of the treatment procedures (HHP at 200 MPa 15 min^{-1})

		Percentage difference					
	Treatment temp (ºC)	0 H	6 H	18 H	24 H	30 H	48 H
ST, OP	8[a]	-18	-4	0	-11	2	-3
ST, AP	8[a]	-2	5	-2	-18	16	-5
ST, OP	50[a]	1	-237	1034	7	-30	-9
ST, AP	50[a]	0.5	-19	5	36	252	5
PT, OP	8[a]	19	113	-49	23	-3	-10
PT, AP	8[a]	6	-7	3	-242	14	20
PT, OP	50[a]	-47	301	-10	904	64	156
PT, AP	50[a]	-435	25	-196	82	202	-34
ST, OP	8[b]	12	104	-43	15	8	12
ST, AP	8[b]	-0.5	143	-23	4.5	-33	-9
ST, OP	50[b]	168	-1	-1	-1.5	1	6
ST, AP	50[b]	104	-5	9	118	12	-23
PT, OP	8[b]	-21	-5	4	-2	0	-7367
PT, AP	8[b]	75	-8	14	-22	-5	5
PT, OP	50[b]	73	-8	-5	231	-2602	-45
PT, AP	50[b]	23	1150	166	214	3	304

ST - supernatant; PT – pellet; OP - organic phase; AP - aqueous phase; H - time (hours)
([a]) - low bacterial load; ([b]) - high bacterial load

and the subsequent storage conditions. Subsequent to injury, bacteria could regenerate during storage, which may have an effect on the microbiological quality of foodstuffs (Bozoğlu, Alpas & Kaletunç, 2004) – not only as an infectious agent, but as a toxic product as well. *E. coli* O111 from the late exponential phase (after 14-16 hours of incubation) were pressurised and immediately subcultured in fresh medium. The exponential growth phase commenced after six hours (all cells) of incubation followed by the stationary phase at 48 hours.

Decreased toxicity (positive PDs) was observed in the following instances: 50ºC[a & b], PT, AP at the lag and late stationary phases; 8ºC[a] and 50ºC[a], PT, OP at the lag and exponential phases; 50ºC[a & b], ST, AP at the early and mid-stationary phases and 8ºC[a & b], ST, AP at the lag phase.

It is possible that sublethal HHP damage resulted in lipid A monomers that were not detected by the leukocytes, resulting in the negative PDs observed. Structural repair resulting in dephosphorylated LPS could lead to non-activation of the leukocytes. Should partial LPS be present in either pentaacyl or tetraacyl conformation, this would lead to reduced toxicity. LPS structures present in the cylindrical shape could also have resulted in lower toxicity and inability to produce IL-6. The formation of lamellar aggregate structures could also result in lower toxicity, as observed with the pressurised cells. HHP in these instances resulted in decreased production of IL-6 by the pressurised cells, indicating the effect that various structural changes may have on the ability of the LPS to challenge porcine

whole blood for IL-6 expression.

Increased toxicity (negative PDs) was observed in the following instances: 8ºC[a & b], PT, AP at the early stationary phase; 8ºC[b] and 50ºC[b], PT, OP at the late stationary phase; 8ºC[b] and 50ºC[b], ST, AP at the lag phase and 8ºC[a] and 50ºC[a], ST, AP at the lag and exponential phases.

Variations in IL-6 expression, as seen from the results, are a function of the physiological status of the cell in response to sublethal HHP and cell death. In instances where negative PDs were observed, it can be assumed that the lipid A structure was able to stimulate the release of IL-6 from porcine whole blood. LPS present with two phosphate groups and six acyl chains (Brandenburg, Andrä, Müller, Koch & Garidel, 2003) will constitute the optimal LPS structure, resulting in the higher toxicity observed in these instances. LPS structures present in the conical shape are optimal for Toll-like receptor 4 (TLR4) activation (increased toxicity) resulting in increased IL-6 production. The presence of cubic inverted aggregate structures of the LPS could also constitute higher toxic activity, thus increasing IL-6 production.

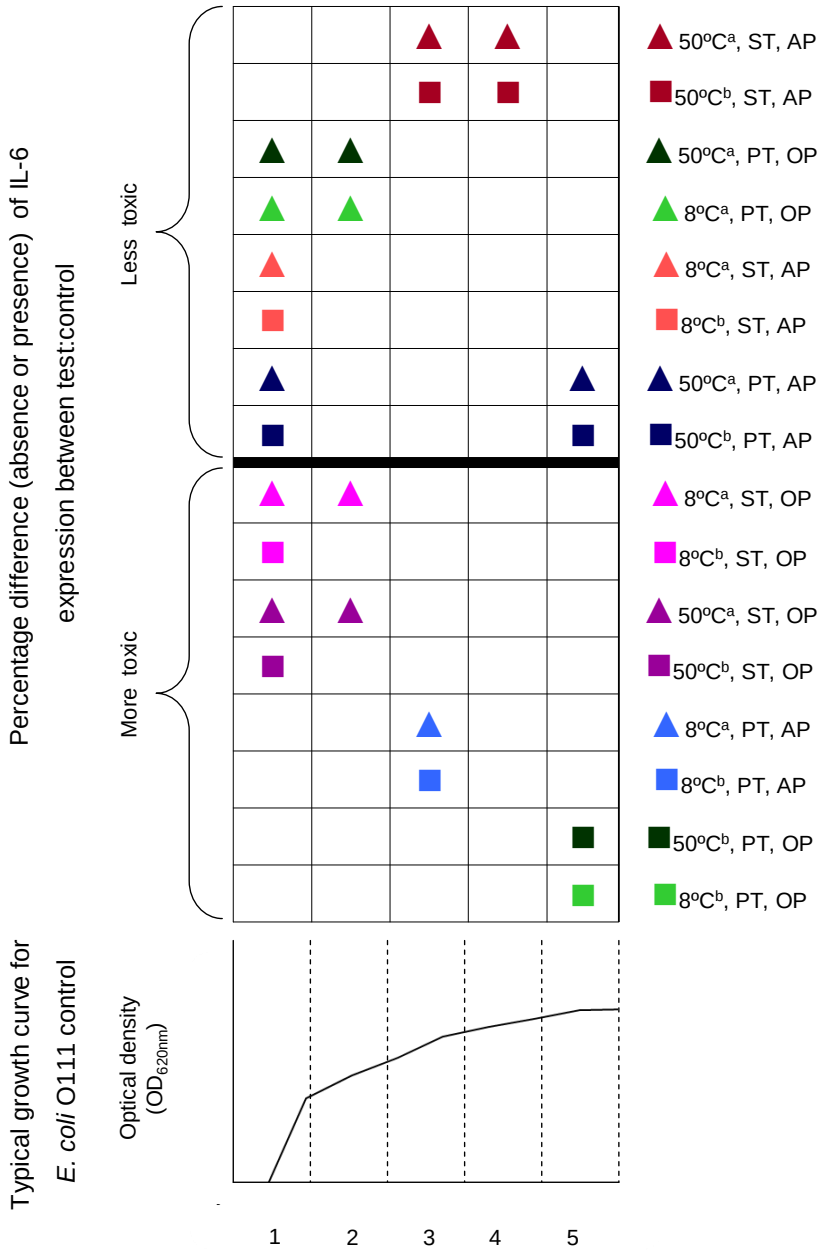
In this study, a PD of -435 was observed for 50[a], PT, AP (immediately after pressurisation). This could be explained by a higher structurally altered (in response to HHP stress) polysaccharide portion in the aqueous phase, resulting in increased toxicity. The increased toxicity observed in the aqueous phase supports

the findings of Abraham, Venter, Lues, Ivanov & de Smidt (2009) who proved that toxicity is not only influenced by lipid A but also by changes in the O-chain in response to structural stress adaptations.

The PDs observed with the 8 ºC treatment parameters were generally lower than those observed for the 50 ºC treatment parameters. Differences in IL-6 expression were still eminent up to the late stationary phase, indicating a higher LPS content in the organic phase. Overall, it would appear that HHP resulted in LPS being less able to stimulate IL-6 production [See figure 5.1 for an illustration of a typical bacterial growth curve and the related toxicity observed in each of the phases].

It is evident that HHP had an effect on LPS structure as variances in IL-6 expression between the two phases were observed, thus the quantity of the LPS does not correlate with the toxicity. It is therefore advisable that for example the pharmaceutical industry must not only consider LPS toxicity in terms of quantity but also evaluate toxicity qualitatively. Further research into the use of whole blood porcine IL-6 production could prove beneficial for both the food and pharmaceutical industries.

This novel test could parallel with human whole blood culture test systems to evaluate the pyrogenicity of different matrixes. However, the response of porcine whole blood to LPS-induced IL-6 expression does not exactly mimic the human *in*



Symbols below the line indicate higher toxicity whereas symbols above the line indicate lower toxicity, as observed for the different bacterial growth phases. ([a])–low bacterial load; ([b])–high bacterial load; PT=pellet; ST=supernatant; OP=organic phase; AP=aqueous phase; 1=lag phase; 2=exponential phase; 3=early stationary phase; 4=mid-stationary phase; 5=late stationary phase.

Figure 5.1 Illustration of a typical bacterial growth curve and the related toxicity observed in each of the phases.

vivo response to IL-6 production. There is also the need to quantify the efficacy of the IL-6 porcine whole blood test system against other Gram-negative bacteria, Gram-positive bacteria, viruses, fungi and yeasts. The use of fresh blood does, unfortunately, have certain limitations e.g. there may be limited amounts of porcine blood available from a single donor, hence the need to use pooled blood. Research into the use of pooled cryopreserved porcine blood for IL-6 determinations may address the limitations and may result in a cost-effective alternative to the LAL. Similar to LAL, the results from the IL-6 assay also proved that HHP had an effect on the structure of LPS which influenced toxicity. The quality and quantity of LPS in this instance had an influence on the ability of whole porcine blood (after being challenged with LPS) to express IL-6.

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CHAPTER 6

6. EVALUATION OF AN *IN VITRO* PYROGEN ASSAY BASED ON THE EXPRESSION OF IL-6 FROM PORCINE WHOLE BLOOD: COMPARISON WITH THE LIMULUS AMEBOCYTE LYSATE

6.1 *LIMULUS* AMEBOCYTE LYSATE

LPS forms a major component of the outer membrane of Gram negative bacteria (Caroff, Karibian, Cavaillon, & Haeffner-Cavaillon, 2002). It is unique and highly conserved bacterial surface molecules that engage with the innate immune system of the host via pattern recognition receptors on a range of host cells (Hodgson, 2006). The LAL is a common method used for endotoxin measurement and is based on the initiation of the coagulation cascade in the LAL (Tanaka and Iwanaga, 1993).

The LAL assay is used as an *in vitro* end-product endotoxin test for human and animal parenteral drugs, biological products and medical devices. It is however advised that this product is not intended for *in vitro* determination of endotoxemia in human (Cambrex). The test procedures are based on those described in the United States (U.S.) Food and Drug Administration (FDA) guidelines whereby it 1) established endotoxin limits for pharmaceuticals and medical devices, 2) validated the use of LAL as an end-product endotoxin test, and 3) developed a routine testing protocol (U.S. Department of Health and Human Services, FDA).

Even though the LAL is the assay of choice to determine endotoxin, it is known that numerous factors do however influence final outcome of the LAL assay, and it does not exactly parallel *in vivo* pyrogenic activity (Lee, Sung, Han, Lee *et al*., 2005). Even though the LAL is highly sensitive, it is not able to detect non-endotoxin pyrogens (Hoffmann, Peterbauer, Schindler, Fenrich, *et al*., 2005) and it also reacts with 1,3-ß-glucans of fungi (Iwanaga, 1993). Furthermore it is not possible to obtain a reliable and repeatable estimate of endotoxin in serum and plasma samples since the reaction of endotoxin with LAL is susceptible to either inhibition or enhancement due to contamination with fungal beta glucans or factors present in blood (Hausmann, Yulzari, Lewis, Saisky, & Douvdevani, 2000; Hodgson, 2006). The chemical and physiochemical structure of endotoxin also influences the LAL reaction (Rietschel, Brade, Brade, Brandenburg, 1987; Abraham, Venter, Lues, Ivanov, & de Smidt, 2009).

The LAL assay is generally based on the bacterial endotoxin catalyzing the activation of a proenzyme in the LAL (Young, Levin & Prendergast, 1972). The initial rate of activation is determined by the endotoxin concentration and structure. The activated enzyme catalyzes the removal of pNA from a colorless substrate Ac-Lle-Glu-Ala-Arg-pNa. pNA released is measured photometrically at 405-410 nm after the stop reagent is added. The correlation between the absorbance and endotoxin concentration is said to be linear in the 0.1-1.0 EU ml^{-1} range. Endotoxin concentration is then calculated from the absorbance values of solutions containing known amounts of endotoxin standard (R&D Systems).

6.2 PORCINE IL-6

Cytokines regulate the cellular immune response and are produced by a variety of cells, notably monocytes, lymphocytes and macrophages. Therefore the presence of cytokines usually yields useful information on the pathological processes of various diseases such as sepsis, cancer, endotoxic shock and acquired immuno deficiency syndrome (Molina, Gamboa, Benavides, León, Guerra, & Padilla, 2006). Interleukin-6 is an example of a pleiotropic cytokine that regulates the immune response, acute phase reaction, and hematopoiesis (Scamurra, Arriaga, Sprunger, Baarsch, & Murtaugh, 1996).

Typically IL-6 is measured in plasma and serum by means of a human enzyme linked immunosorbent assay (ELISA) (van Leeuwen, Westra, Limburg, van Riel & van Rijswijk, 1995) or the Luminex analysis (Hergenroeder, Moore, McCoy, Samsel *et al*., 2010). The Quantikine® porcine IL-6 immunoassay is a solid phase ELISA intended to measure porcine IL-6 in cell culture supernates, serum and plasma. It contains *E. coli*-expressed recombinant porcine IL-6 in addition to antibodies raised against the recombinant factor. This assay is able to quantitate recombinant porcine IL-6 and can also be used to determine the relative quantity of for natural porcine IL-6 (R&D Systems).

6.3 MATERIALS AND METHODS

6.3.1 *Limulus* Amoebocyte Lysate Assay

Refer to materials and methods in chapter 4 (section 4.5.2: p125) for a description on the methodology of the LAL assay.

6.3.2 Porcine Whole Blood Cell Culture Assay

Refer to material and methods in chapter 5 (section 5.3.1: p145) for a description on the methodology of whole blood challenge with LPS.

6.3.3 Porcine Interleukin-6 ELISA

Refer to material and methods in chapter 5 (section 5.3.2: p147) for a description on the methodology of the Porcine ELISA.

6.4 RESULTS AND DISCUSSION

6.4.1 IL-6 Expression After Challenge With LPS Containing Samples

Samples and endotoxin standards at a concentration of 1 EU were used to stimulate heparinized porcine whole blood for IL-6 expression. For the porcine IL-6 it is indicated that within the parameters of intra-assay and inter-assay precision the maximum coefficient of variation (C.V.) was 10%. Within the parameters of this study, the average C.V. observed for all the standards assayed in the 8 microtitre

plates was 4.3 (concentrations of the standard dilutions from which the calibration curve were drawn: 2 500, 1250, 625, 312, 156, 78.1 and 39.1 picogram). The optimum C.V. for the LAL should be less than 10%. Within the parameters of this study, average C.V. observed for all the standards assayed in the 8 microtitre plates was 9.1 (endotoxin units of the standard from which the calibration curve were drawn: 1.0, 0.5, 0.25 and 0.1). [See table 6.1 for instances where picogram amounts of IL-6 were detected (not extrapolating below the lowest standard which is 39.1 pg ml^{-1}) in the test samples and compared with the corresponding LAL values (endotoxin units)].

Dehus, Hartung & Hermann (2006) reported that in human whole blood a 1 000 times more LPS from *Pseudomonas aeruginosa* was necessary to induce IL-6 expression in amounts comparable to LPS from enterobacteriaceae (e.g. LPS 10 ng ml^{-1} from *Salmonella abortus equi* 46 ± 5.3 ng ml^{-1} IL-6 vs. LPS 10 µg ml^{-1} from *Pseudomonas aeruginosa* 55 ± 6.3 ng ml^{-1} IL-6). From the results it is observed that in this study there is no correlation between the IL-6 value measured and that of the LAL value in any of the treatment parameters. These results concur with those of Dehus *et a*l., (2006) whereby the whole blood assay does not always correlate with the *Limulus* test.

Table 6.1: Values for porcine IL-6 and LAL obtained from selected treatment parameters

Hours	Treatment parameter	IL-6 concentration (pg ml^{-1})	LAL (EU ml^{-1})
0	T50ºC[a], PT, AP	0.16	0.007
6	T50ºC[a], PT, OP	960	3
	T50ºC[a], PT, AP	396	7
18	T50ºC[a], PT, AP	203	14
24	T50ºC[a], PT, OP	974	-1
	T50ºCa, PT, AP	51.5	12
30	T50ºC[a], PT, OP	1760	6
	T50ºC[a], PT, AP	698	7
48	T50ºC[a], PT, OP	364	-0.13
0	T50ºC[b], PT, OP	0.08	0.005
24	T50ºC[b], PT, OP	1568	3.7
	T50ºC[b], PT, AP	2912	0.09
18	T50ºC[a], ST, OP	819	-0.07
6	T8ºC[b], ST, AP	1184	-0.78
24	T50ºC[b], ST, AP	814	15

T – test; ST - supernatant; PT - pellet: OP - organic phase; AP - aqueous phase;
([a]) - low bacterial load; ([b]) - high bacterial load

6.4.2 Additional Materials And Equipment Required

In addition to the material supplied in the test kits (LAL and IL-6), additional materials and equipment are also needed to perform the analysis. Before the IL-6 ELISA can be performed there is also the additional procedure of collecting blood from swine and challenging the porcine whole blood with the LPS containing

samples. [See table 6.2 for a summary of the additional materials and equipment (not supplied in the test kits) needed for the LAL assay, the IL-6 ELISA and the LPS challenge of the porcine whole blood].

Table 6.2 Additional materials and equipment needed for the LAL and IL-6

Additional materials and equipment for LAL	**Additional materials and equipment for IL-6**
Microplate reader	Microplate reader
Automatic- and 8 channel pipettes and pyrogen free tips	Automatic- and 8 channel pipettes and pyrogen free tips
LAL reagent water	Pyrogen free water
Stopwatch	Stopwatch
Endotoxin free glass tubes	**Additional materials and equipment for LPS challenge**
Vortex mixer	Heparinized vacutainer® tubes
Multi-Blok heater	15 Gauge needle
Reagent reservoirs	Holder/adapter
Pyrogen free microplates	Alcohol wipes
Acetic acid stop reagent (25% v/v)	Needle disposal unit
0.1 N Sodium hydroxide	Gloves
0.1N Hydrochloric acid	Container for transport of blood to laboratory
	Pyrogen free glass container for preparation of pooled blood
	Sterile, pyrogen free saline
	Pyrogen free microplates
	Carbon dioxide (CO^2) incubator

As both test kits in this instance was used to detect LPS, care must be taken at all times only to use pyrogen free equipment and -water. Performing the porcine the IL-6 is a lengthy process when compared to the LAL. [See table 6.3 for a comparison of the estimated turn around times (TAT) for LAL and IL-6]. Furthermore, the supernates needs to be carefully pipetted from the microwells into the precoated IL-6 ELISA kit wells not to disturb and transfer any blood products

which could interfere with the analysis. The IL-6 ELISA still requires products from animal origin (ethical considerations) and there is a need for a skilled animal phlebotomist to collect the blood in a sterile manner in order to avoid contamination from organic dust from the environment which could lead to false activation of the mononuclear cells by LPS in the dust. It must also be determined before

Table 6.3 Estimated turn around times for LAL and IL-6

Estimated TAT for LAL		**Estimated TAT for IL-6**	
Procedure	Approximate time (hours)	Procedure	Approximate time (hours)
Preparation of reagents, standards and controls	1	Preparation of reagents, standards and controls	0.5
Wash and incubation steps	0.25	Collection, transport and processing of blood	4
		Incubation process (LPS challenge of blood)	16-24
		Wash and incubation steps	4.5
Total	**1.25**		**25-33**

hand when swine will be available, thus scheduling and coordination between the laboratory personnel and the animal phlebotomist must be taken into consideration. The animal phlebotomist must also be trained in handling of the specimens, since to much manipulation and incorrect storage of the blood specimens can result in undesired stimulation of the cytokine expression pattern, resulting in false positive values. The use of whole blood has the advantage of fewer *ex vivo* manipulations compared to the use of mononuclear cell preparations, thereby reducing the accumulation of undesired stimulatory effects (Duvigneau,

Hartl, Teinfalt & Gemeiner 2003). If animal facilities are not situated in close proximity to the laboratory, this will result in delay in processing of the samples. Porcine blood should be processed within 2 hours to prevent undesired stimulatory effects on the cytokine expression pattern. Collection, transport, dilution of blood (cell preparation) and overnight incubation, therefore makes the IL-6 ELISA test laborious and time consuming. In a laboratory where emphasis is on a fast turn around time (TAT) and high volume work load this methodology is to time consuming and laborious. Advantages however are that the porcine IL-6 will also detect non lipopolysaccharide pyrogens, it can thus be used to determine the presence of Gram positive contaminants as well.

Even though the LAL is not the ideal test, it remains the method of choice for the detection of LPS in parenteral drugs and food substrates. The availability of cryopreserved porcine blood purchased as part of the porcine IL-6 ELISA kit would render this test more favorable. The cost of implementing the porcine IL-6 as a routine test would however be high. The use of the porcine IL-6 also needs to be validated and standardized against other *in vitro* test systems using human blood. These validation tests should not only be performed on laboratory media but should include actual high hydrostatic pressure treated food substrates. As mentioned by de Boer and Beumer (1999) the sample matrix could influence the results whereby background biota, natural substances or debris can interfere with the test method and invalidate the test results. Furthermore the potency of post HHP treated *E. coli* O111 LPS to induce IL-6 and correlation with endotoxin

concentration needs to be investigated. It can be concluded that within the parameters of this study, no correlation between IL-6 expression in porcine whole blood (after LPS challenge) and the LAL assay can be made. This however does not negate the use of the porcine IL-6 assay as further research and development could lead to the successful implementation of this test methodology for the detection of pyrogenic contaminants in matrixes such as medical products (parenteral drugs) or equipment, the detection of pyrogens in organic dust samples, or the implementation thereof as an alternative to the LAL.

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CHAPTER 7

7. CONCLUDING REMARKS

7.1 BACKGROUND

For many years, improvement in food production and preservation technologies have contributed to food safety systems and in most developed countries was perceived by many to be efficient in the prevention of food borne disease. Food borne diseases can result in severe illness in the young, old and the immunocompromised. Numerous problems are prevailing, one of these being the high level of food borne microbiological diseases which seem, for some pathogens, to have increased over the last decades. This has led to the development of alternative technologies to the conventional preservation methods. High hydrostatic pressure (HHP) is one of these alternative methods of food preservation that involves the application of high pressure to achieve microbial inactivation without changing the organoleptic characteristics of food. The numerous applications of HHP in the food industry reflect the success story of this preservation technology. Although there are certain limitations to its application, it is still considered as a favorable alternative to traditional thermal processing. First considered in the food industry for its worth in the inactivation of micro-organisms, it has now proved its value in other areas of food processing. E.g. it was found that pressures of around 200 MPa is sufficient to shuck oysters, thereby reducing the laborious process of hand shucking. Developments in the sphere of HHP have led to the implementation of hurdle treatment regimes, whereby not only time-temperature-pressure are the only variables, but where the use of e.g. bacteriocins

and antibiotics, and the manipulation of pH and salt concentration augmented the effect of HHP as a food preservation technology.

7.2 SUMMARISED PROCEDURES

Three bacterial strains, known for their pathogenicity in the food industry, were selected for pressurisation studies. These organisms, *Escherichia coli* O111, *Staphylococcus aureus* ATCC 25923 and *Listeria monocytogenes* (UAFSBCC), were subjected to 200 MPa pressure at temperatures of 8 ºC and 50 ºC respectively for 15 minutes. Different enumeration methods were used to assess cell damage and evaluate the growth phases of the surviving cells in order to shed light on subsequent cellular repair after sub-lethal injury. The influence of sublethal HHP on the liberation and toxicity of bacterial endotoxins (free and cell wall bound) was assessed by means of the LAL, and a porcine IL-6 ELISA assay was evaluated as an alternative for the customary LAL as a biomarker for pyrogenic substances in matrixes. The use of the chloroform:methanol LPS extraction (chapter 4), is a novel method whereby differences in toxicity (structural adaptations in response to HHP stress) in organic and aqueous phases was determined.

7.3 ORGANISM SUSCEPTIBILITY TO HHP

Staphylococcus aureus (chapter 3) proved to be the most resistant organism and *E. coli* proved to be the most sensitive (chapter 2). The selection of the most resistant strain as a parameter for optimum microbial inactivation is essential whereby sub-lethally injured bacteria can pose a bona fide hazard in the food processing industry as they are able to repair and proliferate under favorable conditions, leading to spoilage of the food matrix. The survival of injured cells are influenced by various factors such as selection of recovery medium for bacterial cultivation (enriched medium vs. selective medium), and storage conditions (refrigeration vs. on shelf storage of the product) (chapter 2 & 3). Bacterial concentration (chapter 2) in the matrix will also determine the magnitude and duration of the HHP treatment as it was proven that the inactivation rate and bacterial concentration are inversely proportionate to each other. This study was performed under laboratory conditions whereby the pathogens and substrate were controlled. When extrapolating data to other matrixes it must be taken into consideration that e.g. a high protein environment (as found in oysters) could contribute to the baro-resistance of bacteria in the matrix.

7.4 EFFECT OF HHP ON LPS TOXICITY

It is well known that endotoxins are a product of Gram-negative bacteria, hence the preference for *E. coli* O111 to perform toxicity studies (chapter 4). It was observed that HHP had an influence in the overall toxicity (as measured by the LAL) of *E. coli* O111 measured after only 6 hours of incubation for most of the treatment parameters. In view of these results, not only will the pressurised product still be contaminated with bacteria (chapter 2), but it will result in a more toxic product. This change in toxicity could be the result of structural outer membrane adaptations in response to HHP and temperature stressors (hurdles). It can thus be reasoned that even though *Listeria monocytogenes* is able to produce an endotoxin-like substance in its cell wall, toxicity will be less than that observed for *E. coli* O111 as *Listeria monocytogenes* is known to be more resistant to HHP stress than *E. coli* O111.

LPS structural membrane adaptation caused by HHP could also influence the response of the immune system to LPS. From the results (chapter 5) it is evident that sublethal HHP cellular damage and LPS structural changes (dephosphorylation and lipid A monomers) could influence the ability of LPS to stimulate leukocytes to produce IL-6. As observed with the LAL, it is evident that HHP had an effect on LPS structure, in this instance LPS was less able to stimulate IL-6 production. However, no correlation between the LAL activity and

porcine IL-6 expression could be made due to the structural orientation of the LPS, and different binding sites and modes of activation of the LAL and IL-6 expression.

7.5 OTHER APPLICATIONS OF HHP

The advancement of HHP technology requires knowledge from many fields of study, from mathematics, physics, biology, chemistry, engineering, aquaculture, agriculture to medicine. Advantages to the food industry and consumer alike are the minimum change in organoleptical- and nutritional quality of a safer food product with an extended shelf life. Other applications of HHP could include the sterilization of starter cultures (e.g. in the cheese making process) whereby any bacteriophage contaminants could be inactivated whilst leaving the Lactobacilli active. In cases where microbiological culture media are prepared in bulk for shipment to smaller satellite laboratories, there is always the possibility of bacterial, viral and fungal contamination of the media. HHP could therefore be employed in the sterilization of especially heat sensitive microbiological culture media.

In July 1988 the UK banned the use of ruminant proteins in the preparation of animal feed due to the presence of a highly stable, heat, freeze and drying resistant agent. This agent, although it is still uncertain whether it is a prion (misfolded protein) or a virus-like agent, is the cause of bovine spongiform encephalopathy, a disease affecting both humans (Creutzfeldt-Jakob disease) and

animals (mad-cow disease) (WHO, 2002). This disease was also transmitted through the use of vaccines prepared from pooled sheep brains, spinal cords, and spleens (Giles, Glidden, Beckwith, Seoanes, Peretz, DeArmond & Prusiner, 2008). As it has been proven that HHP effectively inactivate prions and viruses, the use of HHP to decontaminate carcasses will not only inactivate bacterial contaminants, but could also be successful in inactivating any prions and viruses. This would render a product which could again be used in the production of animal feed. This application could be extended for the sterilization of heat and irradiation sensitive vaccines and biopharmaceuticals. The effect HHP have on the folding and unfolding of proteins could be exploited for the preparation of various vaccines. The use of HHP in the medical field is reaching far beyond than would be expected from a food preservation technology. Knowledge on the effect of high hydrostatic pressure on living cells (*in vitro* and *in vivo*), could revolutionize the current application of HHP in the medical field whereby *ex vivo* inactivation of pathogens in blood products could also be the norm rather than the exception.

7.6 FUTURE RESEARCH

As this study only evaluated the influence of sub lethal high hydrostatic pressure on bacterial cell viability, repair, and the influence on toxicity, the following aspects could be prospects for future research:

1. The influence of metabolic adaptation and cellular repair on the lipopolysaccharide structure of Gram-negative organisms and the lipoteichoic acid (LTA) structure of Gram positive organisms needs further clarification. GC-MS analysis of 3-hydroxy fatty acids could provide valuable information on the LPS profile in the extractions when performing a risk analysis on food preserved with HHP.

2. The relationship between the LPS structure and toxicity of post HHP *E. coli* O111 also needs further investigation.

3. The influence HHP may have on the enzymes responsible for Kdo biosyntheses and membrane structure.

4. A study on the liberation of endotoxins and pyrogens post HHP needs to be performed on organisms in actual food matrixes.

5. The efficacy of the IL-6 porcine whole blood assay needs to be validated and standardized against the human whole blood IL-6 assay.

6. The establishment of cryopreserved porcine blood pool for IL-6 determinations.

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