AN ASSESSMENT OF THE LIPOPOLYSACCHARIDE TOXICITY OF ROUGH AND SMOOTH *ESCHERICHIA COLI* STRAINS CULTIVATED IN THE PRESENCE OF *ZYGOSACCHAROMYCES BAILII*

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DECLARATION OF INDEPENDENT WORK

I, LERATO BONOLO MOGOTSI, do hereby declare that this research project submitted to the Central University of Technology, Free State for the degree MASTERS TECHNOLOGY: ENVIRONMENTAL HEALTH is my own work and has not been submitted before to any institution by myself or any other person in fulfillment of the requirements for the attaimnent of any qualification.

SIGNATURE OF STUDENT **DATE**

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"Become a possibilitarian. No matter how dark things seem to be or actually are, raise your sights and see possibilities - always see them, for they're always there." - Norman Vincent Peale.

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SUMMARY

In nature microorganisms do not exist alone, but in association with one another. These kinds of associations can also be found in food industries, where cells of the same or different species can attach to pipes (biofilm formation) and a variety of surfaces in food processing environments and in food product such as yoghurt which can contain both yeast and bacteria originating from the starter culture as well as fruit. To control food spoilage organisms and food-borne pathogens preventative measures such as good manufacturing processes, the use of sanitizers and preservatives as well as hazard analysis critical control points (HACCP) are crucial in food industries. Sanitation of the working surface, floors, pipes, containers and equipment is a stepwise application of a detergent, acid or alkali rinse, a disinfectant treatment followed by final rinsing. If rinsing of the sanitizer is not done properly it may end up in the product in sub-lethal doses. In this study the influence of Liquid Hypochlorite (LH) and Liquid Iodophore (LI) sanitizers on organism growth and toxicity was evaluated. The organisms investigated included *Escherichia coli* 0113, *Escherichia coli* 026 and *Zygosaccharomyces bailii* Y-1535 in yeast malt broth, which was supplemented with LH and LI at sub-lethal concentrations 0.05% LH, 0.2% LH and 0.075% LI. Subsequently, bacterial and yeast growth responses as pure cultures and in combination (*E. coli* + *Z. bailii)* were measured as colony forming units and optical density values. Incorporation of the sanitizers in the growth media resulted in different levels of growth inhibition. Z. *bailii* proved more robust and the growth rate was not influence significantly by the addition of sanitizers or communal growth with either *E. coli* strains. The growth rate of both *E. coli* strains decreased where grown in combination with Z. *bailii* as well as in the presence of sanitizers, with the most influence exerted by LH. Changes in endotoxicity following the growth of the test samples (stressed cells) and the control (unstressed) were

measured by the *limulus* amoebocyte lysate (LAL) and porcine IL-6 ELISA methods. Where *E.*

coli strains were cultured together with Z. *bailii* the toxicity of tire mixture showed a decrease over time when measured with the *limulus* amoebocyte assay method. Interestingly the communal growth of the *E. coli* strains and Z *bailii* produced different toxicity profiles when the IL-6 porcine method was used, hi both cases, where *E. coli* strains were cultured together with Z. *bailii* the toxicity of the mixture showed an increase over tune when measured by this assay.

Other than a similar toxicity profile for *E. coli* 0113 grown in pure culture, the comparison between results obtained using the LAL or porcine IL-6 methods yielded no correlation in determined toxicity. It was established that LH and LI sanitizers as well as communal growth had an influence in the toxicity of LPS/EPS and the method used to determine such toxicity should be carefully considered.

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Chapter 1

Literature Review

1.1 General Background

Food safety is of great importance to the consumer, food industry and economy of a country (Jevsnk *et al.*, 2008) Annual cases of food-bome diseases are escalating, thus the economic and human distress caused by this can no longer be neglected (Todd, 1996 and Raspor, 2004). For instance, countries such as Egypt had a marked decline in the quantity of tourists during the last couple of years as a direct result of unsafe food.

Foodborne diseases are toxic or infectious by nature and can be caused by agents that enter the body through ingestion of contaminated food or water. The main factors contributing to unsafe food can be physical, chemical or biological, such as contamination by pathogenic microorganisms during cultivation, harvesting, processing or storage/transport (Gram *et al.*, 2002). In most cases the clinical picture of food-bome diseases may be mild when symptoms include vomiting, nausea and self limiting diarrhoea, but can also be severe when there are serious cases of nervous system disorders and severe diarrhoea (Nortemans and Floogenboom- Verdegaal, 1992) The situation is equally serious in developing countries where infant diarrhoea causes many illnesses and deaths (Kaferstein, 1988). Certain members of the population such as pregnant women, immune-compromised persons, infants, children and elderly people are particularly vulnerable (Kaferstein, 1988). Every year the World Flealth Organisation (WFIO) receives reports of food-borne diseases all over the world. These reports have indicated an increase over the past years with biologically contaminated food being the major cause of illness (Nortemans and Hoogenboom-Verdegaal, 1992).

The type of microorganisms or biochemical reactions is dependent on the food or environmental factors (Fluis int' Veld, 1996). Food spoilage, on the other hand, does not usually pose a direct

threat to the consumer. It is characterised by any change in the food product that renders it unacceptable for human consumption (Gram *et al.*, 2002). Microbial spoilage is the most common cause of spoilage and may manifest itself as slime colonies, textural changes or as off- flavours and, despite modern food technology and vast preservation techniques available, can lead to serious economic losses (Loureiro and Quero, 1999). Moreover, the number of organisms surviving the environmental conditions present in food processing is on the increase (Martorell *et al.*, 2004). Therefore, both these occurrences (food-borne diseases and food spoilage) are problematic in various different sectors of the food industry and validate continuous investigation.

The overall impact on product quality and safety will depend upon the number of organisms present; for infectious food-borne pathogens, this number may be only a few cells per g or ml, whereas food spoilage microorganisms need to reach a population of millions of cells to have an adverse effect on the sensory and physical attributes of foods (Bower, 1996). From the factors mentioned that can influence the safety and quality of food products, the greatest concern is microorganisms.

Prokaryotes like *Escherichia coli* which form part of the normal microbiota of the gastrointestinal tract of a healthy person and many warm-blooded animals can be associated with food-borne diseases, such as hemorrhagic colitis (Kuntz and Kuntz 1999). Strains that cause diseases in humans have been divided into four categories: enteropathogenic (EPEC), enteroinvasive (EEEC), enterotoxigenic (ETEC), and enterohaemorrhagic (EHEC) (Vijayakumar and Wolf-Hall, 2002). These categories are based on distinct virulence properties, different interactions with the intestinal mucosa, clinical symptoms, and distinct 0:H serotypes (Albert *et ai.*, 1993). The enterohemorrhagic *E. coli* 0157:H7 has received a considerable amount of

attention because of numerous outbreaks and sporadic cases predominantly originate from consumption of various meat and dairy products. *E. co/i* 0157:H7 has a very low infective dose (Roberts, 1988; Kuntz and Kuntz 1999). Though the microbes mainly associated with food- borne disease/poisoning are prokaryotic, eukaryotes do surface and are more related to food spoilage. In the last few decades the relevance of yeasts as food spoilage agents has become greater (Loureiro and Quero, 1999). *Zygosaccharomyces bailii* has been described as the most important of all food spoilage yeasts (Steels *et a I.*, 1999). This yeast originates from fruit trees exude and results in the spoilage of sweetened wine during processing. In addition *Z. bailii* may lead to explosion of canned food as a result of vigorous alcoholic fermentation (Rodrigues *et al.*, 2001).

Production of safe food is based on the use of preventative measures such as good manufacturing processes, the use of sanitizers and preservatives as well as hazard analysis critical control points (HACCP) (Nortermans and Borgdoff, 1997). The sanitation of working surfaces or equipment is a stepwise application of a detergent, acid or alkali rinse, a disinfectant treatment followed by final rinsing (Venter *et al.*, 2006, 2006a). hi a food processing environment, a parallel exits between the resistance of microorganisms and the efficiency of sanitizers. This resistance to sanitizers and preservatives may be attributed to cellular barriers of a microorganism such as an impermeable outer membrane for example, the lipopolysaccharide (LPS) layer hi Gram-negative bacteria that limits diffusion of such molecules into the cell (Davidson and Harrison, 2002) while the EPS which forms the biofilm matrix where a population of microorganisms is embedded (Kumar and Anand, 1998; Bower and Daeschel, 1999)

1.2 Microbial interactions

Food is a complex material and more than often nutrient rich. Thus, food is generally able to support a diverse number of microorganisms that interact with each other in different ways (Malakar *et al.*, 2002; Loureiro and Quero, 1999). One possible scenario is depicted in Fig. 1.1. The growth of microorganism B decreases when grown in combination with microorganism A. If the microbial interaction exceeds a certain limit, for example the level of > 10^7 CFU ml^{-1}, the food is considered spoiled and unfit for human consumption (Malakar *et al*, 1999).

hi food industries this kind of interaction is found in, for instance in yoghurt, which can contain both yeast and bacteria from fruit as well as the starter culture. Various interactions found in mixed populations of microorganisms have been classified on the basis of effects which may be direct or indirect interactions. Indirect interactions refer to mutualism, commensalism, ammensalism or neutralism (Sieuwerts *et al.*, 2008), while direct interactions refer to predation and parasitism (Bull and Slater, 1982; Sieuwerts *et al.*, 2008). Empirical studies with pure and mixed cultures of the same or different species have been performed, where interaction is measured as functions of physical or chemical characteristics of food or the environment, such as temperature, pH, water activity etc. (Malakar *et al.*, 1999). Quantitative studies have reported on microbial interaction as a function of their environment: Buchanan and Bagi (1997) modelled the decrease in maximum population density of a mixture of *Listeria monocytogens* and *Carnobacterium piscicola* as a function of pH, temperature and NaCl concentration. They concluded that the extent of suppression depended on relative growth rates of individual species. Pin and Baranyi, (1998) analysed the growth response of pure and mixed bacterial cultures found in meat as a function of pH and temperature and used a statistical F-test to show if differences in growth rates in mixed cultures were significant.

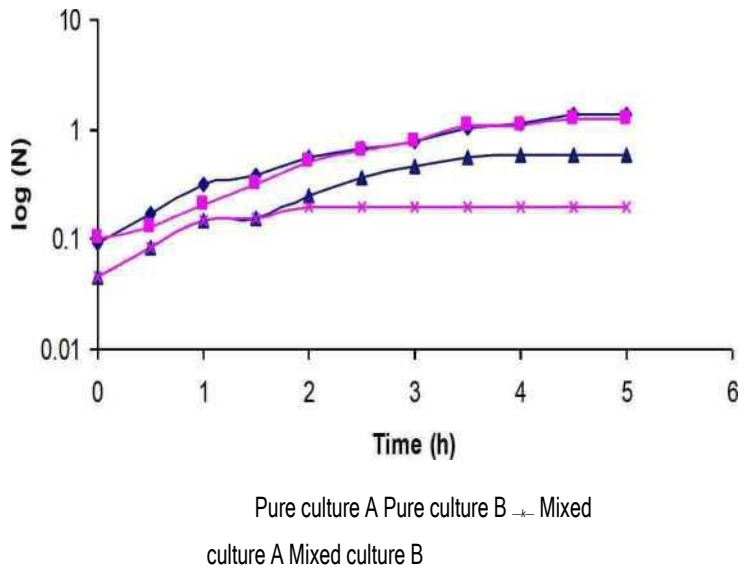

Fig. 1.1 Hypothetical simulation of growth of pure cultures of A and B compared with mixed cultures A and B. Interaction between A and B in the mixed culture decreases the growth of B (Malakar *eta!.*, 1999).

This allowed them to identify which strains were dominant under set pH and temperature conditions. An inherent problem of these studies is that the number of variables and the complexity of the mathematics required by mechanistic models do not match with the quantity and quality of the available data (Pin and Baranyi, 1998). Panikov (1996) used four differential equations to model the steady growth of a mixed population of pseudomonades and enterobacteria. However, further studies are necessary to find less laborious and more accurate ways of studying the behaviour of microorganisms in mixed populations (Pin and Baranyi, 1998).

1.3 Yeast and bacteria associated with food spoilage and contaminations

Although a magnitude of microorganisms have been associated with food safety and spoilage over the past two decades (Cohen, 1998), the yeast and bacterium of interest for this study will be briefly introduced.

1.3.1 *Zygosaccharomyces bailii*

Zygosaccharomyces is a genus of yeast that belongs to the family Saccharomycetaceae, and has been identified as a spoilage agent in the food industry, owing to their resistance to commonly used preservatives such as acetic acid, benzoic acid, sorbic acid and ethanol (Steels 1999). Although these characteristics are also common among yeasts in general, the frequency of spoilage caused by this organism is much higher compared to other spoilage yeasts. According to a study done by Rodrigues *et al* .(2001), unlike other food spoilage yeasts, *Zygosaccharomyces* growing anaerobically in foods and beverages are unlikely to prevent cell growth and alcoholic fermentation in food spoilage situations. Other characteristics of *Zygosaccharomyces* yeasts are fermentation of sugars, osmotolerance, and fructophility (Steels *et* 1999). They can survive and proliferate in foods with high concentrations of fermentable sugars such as in wines, syrup, fruit concentrates, candied fruit, soft drinks, jams, honey, as well as tomato sauces (Thomas and Davenport, 1985). Although *Zygosaccharomyces* are not pathogenic to humans, spoilage by these yeasts results in excessive gas production capable of distorting food packages, causing bottles to explode which can result in serious injury particularly to the eye (Grinbaum *et* 1994).

Zygosaccharomyces consists of eight recognized species, of which three pose serious economic spoilage risks to food manufactures. These include Z *rouxii*, *Z. bisporus* and Z. isolated

from dehydrated fruit, poultry, spoiled wines and dairy products (Hinton, Jr. 2002). The three beverage and food spoilers can be distinguished by sucrose fermentation properties, a presence or absence of growth at 37°C and resistance to acetic acid (Erickson and Mckenna, 1999). Z. *bailii* is particularly troublesome in foods such as mayonnaise, pickles, salad dressings, pickled vegetables, teas and various fruit drinks (Erickson and Mckenna, 1999). A low concentration of cells is sufficient to cause spoilage as Rawsthorne and Phister (2009) found that five cells inoculated into canned carbonated drinks were sufficient to cause spoilage. Z *bailii* also has the ability to produce ascospores, which arise as a result of meiosis when a typical diploid cell becomes transformed into an ascus containing haploid ascospores. The ascospore contains an inner and outer coat making it more resistant to chemical and physical agents than the vegetative cells (Raso *et al.*, 1998).

Extensive research on the physiological behaviour of Z. *bailii* have been conducted in the past, demonstrating the resistance of this organism under different conditions (Table 1.1). Rodrigues *et al.* (2001) studied the oxygen requirements for growth of Z *bailii* in aerobic and anaerobic cultures compared with other yeasts. The study included *Saccharomyces cerevisiae*, since it grows rapidly in anaerobic cultures, and *Candida* as it requires low levels of oxygen for growth and vigorous alcoholic fermentation. Their results demonstrated that nutrient rich foods and beverages with low/limited oxygen content does not prevent growth and gas formation by Z *bailii*. Another study by Joao Sousa *et al.* (1998) investigated the mechanism underlying the transport of acetic acid into the cell and its regulation which is the first step of acid metabolism. The results demonstrated that Z. *bailii* shows high resistance to the environments containing mixtures of sugars and acetic acid and contributed this resistance to the membrane transport of acetic acid and acetyl-CoA synthetase activity. Martorell *et al.* (2006) carried out a full

physiological assessment of Z *bailii* strains isolated from high sugar environments, investigating the effect of preservative and biocide resistance, osmotolerance, ethanol-tolerance, low pH resistance, degree of fermentation, growth temperature and survival of pasteurization. These results indicated that the Z *bailii* strains displayed extreme osmotolerance, resistance to weak acid preservatives, ability to adapt to high glucose concentrations and high temperatures, ability to ferment glucose, growth at low pH and low resistance to biocidal cleaning agents.

Tablel 1.1 Growth at 4°C and 37°C, osmotolerance, growth in low pH, gas production and inhibition temperature of various spoilage yeasts. (Taken from Martorell *et al*, 2006).

Yeast strain	**Growth at 4°C/37°C**	**Glucose (M)**	**Min. pH**	**Inhibition Temperature (°C)**	**Gas production 2% glucose (ml)[a]**	**Gas production 18% glucose (ml)[a]**
Zygosaccharomyces lentiis 398	+/-	3.75	1.9	48.0	29	170
Zygosaccharomyces bailii 594		3.9	2.0	48.5	30	118
Zygosaccharomyces bailii CECT 12001	-/+	4.0	2.2	52.5	29	190
Zygosaccharomyces bailii CECT 12002	-/+	4.05	2.2	52.5	30	186
Zygosaccharomyces bailii 593	-/+	4.0	2.15	52.5	30	111
Zygosaccharomyces bailii 592	-/+	4.25	2.2	52.5	20	133
Zygosaccharomyces rouxii CECT 12003	-/+	>5	2.2	47.5	31.5	28
Zygosaccharomyces rouxii CECT 12004	-/+	>5	2.2	46.5	31.5	102
Candida halophila 396		4.4	2.6	43.0	28	98
Candida magnoliae 397	-/+	4.75	1.75	51.0	30	48

[a] Yeast were incubated at 25 °C

1.3.2 *Escherichia coli*

Escherichia coli belongs to the genus *Escherichia* which contains mostly motile Gram-negative bacilli within the family Enterobacteriacea. This family is described as enteric, facultative anaerobic, Gram-negative rods that ferment lactose with gas formation within 48 hours at 35°C (Bettelheim, 1994). *E. coli* accounts for the majority of normal flora in the gut, and as a result of faecal contamination or contamination during animal slaughter it is found in soil, water and foods (Kaper, 1998). A majority of *E. coli* serotypes are non-pathogenic (Kaper, 1998), but some serotypes can cause illness and *E. coli* is therefore, regarded as a potential pathogenic organism. The pathogenic strains can cause neonatal meningitis, neonatal septicemia, and diarrheal diseases in humans and animals (Neidhardt, 1986).

It has been demonstrated in a number of studies that bacteria can become resistant under certain environmental factors in conditions that are considered lethal for the organism to grow (Bower and Daeschel, 1999). This inherent adaptation is ascribed to several phenotypic changes that generally occur in the outer membrane and includes changes in the macromolecular structure of the lipopolysaccharides (LPS) of Gram-negative bacteria, such as *E. coli* (Shylina and Molozhava, 2004).

By serological means it is possible to divide *E. coli* strains into an increasing number of serogroups and serotypes. Kauffman (1947) developed the initial serotyping scheme on the basis of the antigenicity (somatic or O-antigens for serogroups and flagellar or H-antigens for serotypes). Many strains express a third class of antigens (capsular or K-antigens) which are occasionally used in serotyping (Campos *et al.*, 2004). Since 1885, 170 O serogroups and more than 60 H serotypes have been recognized (Barceloux, 2008). Strains of *E. coli* that cause

diseases in humans can be grouped into six different pathotypes namely; enteropathogenic (EPEC), enteroinvasive (EIEC), enteroaggregative (EAEC), enterotoxigenic (ETEC), diffusely adhere (DAEC) and enterohemorrhagic (EHEC).

1.3.2.1 Enteropathogenic *E.coli* (EPEC)

EPEC has been reported the main cause of diarrhoea in infants in locations such as Brazil, Sao Paulo and South Africa, and in some studies exceeding the rotavirus as etiological agent (Gomes *et al.*, 1989). Transmission of EPEC is *via* the faecal-oral route with an incubation period of nine hours in adults (Benson, 1995). The common serogroups among EPEC strains are: 055, 086, 0111,0119, 0125ac, 0126, 0127, 0128, and 0142 (Nataro and Kaper, 1998).

Historically, EPEC strains were defined incorrectly, particularly in their inability to produce enterotoxins or *Shigella*-like invasiveness. In recent years, the virulence mechanism of EPEC has been better understood (Clarke *et al.*, 2003). Unlike many *E. coli* strains EPEC strains are able to adhere to epithelial cells *in vitro* (Goosney *et al.*, 2000). This attachment of EPEC to intestinal mucosa rather than toxin production is an important mechanism in their pathogenesis (Wilshaw *et al*,2000). The virulence of EPEC depends primarily on the induction of a characteristic
ultrastructural lesion in which the bacteria make intimate contact with apical plasma membrane, resulting in a localized destruction of intestinal brush border (Donnenberg *et* 1989; Francis *et al*, 1991).

1.3.2.2 Enteroinvasive *E. coli* (EIEC)

Enteroinvasive *E. coli* in some cases may produce an illness similar to shigellosis (bacillus dysentery) (Gross, 1990; Benson, 1995) common to both adults and children. The most important serotypes associated with illness in humans among EIEC include: 028ac, 0112, 029,

0152, 0136, 0143, 0144, 0124, 0164 (Doyle 1997). EIEC strains resemble biochemically, genetically and pathogenitically, like ***Shigella***, EIEC are non-lactose fermenting and lysine decarboxylase negative (Doyle ***et al.***, 1997). In addition both EIEC and ***Shigella***, produce heat stabile or heat labile enterotoxins (Flowers ***et al.***, 1992). EIEC results from ingesting contaminated food or water, although person-person transmission is possible (Benson, 1995). Symptoms usually appear 8-24 hours after ingestion of contaminated food with the infective dose of more than $10^6$is. ***coli*** per gram of food (FDA, 2006).

1.3.2.3 Enterohemorrhagic *E. coli* (EHEC)

EHEC ***E. coli*** cause hemorrhagic colitis (HC), haemolytic uremic syndrome (HUS), and thrombotic thrombocytopaenic purpura (TP) in humans. They excrete a potent toxin active against cultured vero cells called verotoxins which are also known as shiga toxins [so named because of their close resemblance to Shiga toxin of ***Shigella dysenteriae*** (Nataro ***et al.***, 1998)]. Hence, EHEC are also known as verocytotoxigenic ***E. coli*** and Shiga toxin-producing ***E. coli*** (STEC).

The most important serogroups among EHEC are OI 11, 026 and 0157:H7. ***E. coli*** 0157:H7 is the most relevant and notorious serotype in foodbome outbreaks (González Garcia, 2002) which can be attributed to a low infective dose (100-200 organisms) and acid tolerance (Buchanan and Bagi, 1997; Byrne ***et al.***, 2002). EHEC are more likely to cause bloody diarrhea and hemolytic syndrome (50-85 %) compared to other non-serotypes. Cattle are the main reservoirs of ***E. coli*** 0157:H7 and as a result, these organisms are often found in soil (Whipp ***et al.***, 1994; Charimba, 2004). ***E. coli*** 0157:H7 and has an incubation period of three to eight days causes a significant mortality rate among children, the elderly, pregnant and immune-compromised individuals (Benson, 1995).

1.3.2.4 Enteroaggregative *E.* (EAEC)

Enteroaggregative *E. coli* are described by their aggregative manner of adherence to EIep-2 cells in culture giving a stacked bricks appearance when observed under the microscope. This bacterium is the most recently identified and described pathogen in the diarrhoeagenic *E. coli* group associated with persistent diarrheal diseases in children and EIIV infected persons in developed and developing countries and is the second most common source of travelers' diarrhea (Nataro *et al.*,1995); Harrigan, 1998). Clinical symptoms of EAEC vary and the incubation period is estimated to be 20 to 48 hours (Benson, 1995; Nataro and Kaper, 1998). Variations in clinical symptoms are due to factors such as host genetic susceptibility and immune response, type virulence among EAEC strain, as well as the concentration of bacteria ingested by the host (Eluang *et al.*,2006). The pathogenicity of EAEC is not fully understood, but studies indicate that this bacterium produces cytotoxins and heat stable enterotoxins after colonizing the intestinal mucosa, mainly in the colon (Nataro *et al.*, 1998; Jenkins *et al.*, 2005).

1.3.2.5 Enterotoxigenic *E. coli* (ETEC)

Among the six recognized diarrheagenic *E. coli,* ETEC are the most common, particularly in developing countries. Enterotoxigenic *E. coli* associated with infant diarrhea in developing countries produce a heat-stable (LT) and/or a heat-labile (ST) enterotoxin (Flowers *et al.*, 1992). Colonization of the proximal small intestine by one of the enterotoxins is required and a short incubation period of 14-50 hours has been reported (Flowers *et al.*, 1992; Nataro and Kaper 1998). There are two types of LT enterotoxin, LT-1 and LT-11 which are antigenitically, physiologically and structurally similar to cholera toxin produced by strains of *cholera* (Wilshaw *et al.*, 2000). Like EIEC, ETEC is associated with poor hygiene and sanitation, commonly found in developing countries. The majority of people visiting developing countries

suffer from ETEC infections. Most of these cases probably occur as a result of water or food contaminated with ETEC in hotels after person to person transmission (Clarke, 2001).

1.3.2.6 Diffuse-adhering *E. coli* (DAEC)

Little is known about the epidemiology and clinical profile of DAEC *E. coli*. They are a heterogeneous group with diffuse adherence pattern on cultured epithelial Hep-2 as well as Hela cells (Servin 2005) There are two classes of DAEC *E. coli*. The first class includes strains of *E. coli* that harbors Afa/Dr adhesins which are associated with urinary tract infections and enteric infections (Servin, 2005). The second class includes strains that are potential causes of diarrhea in infants (Dlamini, 2008).

There is no doubt that *E. coli* strains can be associated with food-borne diseases while Z *bailii* participates in food spoilage. Where microbes are grown in complex communities with adverse environmental conditions, adaptation will be necessary for their survival. It is therefore of importance to investigate the extracellular components of these microorganisms associated with such adaptations; namely extracellular/lipopolysaccharides.

1.4 Extracellular components

1.4.1 Lipopolysaccharides (LPS)

LPS have been historically described as heat stable (up to 100°C), endotoxic, non-proteinaceous, highly variable molecules (Rietschel and Brade, 1992). In general LPS are a family of toxic phosphorylated glycolipids that are a major constituent of the outer membrane of Gram-negative bacteria such as *E. coli* (Freudenberg *et al*, 2001). Although for the most part they are conserved within the bacterial cell, they are constantly released into the environment during growth and cell

lysis (Magalhaes *et al.*, 2007). Structurally LPS consists of three parts: Lipid A, core oligosaccharide and the O-antigen (Fig 1.1) (Freudenberg 2001). Lipid A is known as the endotoxic part of LPS, as it is believed to mediate most of the biological effects of the LPS structure. It also serves as hydrophobic anchor of LPS in the majority of Gram-negative outer membranes (Raetz, 1990, Raetz and Whitfield, 2002). The O-antigen, which is the oligosaccharide region, is linked by a 2-keto-3-deoxyoctonate to the lipid A. This O-antigen contributes to the antigenicity and serospecificity of the molecule (Rietschel *et al.*, 1990).

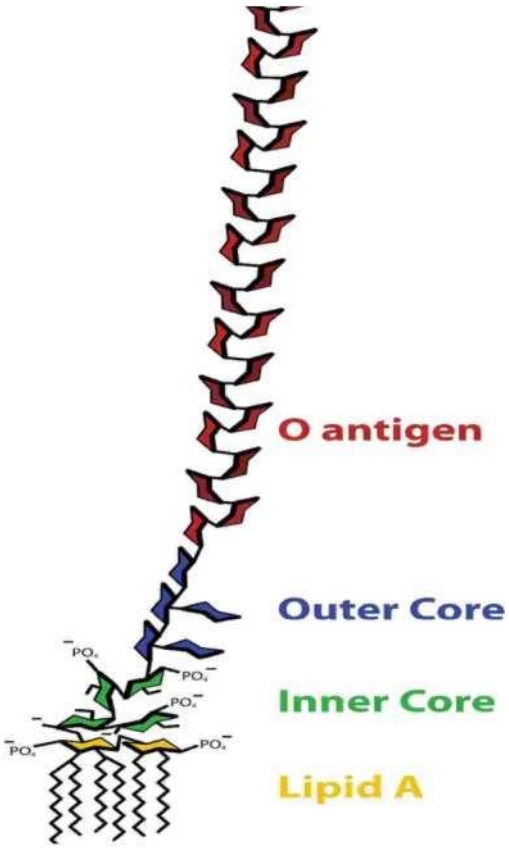

Fig.1.2 The general structure of Gram-negative lipopolysaccharide showing three regions: lipid A, core oligosaccharide and the O-Antigen (Erridge *et al.*, 2002).

LPS play an important role in the following: interacting with their environment and with higher organisms, elimination and recognition of bacteria by host defence systems. LPS also activate the immune system cells with the release of proinflammatory mediators, such as tumor necrosis factor (TNF), interleukin (IL)-6 and IL-1 (Moran, 1997; Magalhaes 2007; Freundenberg *al*., 2008). Strong inflammatory response can result in alterations in the structure and function of organs and cells, changes in metabolic activity, increased body temperature, shock and eventually death (Magalhaes *et al*.,2007; Freundenberg *et al*., 2008).

1.4.1.1 Lipid A

Lipid A is responsible for nearly all of the numerous biological activities of LPS in Gramnegative bacteria. It is a hydrophobic, gluco-configured pyranosidic hexosamine-based residue, which is present as a p-(l-6)-linked homo or heterodimer. The disaccharide carries an a- glycosidic and nonglycosidic phosphoryl group, and in an ester (positions 3 and 3′) and amide (positions 2 and 2′) linkage (R)-3-hydroxy fatty acids, which are generally acylated at their 3- hydroxyl group (Rietschel *et al*.,1994). This structure is more rigid compared to the rest of the molecule (Dixion and Darveau, 2005; Magalhaes *et al*., 2007). The lipid A of all Gram-negative bacteria possesses a similar architectural structure. Variations in structure are found in the type of hexosamine present, degree of phosphorylation, presence of phosphate substituents, and the nature, length, location and number of fatty acyl chains (Moran, 1997). Flowever, despite these variations, compared to other LPS components lipid A exhibits rather low structural variability (Moran, 1997). Removal of a particular group of lipid A leads to a decrease of endotoxicity. For instance, removal of one acyl chain such as a secondary one in position 3′ can even lead to a reduction in endotoxicity by more than three orders of magnitudes (Wang and Quinn, 2009).

Unsaturated fatty acids are rarely seen in lipid A, but have been reported in Enterobacteriaceae grown at low temperature (Erridge *et ah*, 2002). There are no strains that do not contain lipid A.

1.4.1.2 The O-antigen

The polysaccharide side chain is referred to as the O-antigen of bacteria and extends from the core polysaccharide. The O-chain consists of repeating units between one and eight glycosyl residues which vary among strains by means of sugars, sequence, chemical linkage, substitution and ring forms utilized (Erridge *et al.*, 2002). This leads to almost limitless diversity of O-chain structure which is determinative for serological identity of the respective bacterium (Magalhaes *et ah*,2007). At least 20 different sugars are known to occur and many of these are

characteristically unique di-deoxyhexoses. Full length O-chains are present only in smooth type Gram-negative bacteria. In pathogens these O-chains are in direct contact with the host during infection and are, therefore a major antigen targeted by host antibody response (Erridge *et ah*, 2002). These chains protect the bacteria from the effect of numerous antimicrobials compared to rough type strains which have more penetrable cell membranes (Caroff and Karibian, 2003).

1.4.1.3 Core polysaccharide

While the O-polysaccharide is extremely variable, the core oligosaccharide structure attached to lipid A backbone has a conserved structure with an inner core characterized by 2-keto-3-deoxy- octulosonic acid (KDO) and heptose (Keto-deoxyoctulonate) (Petsch and Anspach, 1999). The structure of growing and multiplying bacteria harbours one KDO residue. Studies show that this KDO is required for bacterial viability (Rietschel *et ah*, 1994). The fact that KDO synthesis is essential for Gram-negative bacterial viability gives an opportunity for drug designers to design

drugs targeting the KDO synthesis enzymes, which will also be specific to a wide variety of Gram-negative organisms (Erridge *et al.*, 2002).

The outer core consists of common hexose sugars such as glucose, galactose, A'-acetyl galactosamine and A-acetyl glucosamine and is more variable than inner core. In five different core types are known, while *Salmonella* species share only one core structure (Magalhaes *et al.*,2007).

1.4.2 Exopolysaccharides

EPS are polymeric substances that contain polysaccharides, proteins, glycoproteins, glycolipids, and nucleic acids (Flemming *et al.*,2007, Li *et al*, 2008). EPS can be associated with the cell surface or excreted in the medium (Dogsa *et al.*, 2005). Functions of EPS include cell aggregation, biofilm formation and protection of cells from extreme environments, facilitation of initial attachment of bacteria on a surface, and also enabling the bacteria to capture nutrients (Veno Poulsen, 1999; Dogsa *et al.*, 2005). Adherent bacterial communities in a polysaccharide matrix can result in hygiene problems in the food industry and are usually highly resistant to antibiotics (Kumar and Anand, 1998). The content of EPS in a biofilm can differ in quantity and composition, due to environmental factors and operational conditions (Li *et* 2008). Most of the studies done on EPS are of bacterial origin and only little is known about yeast EPS, with most research centred around fungal biofilm formation (Cejkova *et* 1997). It is clear that not only the presence of living bacteria/yeast should be considered in food safety, but also the debris (LPS) left by their death, which still has the ability to function as a toxin.

1.5 Rationale

Food spoilage and food-borne disease remains to be a great concern worldwide, especially in developing countries and Organisms associated with food spoilage and food-borne diseases pose a considerable threat to human welfare and the economy of countries. In nature, microorganisms exist in association with one another, i.e. yoghurt can contain both yeast and bacteria from starter culture and fruit. These kind of associations can be found in the food industry, where cells of the same or different species can stick to pipes and a variety of surfaces in food processing environments (biofilm formation) (Flood and Zottola 1995; Sutherland, 2001).

In order to control microorganisms on food contact surfaces, food processors have relied on use of preventative measures such as the use of sanitizers and preservatives to name a few (Nortermans and Borgdorff, 1997). However, improper rinsing of chemicals from food processing equipment may end up in the product in sub-lethal concentrations. This could influence the response of the microorganisms to residual chemicals. Certain molecules could kill the organisms, but what happens with the remaining molecules such as LPS is still unclear. The response of the organisms to these sub-lethal concentrations also varies depending on the strain, type of sanitizer and concentration used.

Previous studies have focused on the effect of sanitation procedures and sub-lethal doses of preservatives on the toxicity of specific microorganisms, but these were conducted on pure cultures. There exists a need to investigate this phenomenon in a setting that better reflects the situation found in industries where microorganisms exist not as pure cultures, but in communities.

Therefore, the aim of this study was to investigate the communal growth of yeast (Z *bailii)* and Gram-negative bacterium (*E.coli)* generally associated with food spoilage or contamination, focussing specifically on the effect of sub-lethal sanitizer concentrations on LPS/EPS toxicity.

1.6. References

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Chapter 2

The influence of sanitizers on the growth of *Escherichia coli* strains cultivated in the presence of *Zygosaccharomyces bailii*

2.1 Abstract

In the food industry sanitizers are used to inhibit or eliminate food spoilage and food-borne pathogens. However, the effect of sub-lethal doses of sanitizers, due to for example insufficient rinsing, on the biology of microorganism is not taken into account. It is evident from literature that LPS shed from stressed cells and debris from ruptured cells has a measurable toxicity, which is subject to change dependent on the stress applied. However, studies regarding the effect of microbial interaction (yeast and bacteria) and stress response in terms of toxicity due to sub- lethal doses of sanitizers on growth are limited. In this study the influence of sanitizers on growth of *Escherichia coli* and *Zygosaccharomyces bailii* in pure cultures, as well as in combinations, were investigated. The organisms were propagated in the presence of two typical commercially applied sanitizers which included Liquid Hypochlorite and Liquid Iodophor. Viability measurements were used to verify the bactericidal and static effect of the applied sanitizers. The bactericidal effect was only found in *E. coli* 0113 in the presence of both sanitizers. Growth rates and effect of pure and communal growth were influenced by various factors such as microbial interaction, media, concentration of sanitizer as well as the type organism.

2.2 Introduction

Food-borne illness continues to be a public health problem in developed and developing countries especially due to improper washing and sanitation in food processing plants, and various other industries (Gibson *et al.*,1999). In most countries safe food production is therefore of high concern in the food industries and legislation has been established to ensure safety of food (Nortemans and Borgdorff, 1997).

In the food processing environments, conditions favour the growth and attachment of a variety of organisms to food processing surfaces (Gibson 1999). Studies with pure and mixed cultures of the same or different species have been done, where interaction is measured as functions of physical or chemical characteristics of food or the environment, such as temperature, pH, water activity, presence of other microorganisms and antimicrobial compounds (Malakar *et al.*, 1999). Food is a heterogeneous environment that can support a variety of microorganisms. The microbial species present in food stuffs such as yoghurt may be present as contaminants *(E. coll* from contaminated milk and food spoilers like Z *bailii* from fruit pulp) or added deliberately as starter cultures (Thomas and Wimpenny, 1997).

Microbial interactions may occur between the microorganisms, which may be either competitive, neutralistic or mutualistic (Gram *et al.*, 2002). Competition generally leads to dominance of one species over another under different environmental conditions and nature of the strains themselves. For example, Thomas and Wimpenny (1997) investigated the competition between food-borne pathogens *{Salmonella* spp.) and food spoilage organisms *{Pseudomonas* spp.) under different environmental conditions using viable plate counts. At 30°C *Salmonella* inhibited growth of one of the *Pseudomonas* species, while at 20°C, the situation was reversed.

In the food industries sanitizers are designed either to kill microorganisms (bactericidal) or to inhibit growth (bacteriostatic). There are also bacteriolytic chemicals, but these are not normally used in food industries since they tend to result in the release of toxins into the environment when cells are lysed (e.g. enterotoxins) (Venter *e* 2006). Sanitizers commonly used by food manufactures include chlorine, chlorine derivatives, iodine derivatives, quaternary ammonium compounds, acidic-anionic sanitizers, hydrogen peroxide, peroxyacetic acid, and acidified sodium chlorite (Thi Hoai Duong, 2005).

Gram-negative bacteria are generally, more resistant to chemicals than Gram-positive bacteria (Russel, 1998). The outer membrane of Gram-negative bacteria acts as a barrier, preventing the entry of antimicrobial agents; this is accompanied by changes in their macromolecular structure known as lipopolysaccharide (LPS) (Macdonell and Russel, 1999; Venter *et al* 2006a). According to previous studies by Venter *et al.* (2006, 2006a) when food-borne pathogens like *E. coli* are exposed to sanitizers it results in inhibition of growth and affects LPS toxicity. However, studies regarding the effect of microbial interaction (yeast and bacteria) and stress response due to sanitizers on their growth and toxicity are limited.

It is, therefore, important to investigate both the effect of communal growth and sub-lethal doses of sanitizer on the growth characteristics of *E. coli* (faecal contaminant indicator) and *bailii* (food spoiler). In order to achieve this aim, the following objectives had to be met: establishing sub-lethal doses of the sanitizers to be used, and comparing growth characteristics of pure and communal cultures grown in the presence or absence of the relevant sanitizers.

2.3 Materials and Methods

2.3.1 Strains used

Two bacterial strains (faecal contaminant indicators) and one spoilage yeast strain were selected for this study. *Escherichia coli* 0113 (smooth strain) and *Escherichia coli* 026 (rough strain) were isolated from food samples and the yeast strain, *Zygosaccharomyces bailii* Y-1535, was obtained from the University of the Free State culture collection. The rough or smooth status of the *E. coli* was confirmed by a salt aggregation test method, which entailed the "salting out" of rough strains with ammonium sulphate (Sorongon *et ah*, 1991).

2.3.2 Media composition and pre-inoculum preparation

Stock cultures of the *E. coli* strains and Z *bailii*were stored at -80°C in 15% glycerol. They were revived on nutrient or yeast malt (YM) agar plates, respectively, at 30°C for 24h-72h. The cells were scraped off the plates and inoculated into 5 ml YM broth of the following composition: malt extract (3 $g.f^{1}$), yeast extract (3 $g.f^{1}$), peptone (5 $g.l'^{1}$), glucose (10 $g.f^{1}$) and KH_2PO_4 (5 $g.f^{1}$) at pH 6. YM medium (20 ml) was inoculated with enough cells to yield an initial OD_{69}onm value of 0.06 and incubated in a 100 ml flask at 30°C with shaking (200 rpm) for 1 Oh- 1211. This was used to inoculate 200 ml fresh YM media in a 500 ml flask to an initial OD^onm value of 0.1. The same growth medium was used for both bacteria and yeast to minimise variability. Although yeast malt broth is an enrichment media for yeast, *E. coli* strains were also able to grow in it.

E. coli was enumerated on Violet red bile MUG agar with the following composition: brain heart infusion (7 $g.f^{1}$), peptone (4 $g.f^{1}$), lactose (9 $g.f^{1}$) bile salts (No. 3)(1.5 $g.f^{1}$), neutral red (0.03 $g.f^{1}$), crystal violet (0.002 $g.f^{1}$), MUG (0.1 $g.f^{1}$), NaCl (4.5 $g.f^{1}$), K_2HP0_4 (1.0 $g.f^{1}$) and agar

(13.0 g.l[1]) at pH 7.4, using the spread plate method. The plates were incubated for 24h at 30°C. Yeast malt with 5% tartaric acid (pH 3.5) (YMT) agar was used for enumeration of Z the lower pH served to inhibit bacterial growth. The plates were incubated for 3 days at 30°C.

2.3.3 Determining sub-lethal doses of sanitizers

Liquid iodophor (LI) and liquid hypochlorite (LH) were purchased from a leading supplier of sanitizers to the food industry. These agents were selected according to the properties displayed in Table 2.1. A preliminary study by using a use- dilution method was performed to determine sub-lethal concentrations (du Preez, 2004). The sanitizers were added to cultivation broth to a final concentration of 0.05% - 0.6% LI and 0.075% - 0.6% LH. The influence of these sanitizers on growth was determined by calculating the change in growth rate (%) of the stressed organisms (sanitizers added) compared to that of the controls (no sanitizers present). The tubes were incubated at 30°C with shaking (200 rpm) and optical density measured at different time intervals. Standardized inoculums of initial OD690nm val ues of 0.1 were incubated in 100ml flasks at 30°C with shaking.

Table 2.1. Description of sanitizers used in this study.

Name	Mechanism of action	Lethal dose	Sub-lethal dose
Liquid Iodophor (LI)	Disruption of protein and nucleic structure	0.15%	0.075%
Liquid Hypochlorite (LH)	DNA synthesis disruption	0.1%	0.2% LH, 0.05% LH

2.3.4 Growth conditions and calculations

Cultivations were performed in 200 ml YM medium in 500 ml flasks at 30°C while shaking (200 rpm). Each strain was cultivated in pure culture and in combinations *(E. coli* + *Z bailii).* Growth was monitored to late exponential phase by measuring optical density (ODggonm) and viable plate counts. The influence of selected sanitizers was evaluated by growing the strains under the same conditions as described for the controls, but with added sanitizers at concentrations as determined in 2.3.3.

Maximum specific growth rates (p_{max}) were calculated by fitting a straight line to the data points that appeared to best represent the exponential growth phase using equation 1 where N1 and N2 = Colony forming units at time 1 (tl) and time 2 (t2). Differences in growth of communal cultures from their corresponding control at two selected time intervals were calculated using equation **2** where **[Tci]** represents concentration (%) of pure culture control (with sanitizer) at time 1 and **[TGI]** represents the concentration (%) of communal growth (with sanitizer) at time 1. A value of zero was considered to indicate no difference in growth when the strains were grown in combination. When a difference between the two concentrations was below zero the strain indicated better growth in combination and the opposite when the two concentrations were greater than zero.

$$Ln \frac{N2}{N1} \quad t2 - tl \qquad \text{eq 1}$$

[rci] - [TCi] = o eq 2

2.4 Results and Discussion

2.4.1 Determining the sub-lethal doses of different sanitizers

The effect of sanitizers at different concentrations on the growth of *E.* 0113, 026 and *Z. bailii* Y-1535 is shown in Fig. 2.1. The incorporation of sanitizers in the growth media resulted in different levels of inhibition of growth of *E.* and Z strains. The concentration of Liquid Flypochlorite (LH) added to the growth medium varied from 0%-0.6%. *E. coli* 0113 (Fig. 2.1A) showed growth inhibition at 0.2% LH and 0.4% LH resulting in a decrease in growth rate of 6.75% and 25% respectively. No growth was observed at concentrations exceeding 0.4%. Growth of *E. coli* 026 was not inhibited at LH concentrations up to 0.1% (Fig. 2.1C). No growth was observed at 0, 2% and 0.4% LH. Z. *bailii* (Fig. 2.IE) showed no significant change in growth rates at 0.05%-0.2% LH with zero growth at 0.4% LH. For liquid Iodophor (LI) the concentrations ranged from 0% - 0.3%. *E. coli* 0113 (Fig. 2. I B) showed a change in growth rate of 17%, 40% and 58% at LI concentrations of 0.075%, 0.15% and 0.2% respectively; above 0.2% no growth was observed. *E. coli* 026 (Fig. 2.ID) showed decreases in growth rate of 47% (0.075% LI) and 78% (0.15% LI). Z *bailii* showed no significant change in growth rate at 0.075% and 0.15% LI while at 0.2% LI and above there was no growth (Fig. 2. IF).

According to these results, the level of inhibition depended on the organism, as well as the type and concentration of sanitizer used. This response of the microorganisms to different sanitizers may be innate, apparent or acquired. Innate resistance is naturally associated with the organism, thus differences in microbial responses to sanitizer concentrations is most likely innate

(Davidson and Harrison, 2002). Mechanisms of innate response may include outer membrane barriers of Gram-negative bacteria such as *E. coli* (Venter 2006).

In this study *E. coli* 026 (rough strain) was more susceptible to 0.2% LH and 0,2% LI while the growth of *E. coli* 0113 (smooth) was less inhibited compared to a control at these concentrations. This was expected since rough strains tend to be more susceptible to disinfectants due to a lack of a complete O-specific chain at the cell surface making them easily accessible to antimicrobials (McDonell and Russel 1999). Percentage differences in growth rates could be explained by the decrease in biomass yield since growth rate is related to growth of the cell (Quintas *et al.*,2005). In Z. *bailii*Y-1535 the growth rates of the stressed cells were similar to

that of their respective control.

Z. *bailii* seems to be more robust compared to the bacterial strains except at 0.2% LI where no growth was observed. These differences are based on the physiological differences between the yeast and bacteria at the given concentrations. Russel (2003) also showed that biocide adsorption and uptake into the cells differed between bacteria and yeast; if the organism has a high affinity for the sanitizer; the level of uptake and adsorption into the cell will be higher. Affinity also depends on the type of sanitizer used (Hugo and Newton, 1964).

Based on the data and the manufacturers' specifications, the following sanitizer concentrations were used as sub-lethal dosages for further study: 0.2% LH, 0.05% LH, and 0.075% LI. Two concentrations of LH were included to allow growth for both *E. coli* strains, essential for determining LPS toxicity.

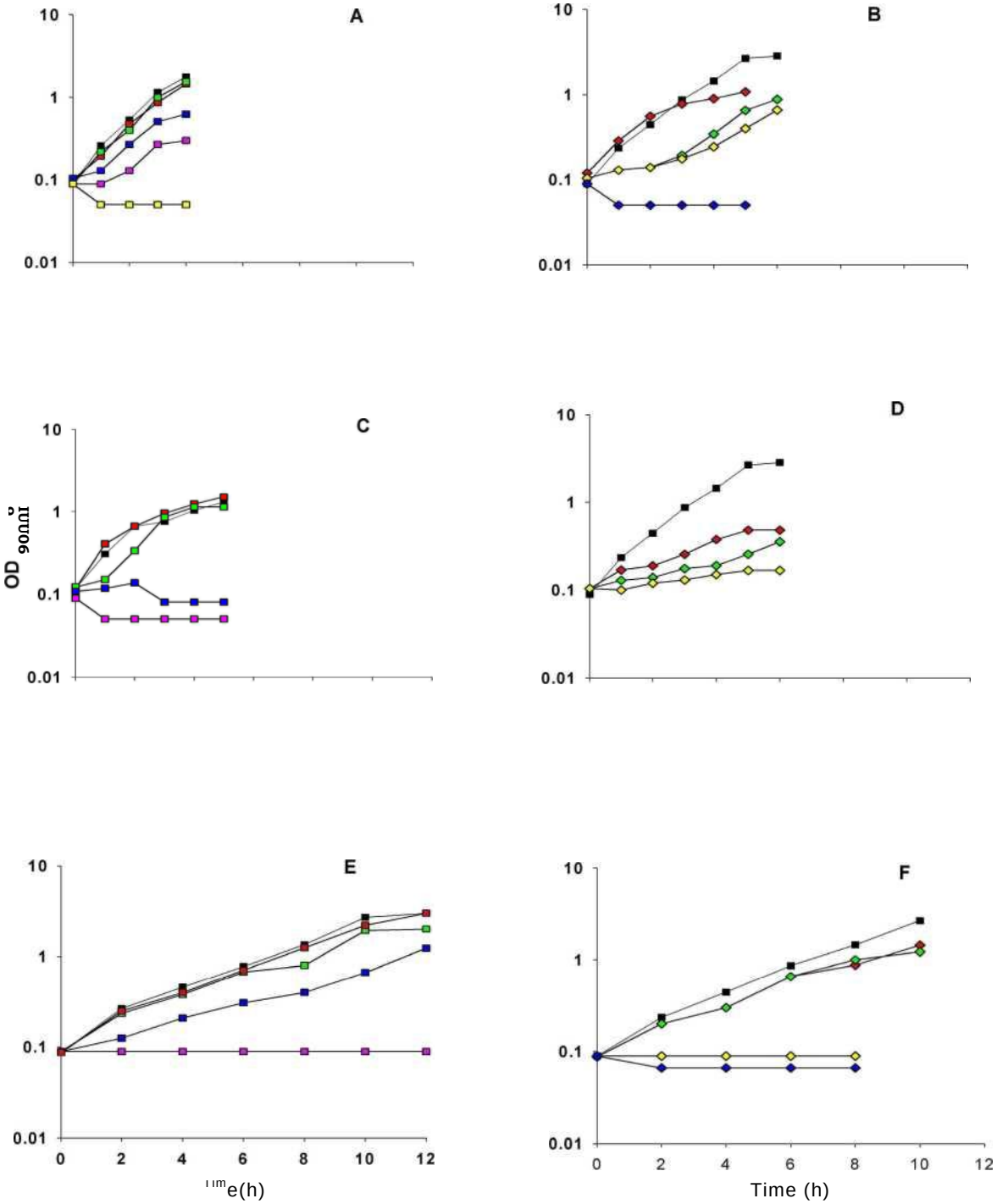

Fig.2.1. Determining the sub-lethal doses of sanitizers on pure cultures of A-B: ***E. coli*** 0113; C-D: ***E. coli*** 026; E-F: Z. ***bailii*** Y-1535 grown in YM at 3 0°C. The following concentrations of sanitizers were added to the growth medium where A,C,E is LH and B,D,F is LI:
—0%—»= 0.05% LH —o— 0.1 %LH ■ 0.2%IH—■— 0.4%LH—o— 0.6%LM —0.075% LI
—0— 0.15% LI —O— 0.2%LI —♦— 0.3%LI

2.4.2 Influence of sanitizers on pure cultures and communal growth

Growth of pure suspensions of *E. coli* 0113, *E .coli* 026 and Z *bailii* Y-1535 was monitored by optical density (OD_{690nm}) and viable cell counts ($CFU.mf^{1}$) are illustrated in Fig. 2.2 (growth curves are representative of independent duplicate experiments). The experiment served to illustrate the influence of sub-lethal sanitizer dosages on the growth characteristics in pure cultures. The sub-lethal concentrations of 0.075% LI resulted in a decrease in growth rates of 1.7%, 1.9% for *E. coli* 026 (Fig. 2.2C, D) and Z *bailii* (Fig. 2.2E, F), respectively, with no change in growth rate noted for *E. coli* 0113 (Fig. 2.2A, B). The addition of 0.2% LFI to the growth medium showed a marked decrease in growth rate (25%) of *E. coli* 0113 while the growth rate of Z. *bailii* increased by 17 %. A lower LH concentration (0.05%) resulted in a decrease in growth rate of *E. coli* 026 of 21% and a minor increase was observed in growth rate of Z. *bailii* (3.5%). Overall results indicate that the growth rate of Z. is affected most by sanitizer LI, while LH was prone to affect *E. coli* 026 and 0113, in that order.

In order to determine whether the sanitizers' modes of action may be defined as bacteriocidic or bacteriostatic, the relationship between viability and optical density was applied to quantify debris formation (Venter *et al.*, 2006). The following ratio was used: Aviability ($CFU.mf^{1}$) per AOD_{690nm} (change from inoculum to harvest which was at the late exponential phase) where a ratio less than that of the control represented excessive debris formation (Table 2.2). By applying this methodology, debris formation by the *E. coli* 0113 control culture was quantified at 3.74×10^{9}. Addition of the LH sanitizer to the growth media resulted in a viability versus OD ratio of 2.60×10^{8}, which indicates a similar level of debris formation to the cells grown in the presence

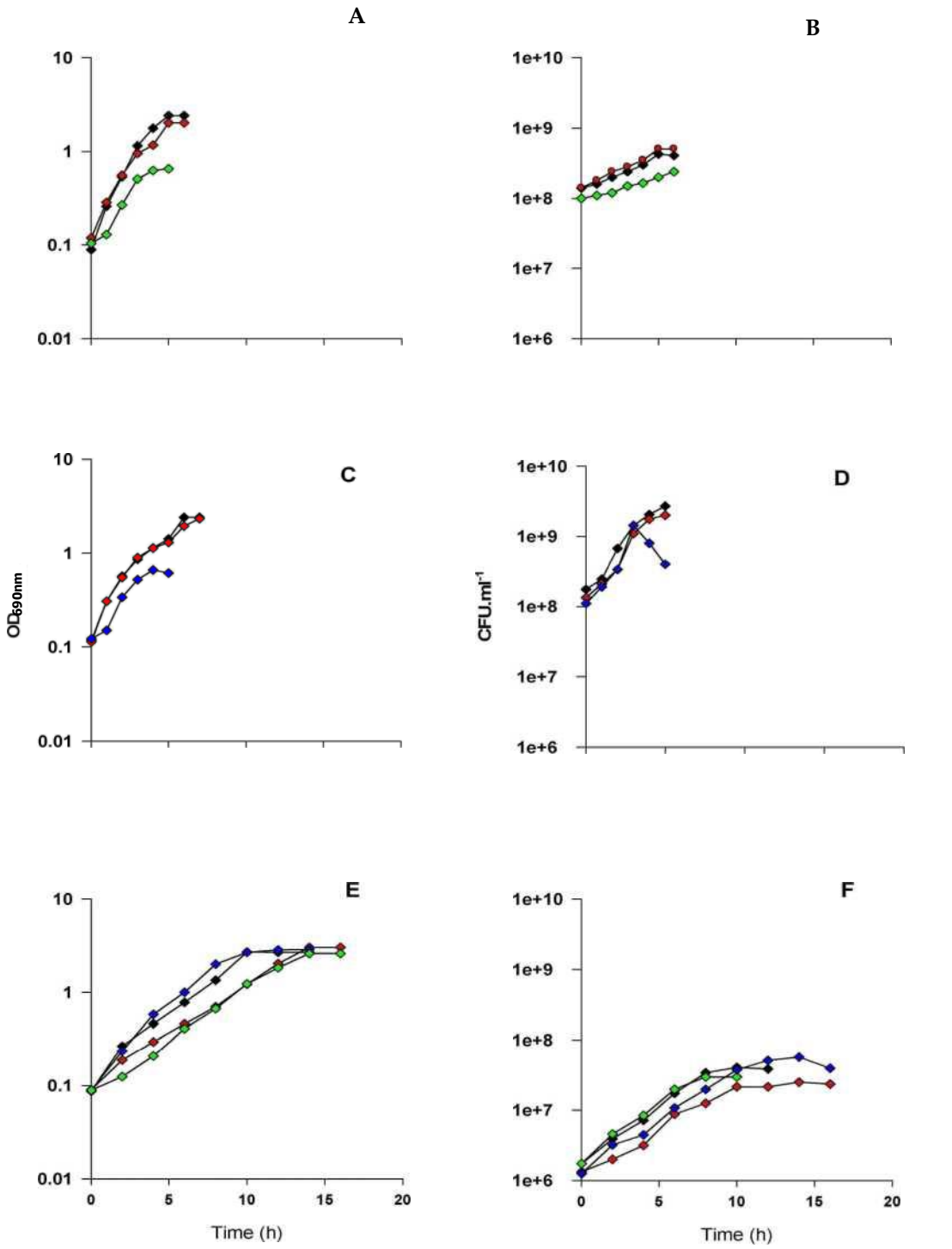

Fig.2.2. Influence of sanitizers on the growth of pure cultures of A-B: *E. coli* **OI 13; C-D:** *E coli* **026; E-F:** *Z bailii* **Y-153 5 with the following concentrations of sanitizers added to the medium: ♦ 0% —o— 0.2% LH ♦ 0.05% LH ♦ 0.075% LI**

Table 2.2. Changes in viability of *E. coli* 0113, *E. c* 026 and Z. y-1535 grown in YM medium containing sanitizers concentrations: 0%, 0.05% **LH,** 0.2% **LH** and 0.075% **LI.**

	Aviability.AOD$_{690III}$„''		
Growth medium	***E. coli* 0113**	***E. coli* 026**	***Z. bailii* Y-1535**
YM Broth (control)	3.4 x 10^9	1.84 x 10^9	1.41 x 10^7
0.075%LI added	4.6 x 10^8	1.13 x 10^9	1.82 x 10^7
0.2%LH added	2.60 x 10^8	-	1.58 x 10^7
0.05%LH added	-	8x 10^9	1.46 x 10^7

of LI (4.60 x 10^8), both a log order lower that the control. The *E. coli* 026 control was quantified at 1.84 x 10^9 Aviability.AOD$_{690nm'}$. For the same strain LF1 resulted in Aviability.AOD$_{690nm}$ ^{1} O Q ratio of 8 x 10 which indicates a higher level of debris formation to that of LH (1.13 x 10) and the control. Z. *bailii* had a Aviability.AOD$_{690nm}$ ^{1} of 1.41 x 10^7 (control). There was also no significant difference in viability *versus* OD ratio of Z *bailii* grown in the presence of 0.05% (1.46 x 10^7) or 0.2% LH (1.58 x 10^7) and 0.075% LI (1.82 x 10^7). When administered at sub- lethal concentrations, the sanitizers therefore demonstrated a bactericidal effect only in case of the *E. coli* 0113.

The effect of sanitizers on the communal growth of each *E. coli* strain grown in combination with Z. *bailii* was examined in relation to growth rate and cell numbers (CFU.mf^1) at selected time intervals (Table 2.3). According to literature, interaction is said to have taken place when the growth rate of the target microorganism in a mixed culture is decreased by 10% (Malakar *et al.*, 1999). Generally, communal growth had marked impact on the growth rates of *E. coli* strains compared to their controls (pure cultures), where growth rates decreased by 79.2% and 78.6% for *E. coli* 0113 and *E. coli* 026, respectively. The growth rate of Z. was also influenced when grown in combination with *E. coli* 0113 (9.9%) and *E. coli* 026 (11.7%), but clearly this

interaction had the greater effect on the bacterial strains. These results are not entirely unexpected since the production of organic acids (Malakar 1999) or ethanol by yeasts in restraining the growth of some microorganisms is not uncommon (Liu 2006). However, it was necessary to establish interaction in order to investigate the survival adaptation of the bacterial strains in terms of LPS.

The influence of sanitizers on the communal growth was measured using equation 2 from the materials and methods. This equation gave an indication of the differences in cell growth ($CFU.mf^{1}$) between test organisms in communal growth (with added sanitizers) and the corresponding control (communal growth; no sanitizer added) at different time intervals (Table 2.3). A positive value indicated that communal growth of the test organism in medium with added sanitizers showed less growth than the corresponding control and a negative value indicated the opposite, in addition, values equal to zero indicated no difference in growth. According to the calculated results, growth was either less or poorer. Less growth was observed only in the following: *E. coli* 0113 (ZB+ 0113), Z. (ZB + 0113) and *E. coli* 026 (ZB+ 026) in the presence LI (ti) as well as *E. coli* 0113 (ZB+ 0113) at 0.2% LH (tl), since their values were closer to zero. These results indicate that cell growth in combination can be influenced by various factors such as microbial interaction and nutrient composition at a specific time interval, and nature of the strain and type of sanitizer used (Thomas and Wimpenny, 1997). Extended exposure to chemicals in the media might have had an effect on growth, which caused the organism to start competing with each other for nutrients, resulting in poor growth of the target organisms (Yoon *et al.*,1977; Giotis *et al.*, 2007). However, LH and LI acted differently at tl, t2 and affected the growth rates differently. Their differences in activity may be in their chemical composition and concentrations used (Thi Hoai Duong, 2005). Bessems (1998) also

reported that the rate of killing of a sanitizer can be related to the type of sanitizer used, the concentration and an increase in time.

Table 2.3. Growth parameters of pure and mixed cultures of *E. coli* 0113, *E. coli* 026 and Z *bailii* Y-1535 grown in YM medium containing sanitizer concentrations: 0% , 0.05% LH, 0.2% LH and 0.075% LI.

		E. coli 0113	*E. coli* 026	Z. *bailii* Y-1535	0113 (*ZB+Ol* 13)	*ZB* (ZB+Ol 13)	026 (ZB+026)	ZB (ZB+026)
	l^max (h)	0.496	0.636	0.333	0.103	0.300	0.136	0.294
	r²	0.99	0.99	0.99	0.99	0.99	0.88	0.96
	Cal.tl	-	-	-	4.6 x 10'⁸	5.75 x 10⁻⁶	3.40 x 10'⁸	3.5 x 10⁶
	Cal.t2				1 x 10¹²	8.5 x 10⁶	1.20 x 10⁹	8x 10⁶
:3 t-	**^max (b)**	0.488	0.629	0.306	0.073	0.259	0.099	0.249
	r2	0.99	0.98	0.98	0.99	0.99	0.99	0.96
	Cal.tl	-	-	-	3x 10¹⁵	8.60 x 10⁸		
£	**Cal.t2**				2 xlO⁴⁰	4.30 x 10⁷		
	Ihnax (b"¹)	0.176	-	0.381	0.23	0.352		
X	**r2**	0.99	-	0.98	0.76	0.99		
-J	**Cal.tl**	-	-	-			2x 10⁹³	1 x 10⁸²
0.05%	**Cal.t2**	-	-	-			3.45 x 10⁸	4.0 x 10⁶
	^max(h⁻¹>	-	0.589	0.376			0.0032	0.355
	r2	-	0.99	0.99			0.71	0.98

ZB :Z *bailii* Y-1535
(ZB + 0113): Z. *bailii* cultivated together with *E. coli* 0113
(ZB + 026): Z. *bailii* cultivated together with *E. coli* 026
ji_{max}: maximum specific growth rate
Cal.tl: Calculated value at tl showing differences in growth of test organism and respective control in CFU.ml'¹
Cal.t2: Calculated value at t2 showing differences in growth of test organism and respective control in CFU.ml'¹

2.5 Conclusions

In light of the results, care should be taken in food industries where contamination of the growth media with sanitizers is likely to occur. Sub-lethal doses of sanitizers where shown to have an influence on the growth; extended exposure to chemicals caused the organism to start competing with each other for nutrients, resulting in poor growth of the target organism. Although this may be important for pathogens, it is not beneficial for food such as yoghurt, which contains yeast. Allowing enough time in food processing to rinse off the sanitizers from equipment can help eliminating these contaminants.

Different strains react differently to different sanitizers. Sub-lethal doses also had altering effects on growth of bacteria and yeasts (sometimes none). Based on the results, 0.075% LI was the most effective sanitizer that can be considered by the food industries because it resulted in less growth in communal growth of *E. coli* 0113 (0113+ Z. *bailii)*, *E.* 026 (026 + Z. *bailii)* and ZB (ZB+ 026).

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Chapter 3

Changes in lipopolysaccharide toxicity as a result of microbial interaction and exposure to sub-lethal concentrations of sanitizers as measured by chromogenic LAL assay

3.1. Abstract

This study focused on the influence of two sanitizers, liquid iodophore (LI) and liquid hypochlorite (LH) on the LPS-related endotoxicity of food-borne pathogenic *Escherichia coli* strains, grown in the presence of spoilage yeast *Zygosaccharomyces bailii*. Changes in endotoxicity of the control (unstressed cells) and the test organisms (stressed cells) were measured by the chromogenic LAL assay at late exponential growth phase. Results indicated that liquid hypochlorite and liquid iodophor influenced toxicity of each organism in a different manner. A significant decrease in toxicity was noted in pure and mixed cultures in the presence or absence of sanitizers. This change in toxicity in response to sanitizers could be influenced by the lipid A structure. Any change in lipid A structure will result in either an increase or a decrease in toxicity levels as measured by LAL. The toxicity trend remained the same in the presence and absence of sanitizer. LI caused an overall decrease in toxicity of the bacteria. Communal growth or sanitizers had almost no effect on the toxicity of *Z. bailii* and also resulted in overall decrease in toxicity of both bacterial strains. Toxicity could still be detected in cell debris even after LI and LH treatment indicating that viability and toxicity are not necessarily connected, and that the toxicity of LPS molecules that remain in food products after sanitation should be considered.

3.2 Introduction

In nature, an interaction between microorganisms is a well known phenomenon. Although most microorganisms are not harmful to humans, there are microbial associations that can cause diseases (Bower and Daeschell, 1999). Effective preventative methods such as sanitizers can be used to control the proliferation of such organisms (Nortermans and Borgdorff, 1997). Microbial testing in most food industries rely on live counts ($CFU.mf^{1}$) and does not take into consideration the remaining debris (LPS) or the effect that microbial adaptation (membrane associated) could have on the toxicity of the surviving organism. This adaptation is ascribed to several phenotypic changes in the outer membrane such as the macromolecular structure (Russel, 1998).

Molecules known as pyrogens have the ability to induce an inflammatory response characterized by among other symptoms, a fever reaction (Daneshian 2008). Strong inflammatory response can result in alterations in the structure and function of organs and cells, changes in metabolic activity, increased body temperature, shock and eventually death (Magalhaes *et* 2007). It is therefore important to monitor and determine the presence of organisms and their products in food and pharmaceutical industries (Qu 1998). Pyrogens and powerful immune stimuli derived from microbes include networks of carbohydrates, peptidoglycan and lipoteichoic acid from Gram-positive bacteria, LPS from Gram-negative bacteria, yeast cells, fungal spores and viral pathogens (Daneshian *et al.*, 2008). The wide range of biological effects attributed to LPS have raised considerable interest.

Lipid A is known as the endotoxic part of LPS, and to mediates most of the biological effects of the LPS structure. The chemical structure of lipid A influences its activity. For example, *Salmonella*-type lipid A can activate mouse cells and inactivate human cells. Different species can therefore discriminate between slight differences in the chemical structure of lipid A (Tanamoto *et al.*, 2001). In other words, lipid A of one bacterial strain differs from that of another even within the same species. Structural differences in lipid A, such as hydroxylation, secondary substitution, as well as presence of reduced amount fatty acids in long- chain LPS may affect its biological activity (Tanamoto *et al.*,2001). Refer to Chapter 1 for more detailed information on LPS structure.

There are two frequently used techniques for endotoxin detection: *limulus* amoebocyte lysate (LAL) assay and the rabbit pyrogen test. The LAL assay is very sensitive and less laborious compared to the rabbit pyrogen test in the detection and quantification of endotoxin (Hansen *et al.*, 1999). The LAL assay is an *in vitro* test utilizing lysate of blood cells of horseshoe crab, which enzymatically acts with endotoxin (Zijlstra *et al.*, 1996). Several other techniques are known, such as the gel clot test (formation of a solid gel), the chromogenic test (formation of colour) and a turbidimetric test (measuring the increase in turbidity) (Zijlstra *et al.*, 1996). However, there are few drawbacks to this method; it interferes with proteins from the cell, and other pyrogens are not detected (Nakagawa *et al.*, 2002; Magalhaes *et al.*, 2007). Different bacterial strains exhibit different potencies of endotoxins in the LAL assay (Hansen *et al.*, 1999), as well as changes in pH, temperature and time of incubation (Hurley, 1995). The reaction of LAL assay is also dependent on the physiochemical structure of lipid A. Studies have shown that lipid A structures producing the highest activity as measured by LAL, include a P-(l,'6)-linked D-glucosamine disaccharide backbone as a binding epitope. However, changing the number,

position or length of the primary or secondary acyl groups can result in dramatic alteration of biologic effects (Dixon and Darveau, 2005).

According to Venter *et al.*(2006, 2006a) the LPS structure of food-borne pathogens like *E.* changes when exposed to sanitizers and also affects toxicity. However, these studies refer to pure cultures of *E. coli* and the effects of microbial interaction and sub-lethal concentrations of sanitizers on the LPS/EPS ultra-structure and toxicity are limited. Therefore this study aimed to investigate the influence of sub-lethal concentrations of typical sanitizers Liquid Iodide and Liquid Hypochlorite on changes in LPS toxicity of *E. coli* 0113 and 026 strains cultivated in the presence of Z *bailii* Y-1535 as measured by a chromogenic LAL assay.

3.3 Materials and Methods

3.3.1 Preparation of strains and treatment parameters

Mid-exponential phase pre-inocula of *Escherichia coli* 0113 (smooth strain), *E. coli* 026 (rough strain) and the yeast strain *Zygosaccharomyces bailii* Y-1535 were inoculated into 200 ml yeast malt media (YM) and incubated at 30°C with shaking (200 rpm) until late exponential phase (lOh for *E. coli* 0113 and 026 and 12h for Z *bailii* Y-1535). Refer to chapter 2; sections 2.3.1-2.3.4 for a detailed description of the propagation of the organisms and preparations of test solutions.

Each strain was cultivated in pure culture and in combination *(E. coli + Z. bailii).* Growth was monitored by optical density (OD690nm) measurement and also viable plate counts on VRB + MUG and YMT agar. The influence of the selected sanitizers was evaluated by growing the strains under the same conditions as described for the controls, but with added sanitizers at sub- lethal concentrations of 0.2% or 0.05% (LH) and 0.075%. (LI). Cultures were sampled (1 ml) at

time intervals Oh and lOh (*E.coli* 0113 and 026) as well as Oh and 12h (Z. *bailii* Y 1535) and stored at -80°C until the toxicity analyses were performed.

3.3.2 *Limulus* amoebocyte lysate assay

LPS toxicity was determined using the chromogenic LAL assay (QCL-1000, LONZA) for all samples for time zero and at the late exponential phase of growth. This quantitative test for Gram-negative bacterial endotoxin was performed by the microplate method as prescribed by the manufacturer. The test kits included *E. coli* endotoxin standards with approximately 50 - 648 endotoxin units (EU) lyophilized endotoxins. Endotoxin standards ranging from 0.1 to 1.0 EU.ml'[1] were prepared and a standard curve was constructed by plotting OD[4]io[n]m versus the EU per absorbance unit.

The absorbance of released p-nitroaniline from the synthetic substrate was read at 410 nm with a microplate reader (Bio-Rad 680). Endotoxin concentration of diluted samples was determined by graphic method as proposed by the manufacturer. The endotoxin concentration was measured by adding the sample (i.e. culture + media) to the assay. To prevent any possible interference on the measurement at 410 nm, the blank for the diluted media was done alone in the assay. The standard curve was evaluated by Bio-Rad 680 software (R2 = 0.977) and used to extrapolate the toxicity value for each sample. The change in toxicity values were expressed as EU.mr\OD[69]on[m] ' and calculated using equation 3:

Atoxicity.OD^om,,"[1] = [toxicityCO x 0.1/OD[69Onm](?4] - [[toxicityft«) x 0.1/OD[690mn](i[0])] ..... Eq 3

Where:
toxicity(/o) = calculated toxicity value at time 0 (EU-mf[1])
toxicityOU = calculated toxicity value at lOh for *E. coli* or 12h for Z. *bailii* samples (EU.mf[1])

OD_{690nm}((x) = OD value of sample taken at lOh for *E. coli* or 12h for Z *bailii* OD_{690nm}(to) = initial OD value at time 0

The OD_{690nm} value of 0.1 was used in the calculation since it is the standardized initial OD value at time 0 (inoculation), thus the lowest OD value for any sample. Therefore, a positive value indicates an increase and a negative value a decrease in toxicity, while the Atoxicity.OD_{690nm} ^{1} value represents the magnitude of change. All equipment, reagents and consumables used were pyrogen free

3.4. Results and Discussion

Gram-negative bacteria release the LPS in free or complex form to the outer membrane when they multiply and when they die contributing to endotoxicity (Rietschel *et* 1996). Furthermore, LPS biosynthesis of bacterial strains is dependent on the growth phase, resulting in changes in the endotoxin content of the cells, which differ from strain to strain (Russel, 1976). As a result of the latter, values were calculated as Atox icity.$OD^{\wedge}{}_{nm}{}^{"1}$ and not viability.

In this study, the strains grown individually and in combinations were subjected to different sub- lethal doses of LH and LI. The cells were grown to late exponential phase after which endotoxicity values of the control (no sanitizer added) and test cultures (stressed, i.e. exposed to LH and LI) were determined by the LAL method. When microorganisms are subjected to chemicals it will be expected that their growth will be inhibited and possible cell death may occur. However, different strains behave differently under such conditions. Subsequent to stress, the organism may grow resistant to the sanitizer as a result of its inherent ability to adapt to a new environment, for example, through the formation of highly resistant structures such as
biofilms, or changes in LPS structure of Gram-negative bacteria (Kumar and Anand, 1998;

Venter *et al.*, 2006). This may affect the microbiological quality of foodstuffs, not as an infectious agent, but as a toxic product.

The change in toxicity values of pure and communal growth are presented in Fig. 3.1. There was an overall decrease in toxicity during growth of both *E.* strains without sanitizer or in the presence of sanitizers. When *E. coli* Ol 13 was grown without sanitizers (control) a pronounced change in toxicity was observed as a decrease. A similar trend was evident in the presence of both sanitizers for *E. coli* 0113 in pure culture. Growth of *E. coli* 026 also resulted in a decrease in toxicity as did exposure to both sanitizers. A trend similar to that of 0113 was observed. Z *bailii* contains EPS as its membrane component and not LPS (Notermans and Middelhoven, 1993), thus no detection of toxicity was expected using the LAL assay. Where Z. *bailii* was grown in pure culture an increase in toxicity over time was observed and no influence by either sanitizers was evident. Although the toxicity values are considerably lower than that detected for the Gram-negatives, this is the first evidence of toxicity detected from eukaryal EPS using the LAL assay. It is tempting to speculate on the similarities that might exist between the targeted section of the LPS and EPS as has previously been described for Gram-positive EPS (Abraham *et al.*, 2009).

Communal growth of the *E. coli* strains and Z. *bailii* did not result in different toxicity profiles. In both cases, where *E. coli* 0113 or 026 was cultured together with Z. *bailii* the toxicity of the mixture showed a decrease over time. This occurrence was evident in the presence of both sanitizers, indicating that it can also be attributed to the communal cultivation. The decrease in

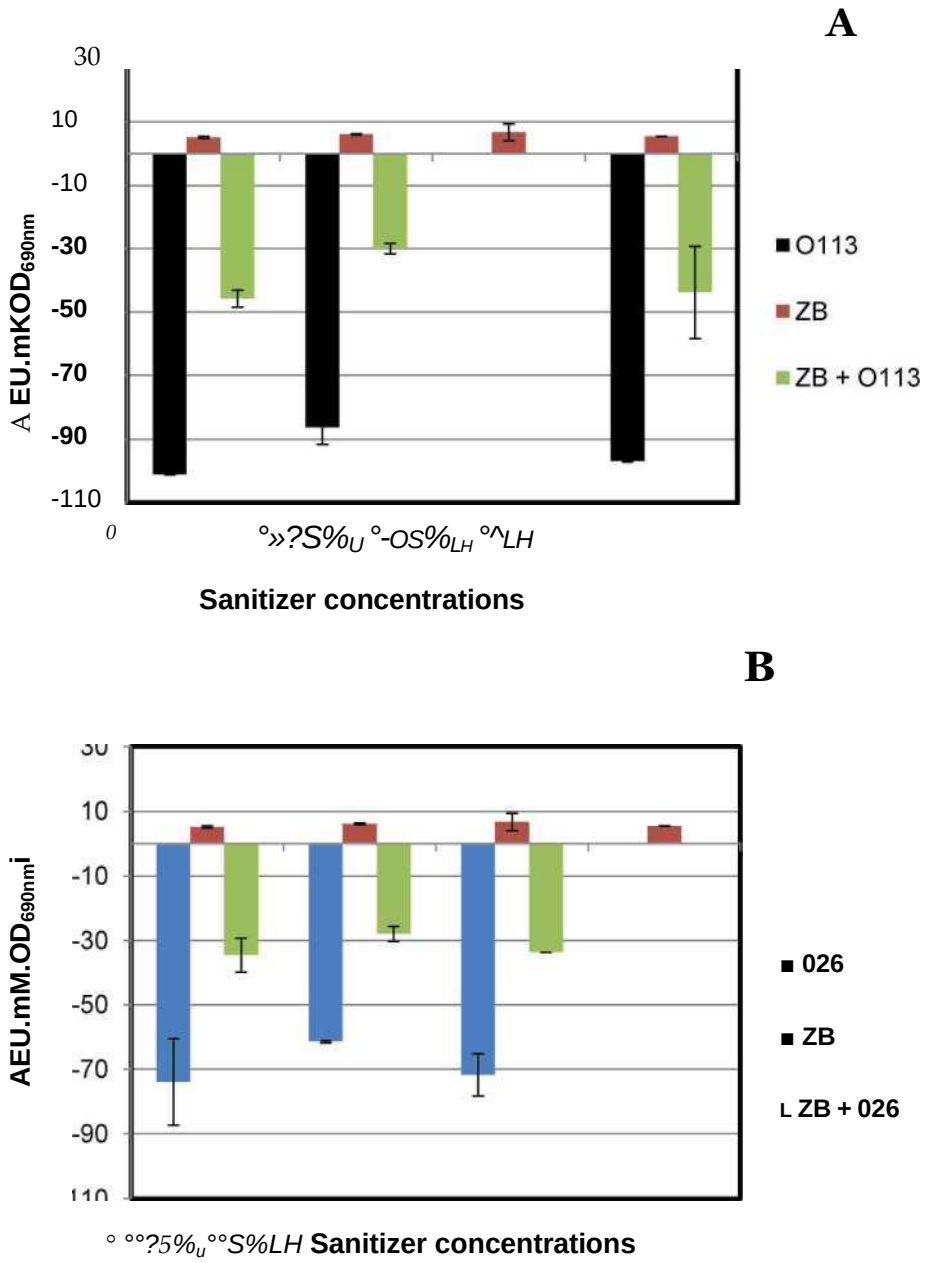

Fig. 3.1. Changes in lipopolysaccharide toxicity of (A) *E.* 0113 , Z *bailii* and grown in combination (ZB + 0113); (B) *E.coli* 026, Z *bailii* and combinations (ZB + 026) expressed as AEU.mr[l].OD$_{690nm}$ [1] following exposure to different sanitizers. Experiments were performed as independent duplicates and the error bars indicate standard deviation

toxicity was similar in both communal growth of 026 and Z. *bailii* in the presence of sub-lethal concentration of sanitizer LI and LH.

The lipid A of LPS has been shown to constitute endotoxic properties (Dixon and Darveau, 2005) and the above change was therefore expected to be reflected in the lipid A structure. The lipid A structure required to produce high LAL activity include: a (3- (l-6)-linked D-glucosamine disaccharide backbone (binding epitope) with phosphorylation at positions 1 and 4 on the disaccharide unit of 3-acyloxyacyl groups. Any change in any part of this structure, such as change in number, position, or length of primary or secondary acyl groups or subtraction of a phosphate group as a result of sub-lethal exposure to the sanitizers, can result in dramatic change in toxicity. This could explain the different responses of the organisms to LI and LH stress. The supra-molecular structure of lipid A could also have an influence on LAL expression. Experimental evidence shows that if the aggregate lipid A structure contains an increased cross- sectional hydrophobic region, compared with that of hydrophilic part, this will yield a conical lipid A shape which will exhibit high endotoxin activity, whereas cylindrical molecules (both regions equal in cross-section) molecules result in lower toxicity (Schromm 2000). Furthermore, the 3-OH fatty acid is a unique component of the lipid A molecule, making them well suited as a chemical marker of LPS (Szponar 2002). It could be that the presence of longer 3-OH FAs may have contributed to higher toxicity levels in pure cultures of *coli* 0113 and *E. coli* 026.

LI and LH displayed different effects on toxicity of rough and smooth variants of *E. coli* 026 and *E. coli* 0113 in pure and communal populations. Generally LH resulted in a change in toxicity per OD_{690nm} higher than LI in all cultivations. These differences can be explained by the different modes of action displayed by the sanitizers. LH is a highly active oxidizing agent and

thereby destroys the cellular activity of proteins. However, their penetration is maximal in an unionized state (McDonnell and Russel, 1999). On the other hand, LI rapidly penetrates into microorganisms and attacks proteins, nucleotides, and fatty acids. This could be a reason why it gave the lower toxicity values compared to LH; by attacking fatty acids, it already interferes with the lipid A structure measured by the LAL method.

3.5 Conclusions

From these results it may be concluded that the toxicity response of the stressed and unstressed cells decreased in both communal and pure cultures. This may be attributed to structural change in the lipid A structure under sub-lethal sanitizer concentrations such as number, position, or length of primary or secondary acyl groups or subtraction of phosphate group.

Growing the two strains together resulted in a decrease in growth. EPS of the yeast cell did not affect LPS toxicity, because the profile was similar to that of the pure cultures. However, this could have been influenced by the type of method used for analysis. Research into communal growth using *limulus* amoebocyte lysate assay in food could prove beneficial for food industries. A structural change in LPS could also influence a response in the immune system, it is therefore of importance to investigate the effect of sub-lethal doses of LH and LI on the immunogenicity of LPS on whole blood cells cytokine expression (IL-6).

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Chapter 4

Changes in lipopolysaccharide toxicity as a result of microbial interaction and exposure to sub-lethal concentrations of sanitizers as measured by the Interleukin-6 (IL-6) porcine method

4.1. Abstract

The current study focused on the influence of commercially applied sanitizing solutions Liquid Iodophore (LI) and Liquid Hypochlorite (LH) on the LPS-related endotoxicity of pathogenic food-borne *Escherichia coli* strains *E. coli* 0113, *E. coli* 026, grown in the presence of the spoilage yeast *Zygosaccharomyces bailii* Y-1535. Toxicity was measured at times zero and the late exponential phase of growth (10h-12h); changes in toxicity of the control (unstressed cell) and test organism (stressed) was measured using the porcine IL-6 method. There was an overall decrease in toxicity in the presence or absence of the sanitizers for *E. coli* 0113, 026 and *Z. bailii*, although the change in toxicity over time was less pronounced for *E. coli* 026 than that of *E. coli* 0113. However, the toxicity of *Z. bailii* increased when the concentration of LH was increased to 0.2%. Communal growth of *E. coli* 026 and *E. coli* 0113 produced different toxicity profiles. In both cases where *E. coli* 0113 or 026 were cultured together with *Z. bailii*, an increase in toxicity was evident over time in the presence of both sanitizers, indicating that it can only be attributed to the communal cultivation.

4.2 Introduction

In nature microorganisms do not exist alone but in association with one another. These kinds of associations can be found within and upon all agricultural products of plant and animal origin as well as in the food industry where cells of the same or different species can stick to the variety of surfaces in food processing environments (Hood and Zottola 1995, Sutherland, 2001). To reduce or eliminate microorganisms on food contact surfaces, food processors have for a number of years relied on physical (hand washing, high pressure sprays) as well as chemical methods (iodophores, liquid hypochlorites) (Hood and Zottola, 1995). However, if cleaning chemicals are not properly rinsed from food processing equipment they may end up in the product in sub-lethal concentrations.

Lipopolysaccharides are the major outer surface structures in Gram-negative bacteria essential for the survival of bacteria. They also serve as one of the primary targets of the innate arm of the mammalian immune system. LPS have been characterized as stimuli for host activation through myeloid cells (neutrophils, monocytes, macrophages) and non-myeloid cells (fibroblasts, platelets) (Dixon and Darveau, 2005). The lipid A is the endotoxic component of LPS and responsible for the pathophysiology of numerous diseases such as fever, headache, nausea, diarrhea tachycardia, tachpnoe, hypotension, organ failure and death (Schromm 2000; Freudenberg *et al.*, 2001). All these reactions are induced by protein molecules known as cytokines (e.g. tumor necrosis-alpha, IL-1 beta, IL-6, IL-8) which are produced after the interaction of the LPS with the humoral and cellular targets (Freudenberg *et al*, 2001). These signals are further transmitted to the brain where thermoregulatory mechanisms are triggered to increase body temperature (Nakagawa *et al.*, 2002). Cytokines that are mostly produced in all tissues and most cells range from 8-30 kDa (Hopkins, 2003). Accumulated evidence shows that

IL-6 may be an important factor in the development of lethal shock caused by combination of tumor necrosis factor and IL-1 induced by bacterial LPS. Therefore, deregulation and abnormal function of IL-6 in some disorders may be typical of this cytokine directly related to pathogenesis (Wang *et al.*, 1991). IL-6 is a multifocal cytokine proven to regulate T and B cell functions and induces maturation of megakaryocytes and acute- phase response proteins (Ryfel *et al.*, 1992).

Studies using natural and synthetic analogues of lipid A have demonstrated a direct relationship between lipid A structure and IL-6 induction. The phosphate groups and the length and number of fatty acyl chains of lipid A play an important role in bioactivity (Wang and Quinn, 2009). The *E. coli* lipid A, containing two phosphate groups and six acyl chains composed of 12 or 14 carbons, is known to be a powerful activator of the innate system (Wang and Quinn, 2009). Removal of a particular group of lipid A leads to a dramatic decrease of endotoxic activity. For example, the cleavage of acid-labile 1-phosphate 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' can lead to a reduction of by more than three orders of magnitude (Gutsmann *et* 2007).

The aim of the investigation was, therefore to determine the influence of typical Liquid Iodide and Liquid Hypochlorite sanitizers on the changes in toxicity of *E. coli* 0113 and 026 strains cultivated in the presence of *Z. bailii* Y-1535 as measured by IL-6 porcine method.

4.3 Materials and Methods

4.3.1 Preparation of strains and treatment parameters

Mid-exponential phase pre-inocula of *Escherichia coli* 0113 (smooth strain), *E. coli* 026 (rough strain) and the yeast strain *Zygosaccharomyces bailii* Y-1535 were inoculated into 200 ml yeast malt media (YM) and incubated at 30°C with shaking (200 rpm) until late exponential phase (lOh) for *E. coli* 0113 and 026 and 12h for Z *bailii* Y-1535). Refer to chapter 2; sections 2.3.1- 2.3.4.

Each strain was cultivated in pure culture and in combination *(E. coli* + Z *bailii).* Growth was monitored by optical density (OD_{690nm}) measurement and viable plate counts on VRB + MUG and YMT agar. The influence of selected sanitizers was evaluated by growing the strains under the same conditions as described for the controls, but with added sanitizers at sub-lethal concentrations of 0.2% or 0.05% (LH) and 0.075%. (LI). Cultures were sampled (1 ml) at time intervals Oh and lOh *(E. coli* 0113 and 026) as well as Oh and 12h (Z. *bailii* Y 1535) and stored at -80°C until toxicity analyses were performed.

4.3.2 Interleukin- 6 (IL-6) porcine assay

Pig blood was randomly collected (after every 5 pigs) from 10 pigs at an abattoir into 500 ml pyrogen free glass containers. Blood was pooled immediately from the 500 ml beakers into heparinized vacutainer® tubes using a gauge needle. To avoid stimulation of cytokine expression during transportation, the pooled samples were kept on ice until processed in the laboratory. The blood was diluted 1:3 with sterile saline and 100 pi of diluted blood was added to 100 pi of undiluted samples in 1.5 ml tubes. This mixture was incubated overnight at 37°C in a CO_2

incubator, to allow blood to separate by itself; thereafter the supernatant was used for analysis as described below.

LPS toxicity on all diluted samples from time zero and the late exponential phase of growth (10h-12h) were determined using Interleukin-6 (IL-6), which is another method for the detection of pyrogens. To prevent any possible interference on the measurement at 540 nm, the blank for the diluted media was done alone in the assay. For the purpose of this study, the porcine IL-6 method was used, since the collection of pig blood does not require ethical clearance. The test was performed by the microplate method as prescribed by the manufacturer. The kit contained the basic components required for the development of sandwich ELISA to measure natural and recombinant porcine interleukin 6 (IL-6). Porcine IL-6 standards ranging 2 500 pg.mf^{1} to 39 pg.mf^{1} were prepared and standard curve constructed by plotting OD_{540nm} against the pg ml"^{1} per absorbance unit.

A polyclonal antibody specific for porcine IL-6 was pre-coated onto a microplate. Standards, controls and samples were added to the wells and any porcine IL-6 present was bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for porcine IL-6 was added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells. The enzyme reaction yielded a blue product that turned yellow when the stop solution was added. The intensity of the color was measured at 540 nm with a microplate reader (Bio-Rad 680); the colour was in proportional to the amount of porcine IL-6 bound in the initial step. Toxicity values were calculated from a standard curve ($R^2 = 0.989$). The change in toxicity was expressed per OD_{690nm} and calculated using equation 3 describe in Chapter 3 section 3.3.2. All equipment, reagents and consumables used were pyrogen free.

4.4 Results and Discussion

When *E. coli* 0113 was grown without sanitizers (control) the change in toxicity showed a pronounced decrease, with the same trend evident in the presence of both sanitizers (Fig. 4.1 A). Control *E. coli* 026 resulted in a decrease in toxicity over time, although the change was less pronounced than that of 0113. Exposure of 026 to 0.05% LH still resulted in a decrease in toxicity, while growth in the presence of 0.075% LI resulted in almost no change in toxicity over exposure time (Fig. 4.IB). Where Z *bailii* was grown in pure culture a decrease in toxicity over time was observed and a smaller change in toxicity (but still a decrease) when grown in the presence of 0.075% LI and 0.05% LH. However, when the concentration of LH was increased to 0.2%, the toxicity also increased over time. This is the first report of toxicity detected using this protocol on yeast EPS.

Interestingly the communal growth of the *E. coli* strains and Z produced different toxicity profiles when compared to the results obtained when using the LAL assay to measure toxicity. In both cases, where *E. coli* 0113 or 026 were cultured in the presence of Z. the toxicity of the mixture showed an increase over time. This occurrence was evident in the presence of both sanitizers, indicating that it may be attributed to the communal cultivation. The largest increase in toxicity was brought about by the communal growth of 026 and Z *bailii* in the presence of sub-lethal concentration of sanitizer LI. It is not unreasonable to consider subtracting the toxicity values obtained for Z. *bailii* from the communal growth value to reflect a more accurate toxicity value. This will, however, not contribute to an explanation for the increase in toxicity values in the case of communal growth. Communal growth might have influenced the liberation of 3- hydroxy fatty acids resulting in a significant change in saccharide composition of EPS (Cadieux, 2007; Abraham *et al.*,2009).

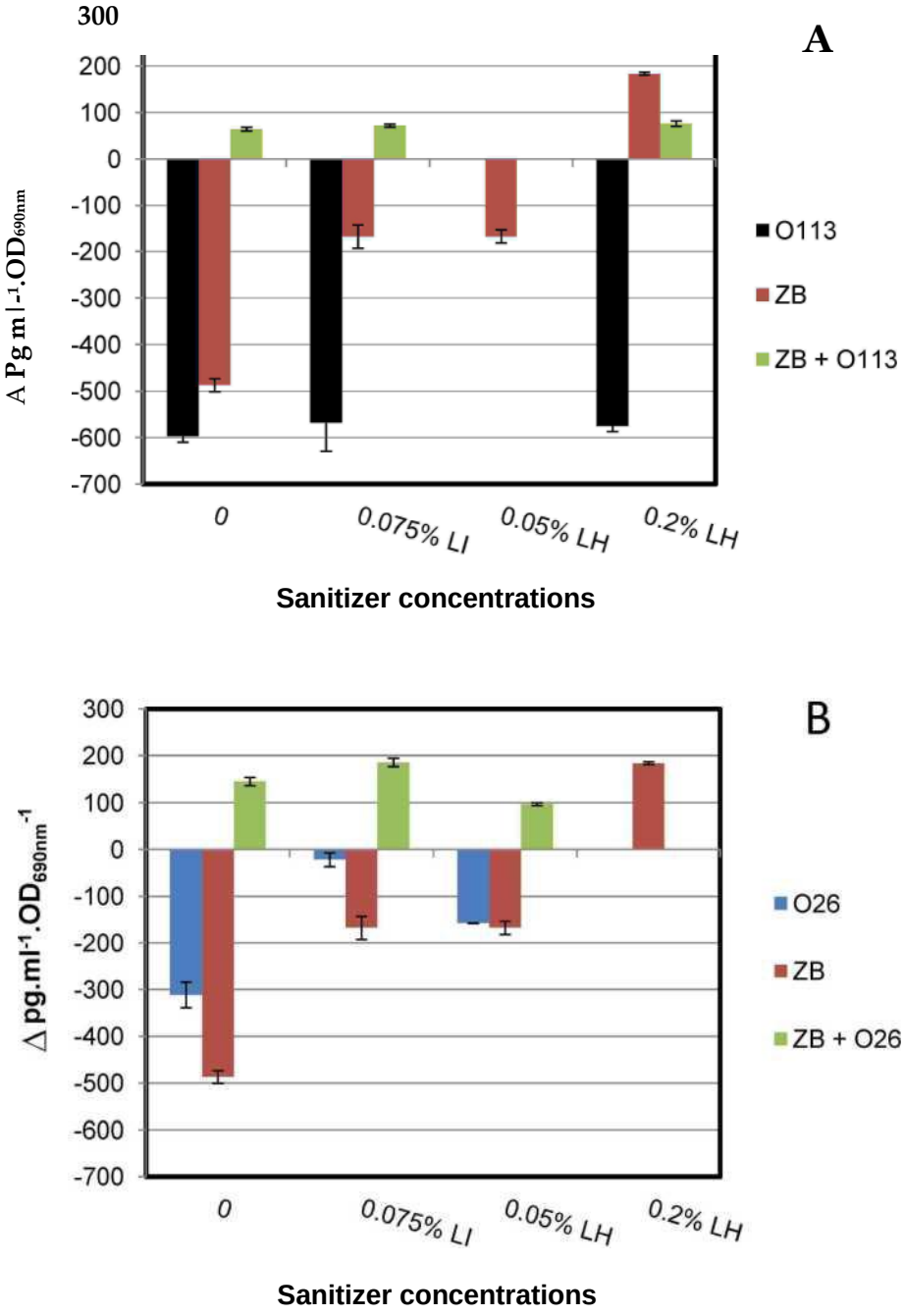

Fig.4.1. Changes in lipopolysaccharide toxicity of (A) *E.* 0113, *bailii* Y-1535 and combinations (ZB+ Oil3); (B) *E. coli* 026, *Z. bailii* and Combinations (ZB + 026) following exposure to different sanitizers expressed as Apg.mr'ODggomn'[1]. Experiments were performed as independent duplicates and the error bars indicate the standard deviation.

Endotoxic activity can be associated with the structural features in the lipid A molecule, which could have led to decreased toxicity values in pure cultures. It has become apparent that the phosphate groups on the glucosamine residues are important, as are the number and nature of fatty acids linked to amino sugars (Lindberg *et ah*, 1990). Should there be any reduction in the phosphate group, this could lead to deactivation of the leukocytes by 100 fold compared to the phosphorylated form (Lindberg *et ah*, 1990). There is also a correlation between lipid A particular aggregate structure and endotoxicity. Lipid A with a lamellar structure may result in lower toxicity as observed in pressurized cells (Brandenburg *et* 2003). In addition, the presence of LPS with a pentaacyl or tetraacyl conformation can lead to reduced toxicity.

Variations in IL-6 expression as seen from the results are a function of the physiological behaviour of the cell, and structural changes in response to subsequent exposure to sub-lethal concentrations of different sanitizers. For example, *E. coll 026* (rough strain) had a change in toxicity over time less than *E. coli* 0113 (smooth), which could be attributed to its LPS being more permeable to the sanitizers which would result in more pronounced structural changes.

4.5 Conclusions

It is evident from the results that communal growth of *E.* strains and Z *bailii* had a marked increase in IL-6 expression under sub-lethal concentrations of liquid iodophore and liquid hypochlorite. This occurrence was observed in the presence of both sanitizers indicating that it may be attributed to communal growth. The change in toxicity over time was influenced by different factors such as the type of organisms, sanitizer concentration and cultural conditions. In the communal growth the largest increase over time was brought about in *E.* 026 and Z *bailii* in the presence of LI. Thus, in the food industries communal growth of a yeast such as Z *bailii* with *E. coli* can be encouraged by the above factors, which would lead to decreased toxicity.

It is known that high doses of LPS can cause toxic effects in the immune system. According to Brandenburg *et al.* (2003) low concentration mediators that are produced in response to LPS are beneficial for the proper functioning of the immune system and its fight against invading microbes. Test systems with human blood whole cells can also be used to test different pyrogens. However, there are many difficulties related to their preparation, and also individual variances of different donors (Nakagawa *et al.*, 2002).

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Chapter 5

Evaluation of an *in vitro* pyrogen assay based on the expression of IL-6 from porcine whole blood in comparison with the *limulus* amoebocyte assay

5.1 Abstract

Lipopolysaccharides are the major outer surface structures in Gram-negative bacteria. They also serve as one of the primary targets of the innate arm of the mammalian immune system. LPS toxicity on all samples from time zero and late exponential phase of growth (1 Oh-12h) were determined using chromogenic LAL, which is a quantitative test for Gram-negative bacterial endotoxin (LONZA, QCL-1000, *limulus* Amoebocyte Lysate) and interleukin-6 (IL-6), which is another method for the detection of pyrogens.

The two methods produced similar trends of toxicity changes only in the case of *E.* 0113 grown in pure culture unexposed and subjected to sub-lethal concentrations of LI and LH. No correlation could be established between the IL-6 or LAL measurements for *E.* 026 or Z. *bailii* for any of the treatment parameters.

5.2 Introduction

LPS elicits a variety of inflammatory responses in the host by activating the immune system, especially through monocytes and macrophages, with the release of a range of pro-inflammatory mediators (Rietschell *et al.*, 1994; Magalhaes *et* 2007). In Chapter 3 and 4 two methods of endotoxin determination were investigated; the *limulus* amoebocyte lysate (LAL) and Interleukin-6 assay (IL-6) methods.

The LAL assay, commonly used for endotoxin determination is based on a gelation reaction of the lysate (Binding *et al.*,2004). LAL is an *in vitro* assay that is most sensitive to endotoxin testing of parenteral pharmaceuticals and medical devices that come in contact with blood or cerebrospinal fluid. It can detect 3 pg ml"[1] that is 0.03 EU ml'[1] of LPS and it reacts with the biologically active component lipid A. However, there are some major drawbacks to this method (Novitsky, 1998; Daneshian *et al.*, 2008; Liebers *et al.*, 2009). The LAL cascade is triggered by (l,3)-|3-D-glucan originating from plants or fungi which are much less pyrogenic, it doesn't detect other pyrogenic molecules, and numerous substances can interfere with the assay(Binding *et al.*, 2004). Examples of these are the presence of inhibitory proteins or serum as well as the possibility that some substances in plasma might mimic endotoxin in the LAL test (DuBose *et al.*, 1980; Laitinen, *et al.*, 2001).

Currently there are a number of procedures designed to prepare plasma samples for LAL testing, In view of the large variety of products, it is not possible to develop one general method for endotoxin removal from all products (DuBose *et al.*, 1980; Magalhaes *et al.*, 2007). As in other biological assays, the activation of the LAL assay also differs depending on the physicochemical structure of the endotoxin and may differ for free and cell bound endotoxins (Laitinen *et al.*,

2001; Lee *et al.*, 2005;). Free endotoxin is significantly more bioactive than cell bound endotoxin (Xuan *et al*, 1997).

The LAL assay is generally based on the endotoxin-initiated reaction catalyzed by pro-enzyme in the LAL. Where the initial rate of activation is determined by the concentration of endotoxin present (Hurley, 1995). The activated enzyme catalyzes the splitting of pNA from the colorless substrate Ac-lle-Glu-Ala-pNA, and the pNA released is measured photometrically at 405 - 410 nm after the reaction is stopped. The concentration of endotoxin in a sample is then calculated from the absorbance values of solutions containing known amounts of endotoxin standard. The correlation between the absorbance and the endotoxin concentration is linear in the 0.1-1.0 EU.mr^{1} range.

Cytokines such as IL-6 are the regulators of the local tissue immune system and are produced by a variety of cells such as monocytes, macrophages and lymphocytes (Matalka 2005). Excessive production of pro-inflammatory cytokines such as (TNF)-a, interleukin (IL)-ip, IL-6 and IL-8 can lead to the development of acute respiratory distress syndrome (ARDS), or multiple organ dysfunction syndrome (MODS) and septic shock (Oda *et* 2005).

IL-6 compared to other cytokines has a blood level that remains consistently elevated in the presence of various diseases (Oda *et al.*, 2005). The blood IL-6 level is usually measured by applying RIA (radioimmunoassay) or ELISA (enzyme-linked immunosorbent assay) (Matalka *el al.*, 2005; Oda *et al.*, 2005). The Quantikine porcine IL-6 is a solid phase ELISA designed to measure porcine IL-6 in cell culture supernatants, serum and plasma. It contains *E. coli*- expressed recombinant porcine IL-6 plus antibodies raised against the recombinant factor. The immunoassay has been shown to be able to quantitate recombinant porcine IL-6 and can be used to determine relative quantities for natural porcine IL-6 (R&D Systems).

The aim of this study was to compare the expression of IL-6 from porcine whole blood with the *limulus* amoebocyte method for the detection of pyrogens.

5.3 Materials and Methods

5.3.1 *Limulus* amoebocyte lysate assay

LPS toxicity on all samples from time zero and late exponential phase of growth (1 Oh-12h) were determined using chromogenic LAL, which is a quantitative test for Gram-negative bacteria endotoxin (LONZA, QCL-1000, *limulus* amoebocyte lysate). Refer to Material and Methods in Chapter 3 (Section 3.3.2) for description on the methodology of the LAL assay.

5.3.2 Interleukin 6 (IL-6) assay

Toxicity on all samples from time zero and the late exponential phase of growth (1 Oh-12h) was determined with interleukin-6 (IL-6), another method for the detection of pyrogens. Refer to Material and Methods in Chapter 4 (Section 4.3.2.) for description on the methodology of the IL- 6 assay.

5.4 Results and Discussion

Table 5.1 shows the picogram amounts of IL-6 in the test samples compared with the corresponding LAL values (endotoxin units). No correlation was observed between the IL-6

IL-6 compared to LAL assay may be influenced by several parameters which have to be standardized. For example, for the whole procedure to be completed starting from preparations of the reagents, standards, controls and samples to the washing and incubation steps it takes approximately lh-3h in the LAL assay. The IL-6 will take 4h-5h due to the collection and processing of blood (by dilution) as well as a 24h incubation process prior to the analysis (LPS challenge of blood), rendering the IL-6 time consuming and laborious. Mohamed (2007) showed that cytokine release is time dependent; suggesting that appropriate collection, storage and handling of blood are essential for the outcome of the test. Furthermore, a cytokine response depends on the environmental and genetic background of the blood, thus showing high interspecies variations in toxicity values (Singh and Schwartz, 2005).

Differences between the LAL method and IL-6 may also depend on the samples used for example communal growth samples, showed low values of LAL assay in spite of high toxicity values in IL-6 because the porcine IL-6 is able to detect other pyrogens (e.g. EPS in yeast). The LAL assay, on the contrary, is highly sensitive to endotoxin activity (Liebers 2009). An increase in toxicity was detected in the *Z. bailii* strain with the LAL method in the presence of all the sanitizers, while with the IL-6 assay an increase in toxicity was detected only with 0.2% LH. This showed that the addition of a high concentration of sanitizer (LFI) lead to the inhibition of cytokine production, indicating that structural adaptation of the cell membrane in response to sanitizers may influence the ability of LPS to stimulate macrophages and monocytes. Gutsman *et al.* (2009) showed that addition of certain antimicrobial agents caused almost complete inhibition of cytokine production, whereas the reduction in the *limulus* assay is much lower.

Substances present in the sample are capable of inhibiting the LAL enzyme. This inhibition can cause an underestimation of the amount of endotoxin present (Dawson, 2005). However, dilution

of the sample can help overcome inhibition, but the degree of dilution necessary to achieve this may vary between strains. This also applies to the blood samples which contain substances that may interfere with the IL-6 assay.

Immunoassay precision is defined as the reproducibility of results within and between assays. This is done to assure that the results obtained throughout the assay are accurate and reproducible. Precision is measured as a ratio of standard deviation to the mean (CV). Two types of precision should be considered, intra-assay and inter-assay precision. For the porcine IL-6 it is indicated within the parameters of intra-assay and inter-assay that the CV was 10%. Within the parameters of this study, the average CV observed for all standards assayed in the eight microtitre well was 10.7 (concentrations of the standard dilutions from which the calibration curve were drawn were as follows: 2 500, 1 250, 625, 312.5, 156, 78.1 and 39.1 picogram). Within the parameters of this study, average CV for the LAL assay observed for all standards assayed in the eight microtitre plates was 14% (concentrations of the standard dilutions from which calibration was drawn were as follows: 1.0, 0.5, 0.25 and 0.1). This was much higher than optimum CV for the LAL which should be less than 10%.

Table 5.1. Toxicity values for porcine IL-6 and LAL obtained from selected treatment parameters

Growth media and sanitizers	Organism	IL-6 (pg mr'.ODisonm"[1])	LAL (AEU.ml[I].OD$_{690nm}$[1])
Yeast malt broth		-597.46	-101.14
Yeast malt broth + LI	*E. coli* 0113	-568,12	-86.27
Yeast malt broth + LH[a]		-575.76	-96.87
Y east malt broth		-311.45	-73.89
Yeast malt broth + LI	*E. coli* 026	-21.83	-61.41
Yeast malt broth + LH[b]		-157.23	-71.69
Yeast malt broth		-487.03	5.19
Y east malt broth + LI	Z *bailii* Y-1535	-167.58	6.17
Yeast malt broth + LH[a]		184.10	6.77
Yeast malt broth + LH[b]		-167.20	5.50
Yeast malt broth		64.38	-45.77
Yeast malt broth + LI	*E. coli* Ol 13 + Z *bailii*	72.24	-29.97
Yeast malt broth + LH[a]		76.65	-43.82
Yeast malt broth		145.60	-34.49
Yeast malt broth + LI	*E. coli* 026 + Z *bailii*	191.854	-27.91
Yeast malt broth + LH[b]		97.05.854	-33.59

a: 0.2% of LH added to the growth medium b:

0.05% of LH added to the growth medium

5.4.1 Additional materials and equipment required

In addition to the LAL and IL-6 assay kits, additional materials and equipment were needed for analysis. Before the IL-6 assay was performed pyrogen free containers, needles and heparin tubes were prepared for collection of blood from swine (See Table 5.2) for a summary of additional material and equipment used in IL-6 and LAL assays for LPS challenge). Blood must be collected by a skilled animal phlebotomist in order to avoid contaminants such as dust that could interfere with the assay. The animal phlebotomist must be well trained in blood collection and correct storage of the specimen (such as mixing the specimen prior to preserving it in ice for transportation, wearing gloves, etc.) to avoid stimulation of cytokine expression. There must be coordination between the laboratory personnel and animal phlebotomist to ensure that when the pigs are available, everything needed for sampling has been prepared beforehand. Porcine blood needs to be processed within 2 hours after sampling to avoid cytokine expression.

Table 5.2 Additional materials and equipment needed for the LAL and IL-6 analysis

LAL	**IL-6**
Vortex mixer	Heparanized vacutainer® tubes
Acetic acid stop reagent (25% v/v)	Gauge needles
0.1N Sodium hydroxide 0.1N Hydrochloric acid	Pyrogen free glass beakers
	Gloves
	Ice and container Sterile, pyrogen free saline
	Needle disposal unit Carbon dioxide ($C0_2$)
	incubator

5.5. Conclusion

Even though the LAL assay is not the ideal method for detection of all pyrogens compared to IL- 6 assay, it remains the method of choice for parenteral drugs and food substrates. It can be concluded that within the parameter of this study there was no correlation between the porcine whole blood test and the LAL assay. There are many factors that can affect porcine IL-6 expression, considering that the use of porcine cryopreserved blood can be of benefit to minimize contamination or IL-6 expression during handling and transportation of blood. Looking at other factors that might interfere with the assay such as the composition of a sample or the species involved, further research and development of other whole blood assays with standardized operation procedures is desirable.

5.6 References

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Chapter 6

Concluding Remarks

6.1. Background to the study

The food processing industry implements preventative measures such as sanitizers to reduce or eliminate microorganisms. However, if cleaning chemicals are not properly rinsed from food processing equipment they can end up in the product in sub-lethal concentrations. In this study two pathogenic bacterial strains *Escherichia coli* 0113 and *Escherichia coli* 026 and one spoilage yeast strain *Zygosaccharomyces ba* Y-1535 were subjected to sub-lethal concentrations of Liquid Iodide (LI) and Liquid Hypochlorite (LH). Different enumeration methods were used to monitor the growth phase in order to shed light on subsequent LPS production after exposure to sub-lethal sanitizer concentrations. The influence of sub-lethal concentrations of the sanitizers on toxicity of yeast and bacterial strains was assessed by means of the LAL and a porcine IL-6 ELISA method.

6.1.1 Concluding remarks on Chapters 2, 3 and 4.

Chapter 2 highlighted different reactions of different strains may act differently to different sanitizers. Sub-lethal doses had different effects on the growth of bacteria and yeasts (sometimes none). *Z. bailii* grew better in the presence of sanitizers compared to the bacterial strains except at 0.2% LI where no growth was observed. *Escherichia coli* 0113 (smooth strain) was more resistant to LH than *E. coli* 026 (rough). Differences in microbial responses to sanitizer concentrations are most likely innate. This was expected since rough strains tend to be more susceptible to disinfectants as they lack the complete O-specific chain at the cell surface, rendering them easily accessible to antimicrobials.

In communal growth sub-lethal doses of sanitizers influenced the growth causing the organisms to start competing with each other for nutrients, which resulted in poor growth of the target

organism. These results indicate that cell growth in combination can be influenced by various factors, such as microbial interaction and nutrient composition at a specific time interval and nature of the strain and type of sanitizer used.

In Chapter 3, the effect of sub-lethal concentrations of LI and LH sanitizers on changes in LPS toxicity of *E. coli* 0113 and *E. coli* 026 strains cultivated in the presence of *Zygosaccharomyces bailii* Y-1535 were measured by chromogenic LAL assay. Growth of *E. coli* 026 and *E. coli* 0113 in both sanitizers resulted in a decrease in toxicity and although the toxicity values differed, the trend remained similar. Z. *bailii* showed a decrease in toxicity over time with no significant change in toxicity when grown in the presence of the sanitizers.

Communal growth of the *E. coli* strains and Z. *bailii* resulted in similar toxicity profiles. In both cases, where either *E. coli* 0113 or *E.coli* 026 was cultured together with Z. *bailii* the toxicity of the mixture showed a decrease over time. The decrease in toxicity was similar in both communal growth of *E. coli* 026 and Z *bailii* in the presence of sub-lethal concentrations of sanitizer LI and LH. This occurrence was evident in the presence of both sanitizers, indicating that it may be attributed to the communal cultivation.

Chapter 4 illustrated the influence LI and LH on the changes in toxicity of *E. coli* 0113 and *E. coli* 026 strains cultivated in the presence of Z. *bailii* Y-1535 as measured by the IL-6 porcine method. Growth of *E. coli* 026 and *E. coli* 0113 resulted in a decrease in toxicity although the change over time in *E. coli* 026 was less pronounced than that in *E. coli* 0113. *E. coli* 026 grown in the presence of LI resulted in little change in toxicity over exposure time. Z. grown in pure culture resulted in a decrease in toxicity over time in the presence of 0.075% LI and 0.05% LH. However, when the concentration of LH was

increased. *E. coli* 026 and Z *bailii* grown in the presence of sub-lethal concentration of sanitizer LI brought the largest increase in toxicity.

Communal growth of the two *E. coli* strains and Z. resulted in an increase in toxicity. In both cases, where *E. coli* 0113 or *E. coli* 026 was cultured together with Z. *bailii* the toxicity of the mixture showed an increase over time. Results indicate that communal growth of *E. coli* and Z. *bailii* could influence the liberation IL-6 from porcine.

6.1.2 Recommendations and future prospects

In light of these results care should be taken in the food industry where contamination with sanitizers is a risk factor. Incorrect dosage is important as it could improve growth of spoiler/pathogenic yeasts. To eliminate such contaminants, food processing equipments need to be thoroughly rinsed.

Extended exposure to chemicals caused the organisms in communal growth to start competing with each other for nutrients, resulting in poor growth of the target organism. Although this may be useful in controlling pathogens, it may inhibit growth of beneficial yeasts such as those found in yoghurt. Different factors could contribute to the differences in response of *E. coli* and Z. *bailii* in the presence of sanitizers, and also resulted in differences in growth and toxicity profiles. It may be useful to document the conditions in the food environment that will contribute to this response, in an attempt to control these organisms most of the research done thus far, has been on organisms in pure culture. In the food processing environment the mixture of microorganisms is commonly found and the variety of combinations is different to pure strains.

It is furthermore doubtful whether the IL-6 or LAL assays alone will be accurate methods to measure toxicity of communal growth as using the LAL assay can underestimate the toxicity of the mixed culture.

Future research should focus on examining the structural changes possibly occurring in the LPS/EPS under sanitizer stressed and communally grown yeast and bacteria using a sensitive method such as GC-MS analysis.