UNIVERSITY OF BIRMINGHAM

Physiology of *Escherichia coli* in Orange Juice: Applications of Flow Cytometry

By

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Abstract

Flow cytometry (FCM) was utilized for monitoring the physiology of *E. coli* cells in orange juice (OJ) as well as a model orange juice (MOJ). Compared to FCM, plate counts highly underestimated the true number of viable cells in OJ. As a part of this study, the effects of the change in major components of OJ on viability of the cells in OJ and MOJ was investigated using FCM. Increase in ascorbic acid and amino acid concentrations of MOJ improved both the culturability and FCM viability of the cells. FCM was also employed for studying the effects of OJ clarification on viability of *E. coli* in OJ. Although, reduction in cloud content of OJ increased the number of healthy cells, however, the removal of cloud particles of larger than 0.7 μm appeared to increase the antimicrobial efficacy of particles of smaller than 0.7 μm. The effects of washing *E. coli* cells with available chlorine, H_2O_2 and organic acids on their subsequent viability in OJ was also investigated. While increase in concentration of sanitizers resulted in a significant reduction in healthy populations, the total number of viable cells either remained constant or increased particularly in case of H_2O_2-washed cells.

Dedication

I dedicate this thesis to

My Daddy, the kindest person in the whole universe and a true academic who inspired me from a very young age to become a scientist

My Mummy, the greatest mother in the world who always trusted in my abilities and encouraged me to follow my dreams

The one and only true love of my life, my wonderful wife Elnaz. Without her support, patience and understanding this thesis would have never existed.

And finally

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Abbreviations

2×LB	2 time concentrated Lennox broth
AR	Acid resistance
BOX	Bi-oxonol
CFU	Colony-forming units
EMBA	Eosin methylene blue agar
FCM	Flow cytometry
FI	Fluorescence intensity
FL1-A	Green fluorescence intensity
FL3-A	Red fluorescence intensity
FOJ	Filtered orange juice
FSC/FSC_A	Forward scatter
GFP	Green fluorescent protein
MAC	MacConkey agar
MOJ	Model orange juice
MRD	Maximum recovery diluent
OJ	Orange juice
PBS	Phosphate-buffered saline
PI	Propidium iodide
SSC/SSC-A	Side scatter
STEC	Shiga-toxin-producing *E. coli*
TSA	Tryptone soy agar
VBNC	Viable but non-culturable
VTEC	Verotoxin-producing *E. coli*

CHAPTER 1

INTRODUCTION

1.1 INTRODUCTION

Nowadays, there is a growing scientific consensus that consumption of fruits and vegetables plays an important role in improving the human health and wellbeing as well as reducing the risk of developing a wide range of maladies (Prynne *et al.*, 2006; Kris-Etherton *et al.*, 2002; Ames, 2001). Moreover, among the general public, the awareness of the importance of healthy diet and the role that fruits and vegetables play in achieving it, is greater than before (Ragaert *et al.*, 2004). Meanwhile, there has also been an extensive negative publicity on the subject of over-processed foods and those containing artificial food additives and preservatives (Kushi *et al.*, 2006). As a result, the demand for minimally-processed fresh-like healthy foods especially mild-treated/untreated fruit and vegetable products is on the rise (Ragaert *et al.*, 2004). One of the most common type of foods, which in recent years has been extensively processed using mild treatment techniques, is fruit juices (Jia *et al.*, 1999; Leizerson & Shimoni, 2005; Sampedro *et al.*, 2009; Patil *et al.*, 2010; Timmermans *et al.*, 2011). However, concurrent to this trend, there has been an increase in the number of food-borne diseases due to consumption of unpasteurized fruit juices. [Table 1.1] shows a summary of the reported cases of food-borne outbreaks resulting from consumption of acidic fruit juices.

Table 1.1: List of reported non-viral food-borne outbreaks with known causative agents resulting from consumption of acidic fruit juice (1922-2013)

Juice	Agent	Country	Year	Cases	Deaths
Apple	*E. coli* O157 (Suspected)	Canada	1980	15	2
	E. coli O157:H7	Canada/USA	1996	70	1
	E. coli O157:H7	USA	1991	23	
	E. coli O157:H7	USA	1996	14	
	E. coli O157:H7	USA	1996	6	
	E. coli O157:H7	USA	1996	1	
	E. coli O157:H7	USA	1997	6	
	E. coli O157:H7	USA	1998	14	
	E. coli O157:H7	USA	1999	25	
	E. coli O157:H7	USA	2005	4	
	E. coli O157:H7	USA	2007	9	
	E. coli O157:H7	USA	2008	5	
	E. coli O157:H7	USA	2010	7	
	E. coli O111 & *C. parvum*	USA	2004	212	
	Salmonella Typhi	France	1922	23	
	S. Typhimurium	USA	1974	296	
	S. Agona	USA	1999	8	
	Cryptosporidium spp.	USA	1993	213	
	C. parvum	USA	1996	31	
	C. parvum	USA	2003	144	
Orange	*Salmonella* Typhi	USA	1944	18*	1
	S. Typhimurium	USA	1989	70**	
	S. Gaminara, Hartford & Rubislaw	USA	1995	62	
	S. Anatum	USA	1999	10	
	S. Enteritidis	USA	2000	88	
	S. Typhimurium & *S.* St. Paul	USA	2005	152	
	S. Muenchen	USA/Canada	1999	423	1
	S. Typhimurium PT 135A	Australia	1999	405	
	S. Panama	Netherland	2008	33	
	Bacillus cereus	France	2003	43**	
	B. cereus	USA	2004	85*	
	E. coli (Enterotoxigenic)	India	1992	6	
	Shigella flexneri	South Africa	1995	14	
Tomato	*C. botulinum*	USA	1935	2***	
	C. botulinum	USA	1965	1***	
	C. botulinum	USA	1969	1***	
	C. botulinum	USA	1974	1***	

Notes:
All unpasteurized except: * unspecified, ** reconstituted, *** canned.
Sources: Danyluk *et al.*, (2012) and
CDC (2013) (http://wwwn.cdc.gov/foodborneoutbreaks/Default.aspx)

Three major points can be inferred from these data. Firstly, the majority of outbreaks have occurred due to consumption of "unpasteurized" apple juice or orange juice (OJ). Secondly, the microorganisms which have been frequently implicated in these outbreaks are *Escherichia coli* (*E. coli*) and *Salmonella spp*. Both microorganisms are known to be highly resistant to acidic conditions generally encountered in these fruit juices (Foster, 2001). And thirdly, while the pertinent microorganism in apple juice-associated food-borne outbreaks has been *E. coli* O157:H7, OJ-associated outbreaks have been mainly due to contamination of OJ with various serovars of *Salmonella*. To the best of the author's knowledge, with the exception of one case in 1992, in which six people became ill after drinking unpasteurized OJ (Singh *et al.*, 1995), no other case of OJ-associated outbreak of pathogenic *E. coli* has been reported.

The latter point is interesting, considering the popularity of OJ and the relative similarity in composition and acidity of these two fruit juices. In 2011, OJ accounted for more than 60% of fruit juice consumed in the UK (AIJN, 2012) [Figure 1.1]. Moreover, according to the NHS Health Survey in England, consumption of fruit juice is greatest among the younger age groups and the elderly (Ogunbadejo & Nicholson, 2010; [Figure 1.2]). It has also been shown that children and elderly persons are at greater risk of *E. coli* infection and developing Haemolytic-Uraemic Syndrome (HUS), one the most severe forms of infection by entero-haemorrhagic *E. coli* (EHEC) (Thorpe, 2004). Therefore, it was decided to study the physiology of *E. coli* in an acidic fruit juice (in this case OJ), taking into account the low dose of infection of EHEC (< 100 cells) (Kaper

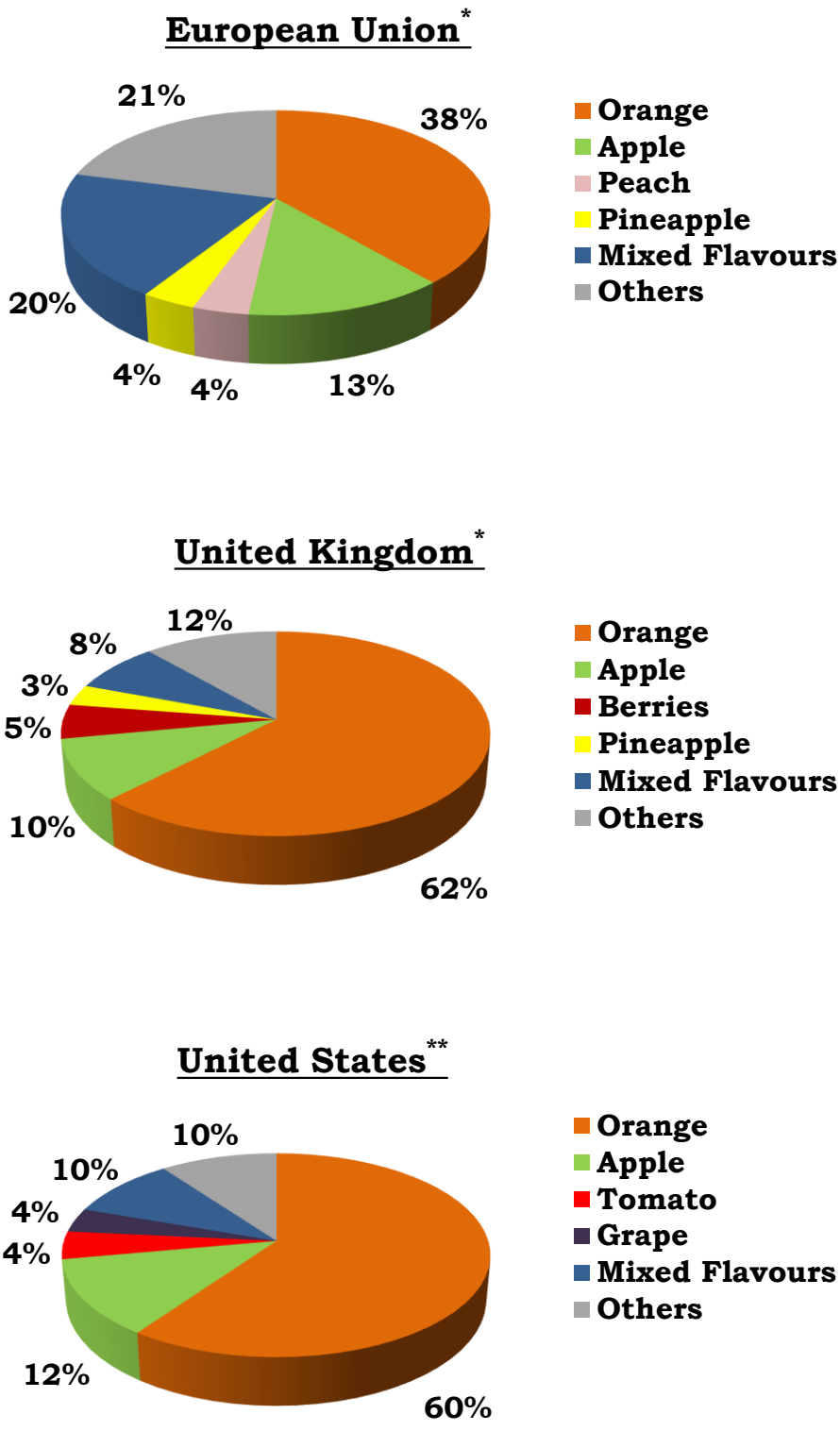


Figure 1.1: Fruit juice consumption in the EU, UK and USA (2010-2011)

Sources:
* : AIJN (2012) Data for fruit juice (100%) and nectar (25-99%) consumption in 2011
**: AAFC (2011) Data for fruit juice (100%) consumption in 2010

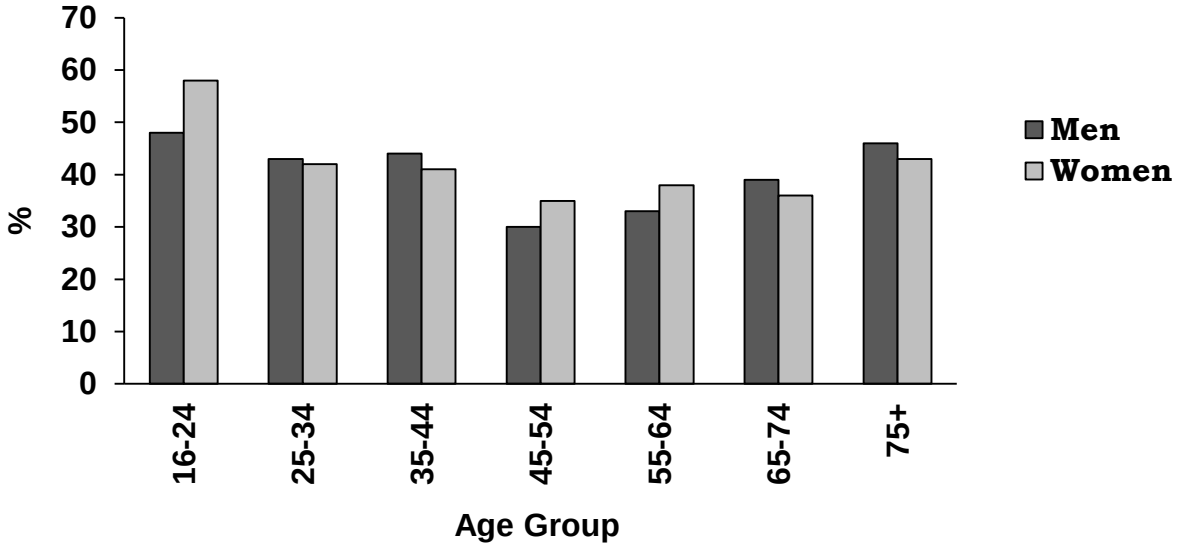


Figure 1.2: Percentage Population who Consume Fruit Juices Daily by Age and Sex in England (2009)

Source: Ogunbadejo & Nicholson (2010)

et al., 2004) and the age groups that are at a greater risk of EHEC infection.

1.1.1 General Characteristics of *E. coli*

Food-borne pathogenic enteric *E. coli* can be classified into six main categories or pathotypes: entero-pathogenic *E. coli* (EPEC), entero-toxigenic *E. coli* (ETEC), entero-invasive *E. coli* (EIEC), entero-aggregative *E. coli* (EAEC), diffusely adherent *E. coli* (DAEC) and shiga-toxin-producing *E. coli* (STEC) (Kaper *et al.,* 2004; Croxen *et al.,* 2013).

The members of STEC pathovar are capable of producing cytotoxins which are generally referred to interchangeably as verocytotoxins (VTs) and shiga-toxins (STs). As a result, this group of *E. coli* is also referred to as Verotoxin-producing *E. coli* or VTEC (Karmali *et al.,* 2010). Entero-haemorrhagic *E. coli* or pathogenic EHEC refers to a subset of STEC which in addition to expressing shiga-toxin (Stx), possess the locus of enterocyte effacement (LEE) cluster of virulence genes (such as *eae*) on the chromosomal pathogenicity island (PAI). These genes are required for attaching and effacing phenotype of EHEC strains and causing lesions on epithelial cells leading to bloody diarrhoea (Croxen *et al.,* 2013; Mathusa *et al.,* 2010). In other words, STEC pathotype could be divided into LEE-positive and LEE-negative STEC.

Not all STEC cause human illness, while all EHEC are considered to be pathogenic. The most common LEE-positive STEC serogroups associated with foodborne diseases are O26, O45, O103, O111, O121, O145 and O157. The

first outbreak of STEC *E. coli* was reported in 1982 and associated with the consumption of undercooked beef patty contaminated with *E. coli* O157:H7 (Riley *et al.,* 1983). While *E. coli* O157:H7 is the most well-known STEC associated with foodborne outbreaks, a wide geographic variability has been reported for the prevalence of O157 and non-O157 STEC infections. For instance while in the United States, Canada and the United Kingdom, *E. coli* O157:H7 is the most prevalent STEC, in other parts of the world such as South America, Australia and Germany non-STEC serotypes account for the majority of the *E. coli* outbreaks (Brooks *et al.,* 2005; Johnson *et al.,* 2006; Mathusa *et al.,* 2010). A list of the major non-O157 outbreaks in the US and worldwide has been reported by Mathusa *et al.,* (2010).

It is important to note that the plasticity of *E. coli* genome makes it difficult to designate some isolates into a certain pathotype. For instance, *E. coli* O104:H4 which was the cause of the 2011 *E. coli* outbreak in Germany is considered to be a hybrid pathogen carrying virulence genes found in both typical EAEC strains (e.g., *aggA*, *aggR*, *set1*, *pic*, and *aap*) and LEE-negative STEC (*stx2*). It is also closely linked to the *stx*-negative EAEC O104:H4 isolated from Central Africa. As a consequence, it has been suggested that this strain should be classified as a member of a new pathotype called entero-aggregative haemorrhagic *E. coli* or EAHEC (Croxen *et al.,* 2013).

From the food safety point of view, the EHEC pathovar is considered to be the most important (Doyle & Cliver, 1990). The most important virulence factors of

EHEC and its mechanisms of causing disease have been described in detail by Law (2000) and Croxen & Finlay (2010). In general, EHEC enters the small intestine and the colon after surviving the extreme acidic condition of the stomach. In the colon, the virulence genes of the bacteria in PAI are turned on, leading to the adhesion of bacteria to the gut lumen, production of shiga-toxins (Stx-1 and Stx-2) and consequent damage to the intestinal epithelium. In the case of severe damage to the epithelium and local blood vessels, STs can enter the blood circulation and be transported to kidney and central nervous system (Gyles, 2006; Law, 2000). The exact mechanism of action of STs is unknown, although it is understood that they inhibit protein synthesis in cells by cleavage of an adenine residue from 28S rRNA resulting in the death of the cell (Law, 2000). The infection can lead to severe clinical complications such as haemorrhagic colitis, HUS, kidney failure and thrombotic thrombocytopenic purpura particularly in children, elderly and immune-compromised individuals (Bell & Kyriakides, 2002).

Various detection techniques are used for detection of STEC as well as differentiation between O157 and non-O157 STEC. In summary, these methods could be divided into cultural, immunological and subtyping methods (Mathusa *et al.,* 2010). The most common growth medium for differentiation between O157 and non-O157 STEC is Sorbitol MacConkey Agar. On this medium *E. coli* O157 appears as clear colonies while non-O157 STEC form pink to mauve colonies. Commercial differential growth media reported for the isolation and confirmation of non-O157 STEC are mainly based on the specific

characteristics of these serotypes such as utilization of carbohydrates (e.g., fermentation of rhamnose by O26 serotype), sensitivity to potassium tellurite (in case of O111) and reactions with selective reagents present in various chromogenic agars. The main principle behind immunological techniques is the immunomagnetic separation of the target STEC using specific antibody-conjugated magnetic beads and the capture of these beads using a magnetic concentrator. Enzyme immunoassays and immunoblot techniques have also been reported for immunological detection of STEC. Differentiation of STEC by phage typing (using strain-specific viruses), serotyping (using O- and H-antigen specific antibodies) and subtyping (e.g., by using pulsed field gel electrophoresis) on the other hand is based on making use of the genetic diversity of STEC serotypes.

1.1.2 Factors Affecting the Survival of *E. coli* in OJ

In order to investigate the physiology of *E. coli* in OJ, it is important to focus on intrinsic and extrinsic factors which could affect its survival in OJ.

pH

Although OJ has been implicated in various food-borne illnesses, nevertheless, its high hydrogen ion concentration (pH 3.2–4.3) prevents the growth of the majority of pathogenic bacteria which are neutrophilic (require neutral pH). This phenomenon combined with the high sugar content of the fruit results in eliminating the competition by bacteria, but instead, promotes the growth of many fungi (moulds and yeasts) as well as various acidophilic bacteria (Kimball,

1999; Kalia & Gupta, 2006).

Water Activity

Water activity (a_w) is defined as the ratio of food's vapour pressure to that of pure water at any given temperature. In other words, a_w is an indicator of the free or unbound water available for microorganisms as well as physicochemical reactions (Barbosa-Cánovas & Juliano, 2007). More than 85% of OJ is made up of water and consequently has a a_w of almost 1.00 ($a_w > 0.99$) (Gabriel, 2008; Fernández-Salguero *et al.*, 1993). As a result, it could potentially support the growth of *E. coli* which requires a_w of more than 0.95 (Bell & Kyriakides, 2002).

Reduction/Oxidation (Redox) Potential (E_H)

Redox potential of food (E_h) is defined as its oxidizing or reducing capability. It depends on intrinsic factors of pH and poising capacity (the capability of food to resist pH changes due to presence of reducing or oxidizing compounds) as well as extrinsic factors of oxygen availability and the atmospheric oxygen tension (Kalia & Gupta, 2006). The presence of oxidizing agents such as oxygen and low pH can increase the E_h of the food. Conversely, exclusion of oxygen, high pH and the presence of reducing agents such as ascorbic acid or reducing sugars can result in a decrease in E_h.

Most acidic fruit juices contain a relatively high concentration of ascorbic acid and reducing sugars (i.e., glucose and fructose) and therefore should theoretically have negative E_h. Nonetheless, due to their low pH and the

presence of oxygen ($E_{0'}$ or standard redox potential of ½ O_2/H_2O = +820 mV) which is introduced during the juice extraction, their E_h is generally positive (Mossel & Ingram, 1955). Alwazeer *et al.*, (2003) reported the E_h of thawed freshly squeezed OJ to be +360 mV. However, they also showed that the growth of yeasts and lactic acid bacteria in OJ can affect its E_h by consuming the dissolved oxygen and production of reducing acids and metabolites. For instance, the growth of *Saccharomyces cerevisiae* and *Lactobacillus plantarum* (present in natural microflora of OJ) in unpasteurized OJ for 72 h at 15 °C led to approximately 200 mV and 250 mV reduction in E_h respectively. From the food safety point of view, this is important considering that this reduction could potentially improve the chance of survival of facultative anaerobic *E. coli* with optimal E_h range of -100 mV to +300 mV (FDA, 2001). Moreover, George *et al.*, (1998) noted that decreasing the oxygen content of growth medium (≤ 1% O_2) and consequently its E_h to less than +111 mV could significantly increase the thermal resistance of *E. coli* and *Salmonella* to mild heat treatment at 59 °C and 57 °C respectively. However, they were unable to ascertain whether the increased heat resistance was due to reduction in O_2 concentration or E_h.

Temperature

OJ-associated spoilage and/or pathogenic microorganisms can grow at a wide range of temperatures. The majority of OJ spoilage moulds and yeasts are psychrotrophs, capable of growing at temperatures below 7 °C with optimal growth at ambient temperatures (20–30 °C). On the other hand, the main food pathogens associated with OJ i.e., *Salmonella*, *E. coli*, and *Bacillus cereus* are

thermotrophs with optimum temperatures of 35–41 °C (Kalia & Gupta, 2006).

Modified Atmosphere Packaging (MAP) and De-aeration

Industrially manufactured OJ is generally subjected to de-aeration step in order to remove the dissolved oxygen. This is generally done in order to extend the microbial and organoleptic shelf-life of the product. As stated above, oxygen can significantly influence the microbial flora by changing the E_h of the food. For instance, Alwazeer *et al.*, 2003 demonstrated that purging OJ (E_h of +360 mV) with N_2 or N_2-H_2 reduced the E_h value to +240 mV and -180 mV respectively. In addition, dissolved oxygen can adversely affect the nutritional and organoleptic quality of fruit juice through oxidation and loss of ascorbic acid and production of browning products (Solomon *et al.*, 1995; Shinoda *et al.*, 2004). Some of these products e.g., furfural and 5-hydroxymethylfurfural (5-HMF) have been shown to inhibit the growth of various microorganisms including *E. coli*, *Klebsiella pneumonia* and *Sacch cerevisiae*. The growth inhibition of the latter is believed to be due to induction and accumulation of reactive oxygen species (ROS) leading to cellular damages (Zaldivar *et al.*, 1999; Boopathy *et al.*, 1993; Allen *et al.*, 2010).

Nutrients

OJ consists of a wide range of compounds, some of which can support the growth and survival of microorganisms. Reducing sugars such as glucose and fructose can be utilized by a wide range of microorganisms as sources of energy. OJ-associated lactic acid bacteria (*Leuconostoc mesenteroides* and *Leuconostoc*

dextranicum) are capable of metabolizing not only fructose but also sucrose, the main sugar present in OJ, resulting in production of a dextran slime (Kimball, 1991). Pectin, another carbohydrate present in OJ, can be hydrolysed by some microorganisms such as *Sacch. cerevisiae* (Sieiro *et al.*, 2012; Gainvors *et al.*, 1994). Organic acids of OJ (mainly citric and malic acids) on the other hand can be metabolised by bacteria from Lactobacillaceae family such as *Lactobacillus plantarum*, an OJ-associated lactic acid bacteria (Kimball, 1999; Perez & Saguir, 2012). OJ also contains various amino acids (e.g. proline, arginine and glutamic acid) which have been shown to protect *E. coli* against different stresses. For instance, Amezaga & Booth (1999) suggested that proline can protect *E. coli* against hyperosmotic stresses. In addition, exogenous supply of arginine and glutamic acid is known to play a major role in amino acid-dependent acid resistance of stationary-phase *E. coli* (Foster, 2004).

Antimicrobial Compounds

OJ is a rich source of essential oil compounds (e.g. limonene), volatile compounds (e.g. ethanol and acetaldehyde) and flavonoids (mainly hesperidin). Many of these compounds have been shown to exhibit antimicrobial effects towards a wide range of OJ-associated microorganisms (Yi *et al.*, 2008; Nannapaneni *et al.*, 2008). OJ also contains relatively high concentration of ascorbic acid (around 500 $mg.L^{-1}$ or 0.05%). Several studies have investigated its role on microbial survival. For instance, Tajkarimi & Ibrahim (2011) and Burnham *et al.*, (2001), reported a significant decrease in survival of *E. coli* O157:H7 in carrot juice upon addition of 0.2% ascorbic acid or soaking apple

slices in a 3.4% ascorbic acid solution respectively. However, the concentrations used in these studies were significantly greater than those naturally found in OJ. On the other hand, Nualkaekul and Charalampopoulos (2011) showed that increasing the concentration of ascorbic acid from 100 to 1,000 $mg.L^{-1}$ in a fruit juice model solution did not affect the survival of *Lactobacillus plantarum*. Possible bacteriostatic and bactericidal effects of ascorbic acid have been attributed to three main mechanisms of increasing the acidity, reducing the E_h and formation of hydrogen peroxide during its auto-oxidation (Eddy & Ingram, 1953).

Organic Acids

The microbiological quality of the OJ depends not only on the pH, but also its acidity. Organic acids play an important role in regulating the pH and acidity level of fruit juices and are considered natural antimicrobial compounds that could kill or inhibit the growth of foodborne pathogens in fruit juices (Uljas & Ingham, 1998; Vojdani *et al.,* 2008). It has even been suggested that one of the possible reasons for the rise in juice-associated outbreaks within the past couple of decades could be due to concurrent natural decline in acidity of the fruits such as oranges and grapefruits (Vojdani *et al.,* 2008).

1.2 OJ Composition and Production

1.2.1 Definition of Orange Fruit and OJ

Orange fruit belongs to the Genus *Citrus* which besides orange fruit includes various important fruit species such as lemon, lime, citron and grapefruit.

Orange is a broad term used for describing three species of *Citrus*: *C. aurantium* (sour/bitter/Seville orange), *C. reticulata* (mandarin orange) and *C. sinensis* (sweet orange). The latter is by far the most important species and is widely used for production of OJ (Salunkhe & Desai, 1984; Sandhu & Minhas, 2007). Sweet orange species itself is divided into four main sub-groups: common white-fleshed, navel, blood and acidless [Table 1.2]. The former is the most important group of orange fruits with regard to the production of OJ and compromises many important cultivars including Valencia which is the most widely used variety in the production of OJ.

According to the Fruit Juices and Fruit Nectars Regulations 2013, OJ is defined as the "fermentable but unfermented" juice of the endocarp of "sound and ripe fresh or preserved-with-chilling" *Citrus sinensis* (L.) Osbeck with no added preservative. The location of endocarp and other sections of orange fruit have been shown in [Figure 1.3]. In this body of work, unless indicated otherwise, OJ refers to the extract of common sweet orange.

1.2.2 Chemical Composition of OJ

OJ is a very complex food consisting of a wide range of nutritional and non-nutritional compounds. The composition can vary depending on the variety of orange, origin, growing season, ripeness, pre-harvest treatment conditions, storage and processing conditions (Robards & Antolovich, 1995; Lozano, 2006). However, they can be classified into major groups such as carbohydrates (mainly sugars), organic acids, proteins and free amino acids, minerals, lipids

Table 1.2: The main cultivars of sweet orange (*C. sinensis*)

Sub-Group	Main Cultivars	Characteristics
Common (White-fleshed)	• Late Season -Valencia (Worldwide) -Pera (Mainly in Brazil) • Mid-season -Pineapple • Early season -Hamlin -Parson Brown -Jaffa -Shamouti	• Valencia is the leading cultivar grown worldwide for juice production, and has excellent juice yield and quality (e.g. deeper colour). It has slightly sour and bland taste at the start and end of its season respectively. • The juice from early and mid-season cultivars generally has lower quality (poor colour, lower soluble solids and bitterness).
Navel	-Washington -Frost Washington -Dream	• Navel oranges are early season orange fruits with outstanding flavour, however, they yield less juice. They are more suitable for fresh fruit market. • They are generally bitter due to high limonene content. • Washington is the most popular cultivar for fresh market, however the juice develop delayed bitterness.
Blood (Pigmented)	-Doblefina -Eutrifina -Moro -Tarocco -Ruby Blood -Maltese Blood -Sanguinello Commun -Ruby Blood	• Mainly grown in Mediterranean region for fresh fruit market. • Pigmented (due to production of anthocyanins) when grown in cool Mediterranean region and weakly pigmented in warmer climate e.g. in Florida. • Can develop post-extraction bitter taste. • Removal of muddy colour can be achieved with activated charcoal however it results in loss of ascorbic acid. • Compared to sweet oranges, blood oranges have a much higher acidity (1.09 $g.L^{-1}$ and 10.17 $g.L^{-1}$ respectively).
Acidless	-Dolce (also known as Douce or Sucrena)	• Not suitable for OJ production due to low acid and high sugar content which can promote the growth of bacteria.

Adapted from: Kimball *et al.*, (2004); Sandhu & Minhas (2007); Rutledge (1996); Moufida & Marzouk (2003)

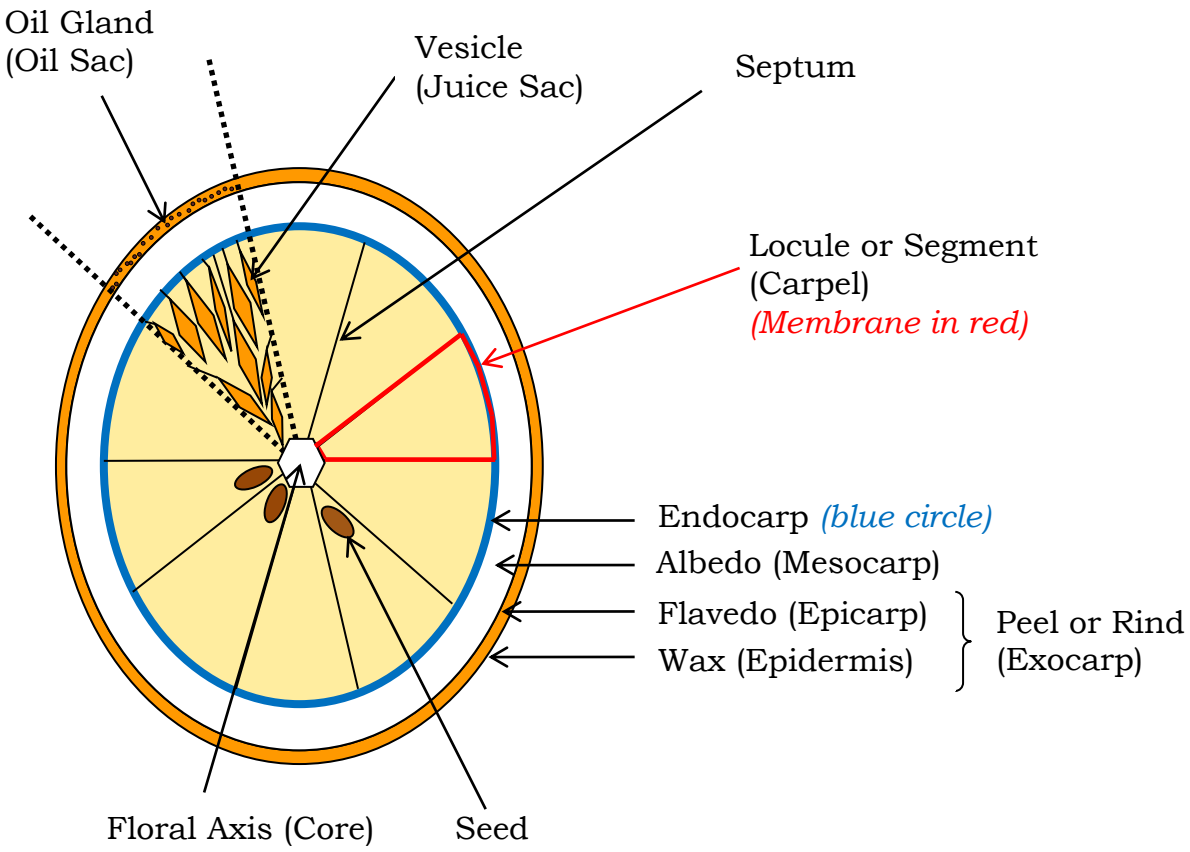


Figure 1.3: Schematic transverse section of orange fruit

Flavedo (epicarp) is the peel of orange fruit and is rich in peel oil which is located inside the oil glands. The peel itself is covered with a thin wax (epidermis). The albedo is the white spongy tissue located between the peel and the edible part of the fruit (endocarp). The latter consists of approximately twelve segments (carpels) which are separated from each other by a thin membrane (septum). The juice sacs (vesicles) and seeds are located within the carpels.

Adapted from Di Giacomo & Di Giacomo (2002)

and fatty acids, vitamins, phenolic compounds, essential oils and volatile compounds. Proximate chemical compositions of OJ has been shown in [Table 1.3] and discussed in more detail below.

Carbohydrates and Sugars

Excluding water which constitutes more than 85% of citrus juices, sugars are the predominant component of OJ. Principally, sugars present are sucrose, glucose and fructose approximately in the ratio of 2:1:1. However, variety and seasonal changes can affect the sugar composition of OJ. For instance, unlike the Valencia variety which has a relatively stable sugar content throughout the season, the amount of sugar increases in early and mid-season varieties mainly due to increase in their sucrose content. Sugar composition can also be affected by factors such as heat and/or acid-catalyzed inversion (hydrolysis) of sucrose to fructose and glucose during heat treatment and storage (Robards & Antolovich, 1995).

According to internationally-recognized German "RSK Standards" which set the criteria for unadulterated OJ, fructose is normally present in greater quantities than glucose, and therefore the fructose to glucose ratio is typically less than 1 (ranges 0.85 to 1, mean 0.92; RSK, 1987). Yet freshly squeezed OJs with glucose to fructose ratios of greater than 1 have also been reported (Villamiel *et al.*, 1998; Kelebek *et al.*, 2009). Other sugars naturally found in OJ in trace amounts include galactose, mannose, rhamnose, xylose, trehalose and inositol (myo-, chiro- and scyllo-) (Robards & Antolovich, 1995; Villamiel *et al.*, 1998;

Table 1.3: Proximate Composition of freshly-squeezed OJ

	Literature [Range] ($g.L^{-1}$)	RSK[a] Mean [Min, Max] ($g.L^{-1}$) –
Carbohydrates		
- Sugars	**65.1–120.2[b]**	**N/S**
Sucrose	29.4–61.4[b]	33.0 [N/S, 47.0]
Glucose	13.8–33.2[b]	28.0 [20.0, N/S]
Fructose	17.0–34.2[b]	30.0 [22.0, N/S]
- Pectin (Fibre)	**3.4–10.1[c]**	**N/S**
Organic Acids	**5.7–18.1[d]**	**N/S**
Citric Acid	5.1–14.3[d]	9.4 [7.6, 11.5]
Malic Acid	0.4–4.0[d]	1.7 [1.1, 2.9]
Minerals	**3.0–5.0[c]**	**4.0 [2.9, 4.8]**
Potassium	1.2–3.0[c]	1.9 [1.4, 2.3]
Phosphorous	0.1–0.3[c]	0.5 [0.3, 0.6][e]
Protein (N×6.25)	**4.0–11.0[c]**	**N/S**
Lipid	**0.8–1.0[c]**	**N/S**

Notes: and Sources
N/S: Not Specified
(a) RSK (1987) (Internationally accepted standard for unadulterated freshly squeezed OJ)
(b) Niu *et al.*, (2008); Kelebek *et al.*, (2009); Kelebek & Selli (2011); Villamiel *et al.*, (1998)
(c) Robards & Antolovich (1995)
(d) Capilla *et al.*, (1988); Niu *et al.*, (2008); Kelebek *et al.*, (2009); Kelebek & Selli (2011)
(e) Values are for phosphate (PO_4) instead of phosphorous.

Sanz *et al.*, 2004). In addition to sugars, non-sugar polysaccharides such as pectin and dietary fibre are also present in OJ (Corredig *et al.*, 2001; Croak & Corredig, 2006). The possible roles of sugars and pectin on physiology of *E. coli* have been discussed in more details in Chapter 3 and Chapter 4 respectively.

Major Organic Acids

Organic acids are the main source of OJ's acidity and therefore play an important role in determining its microbiological quality. For example, as stated in [Table 1.3], low acid content of acidless OJs can promote the growth of bacteria and therefore is not commonly used for production of OJ. In addition, from the organoleptic point of view, total soluble solids (Brix) and/or sugar to acid ratios are important parameters used for describing the tartness or sweetness of the product (Robards & Antolovich, 1995). Similar to other citrus fruits, the main acids present in OJ are citric acid followed by malic acid (Belitz & Grosch, 2009). Matured and late-season oranges generally have lower acidity mostly due to a decrease in their citric acid content; on the other hand, malic acid content remains relatively constant throughout the season (Sinclair & Ramsey, 1944; Robards & Antolovich, 1995). In general, citric to malic acid ratio has been shown to vary depending on the fruit's origin, variety and extraction techniques (e.g. the number of pressings during the extraction stage) (Saccani *et al.*, 1995; Saavedra *et al.*, 2001).

Another important organic acid found in OJ is isocitric acid which plays an important role in assessing the authenticity of OJ. This is mainly because,

unlike citric to malic acid ratio, the ratio of citric to isocitric acid is generally constant, notwithstanding the maturation-dependent changes in concentration of citric acid (RSK, 1987; Robards & Antolovich, 1995).

Ascorbic and Other Minor Acids

Other non-volatile organic acids such as oxalic, quinic, tartaric, fumaric, succinic, *cis*-aconitic and ascorbic acids have also been detected in OJ (Lee, 1993). Among these, ascorbic acid is the primary vitamin of OJ (vitamin C) and has been shown to exhibit antioxidant properties (Ames *et al.*, 1993). The ascorbic acid content of OJ is around 500 ppm (Silva, 2005); nonetheless, it can be significantly affected through processing and storage condition.

Besides its health benefits (Ames *et al.*, 1993; Padayatty *et al.*, 2003), ascorbic acid has been shown to affect the viability of microorganisms. For example, the antimicrobial activity of ascorbic acid especially in combination with organic acids was demonstrated by Tajkarimi & Ibrahim (2011). On the other hand, Van Opstal *et al.*, (2006) showed that ascorbic acid could reduce the bactericidal effects of lactoperoxidase against *E. coli* and *Shigella spp.*.

Amino Acids and Other Nitrogen-Compounds

OJ contains various nitrogen-containing compounds, including free amino acids, proteins (mainly carbohydrate metabolising enzymes) as well as various biologically active amines such as synephrine (Belitz & Grosch., 2009). The latter two groups are minor constituents of OJ which have been extensively

discussed by Vieira *et al.*, (2007, 2010) and Echeverria & Valich (1988).

With regard to amino acids which are the most prevalent nitrogen-compounds in OJ, almost all naturally occurring amino acids are present in OJ. However, the most abundant ones which account for more than 95% of total free amino acids in OJ are proline, arginine, asparagine, aspartic acid, γ-aminobutyric acid (GABA), glutamic acid, serine and alanine [Table 1.4]. The concentration of each amino acid can vary depending on factors such as cultivar, fruit's origin, maturation stage and the juice's processing techniques, mainly the method of extraction and heat treatment. Nevertheless the ratio of certain amino acid remains relatively constant. For instance, the ratios of certain amino acids (e.g., GABA to asparagine) have been found to remain relatively constant and consequently can be used for detecting OJ adulteration (Singhal *et al.*, 1997; Robards & Antolovich, 1995).

Free amino acids of OJ, particularly proline and arginine, are of major interest due to their interaction with ascorbic acid as well as sugar compounds and their consequent involvement in production of browning products (Roig *et al.*, 1999; Murata *et al.*, 2002; Shinoda *et al.*, 2004). Some of these products, such as furfural, and HMF have been shown to cause DNA damage in *E. coli* and *Salmonella* (Zheng *et al.*, 2012). In addition, two major amino acids present in OJ (arginine and glutamate) have been shown to play an important role in the acid stress response of stationary-phase *E. coli* (Lin *et al.*, 1996; see Section 1.3.2).

Table 1.4: Amino acid composition of OJ (fresh, from concentrate and RSK standards) (mg.dL^{-1})

	Reported Values in the Literature[a]			RSK Standard[e]	
	Freshly Squeezed[b]	From Concentrate[c]	Average (Fresh & concentrate)[b,c]	Mean	Range
Amino Acids					
Proline	79.4 ± 1.7	176.8 ± 22.1	131.7 ± 58.8	80.0	44.9–130.1
Arginine	56.4 ± 18.8	58.6 ± 19.9	57.9 ± 17.6	70.0	43.6–104.5
Aspartic	27.4 ± 0.7	21.7 ± 7.8	23.6 ± 6.8	28.0	22.6–39.9
Asparagine	30.5 ± 12.9	45.3 ± 6.0	40.4 ± 10.7	45.0	22.5–59.4
Glutamic	14.5 ± 3.8	10.6 ± 1.5	11.9 ± 2.8	11.8	7.3–16.2
Serine	12.8 ± 1.2	14.6 ± 2.1	14.0 ± 2.0	16.4	10.5–18.9
Alanine	10.8 ± 0.0	9.9 ± 1.6	10.1 ± 1.4	9.9	6.2–13.4
GABA (γ-aminobutyric acid)	23.7 ± 0.0	26.0 ± 2.6	25.5 ± 2.5	24.0	17.5–36.1
Ratios[g]					
GABA/Arginine	0.42	0.44	0.44	0.34	
Arginine/Asparagine	1.85	1.43	1.29	1.56	
GABA/Asparagine	0.78	0.63	0.57	0.53	

Notes and Sources:
(a) The values are the mean ± standard deviation of the values reported in the literature.
(b) Sources: Robards & Antolovich (1995); Gómez-Ariza *et al.*, (2005);
(c) Sources: Capilla *et al.*, (1988); Aristoy *et al.*, (1989); Kimball & Norman (1990); Heems *et al.*, (1998)
(d) Source: Robards & Antolovich (1995)
(e) Source: RSK (1987) (Internationally accepted standard for unadulterated freshly squeezed OJ)
(f) Ratios of the mean values

Inorganic Components

Approximately 0.4% (w/v) of OJ is made of inorganic compounds. With the exception of potassium and phosphorous which account for nearly 60% of minerals in OJ, the concentration of other inorganic compounds in OJ is negligible (Robards & Antolovich, 1995). The mean potassium content of OJ is approximately 0.19% (RSK, 1987) in the form of potassium citrate corresponding to approximate potassium citrate to citric acid molar ratios of 1 to 3. Consequently, this combination results in establishment of a citrate buffer environment in OJ (Lanford, 1942).

Fatty Acids and Sterols

OJ contains around 0.1% fatty acids which account for nearly all the lipid present in the juice, excluding essential oils. The fatty acid composition of OJ has been extensively discussed by Lawrence & Charbonneau (1988) and Nordby & Nagy (1969). According to Moufida & Marzouk (2003), the majority of these fatty acids are comprised of palmitic acid as well as unsaturated 18-carbon fatty acids. Zheng *et al.*, (2005) suggested that unsaturated fatty acids could inhibit the growth of *E. coli* and *S. aureus* by targeting their fatty acid synthesis, through inhibition of FabI production, an enzyme responsible for fatty acid chain elongation. However, as it was shown by Marounek *et al.*, (2003) the concentration required for antimicrobial activity against *E. coli* is much higher than those naturally found in OJ.

Volatile Compounds and Essential Oils

The volatile constituents and essential oils in processed and/or unprocessed OJ has been extensively studied (Nisperos-Carriedo & Shaw, 1990; Moshonas & Shaw, 1994 & 1997). These compounds are important, not only because of their great role in organoleptic properties of OJ (Bazemore *et al.*, 1999; Perez-Cacho & Rouseff; 2008; Kelebek & Selli, 2011), but also due to their well-known antimicrobial activities against a wide range of microorganisms including *E. coli* and *Salmonella*.

[Table 1.5] shows the most abundant volatile compounds and their mean concentration in OJ; nevertheless, many factors can affect the composition of volatile compounds in OJ. These include seasonality, variety, storage and processing conditions (for example, the method of juice extraction or heat treatment) and even the type of packing material (Moshonas & Shaw, 1989 & 1994; Maccarone *et al.*, 1998; Nisperos-Carriedo & Shaw, 1990; Hernandez *et al.*, 1992, Jordán *et al.*, 2005). Three main sources of volatile compounds in OJ are juice sacs, globular bodies inside the juice sac and peel. The former mainly contains the water-soluble volatile compounds, whereas the latter two are the major sources of juice oil and peel oil respectively (Brat *et al.*, 2003). In general, the majority of hydrophobic terpene hydrocarbons such as limonene and valencene are present in the pulp and cloud of OJ (coarse and colloidal particles of the insoluble-phase of OJ respectively). On the other hand, OJ serum (aqueous phase) is the main contributor of the hydrophilic compounds such as ethanol, methanol and acetaldehyde [Figure 1.4].

Table 1.5: The concentration of main volatile compounds in OJ (mg.L^{-1})

Volatile Compound	Mean[(a)]	Min[(a)]	Max[(a)]
Monoterpenes			
Limonene	87.31	14.24	170.00
β-myrcene	3.10	3.10	3.10
p-cymene	1.34	0.10	2.22
β-pinene	0.84	0.60	1.00
Sabinene	0.70	0.70	0.70
α-pinene	0.07	0.04	0.09
Sesquiterpene			
γ-Terpinen	2.95	0.21	7.70
Valencene	1.51	0.02	3.00
α-selinene	0.31	0.01	0.83
Esters			
Ethyl 3-hydroxy hexanoate	0.84	0.54	1.41
Ethyl acetate	0.75	0.20	1.80
Ethyl butanoate	0.64	0.10	1.18
Ethyl octanoate	0.35	0.16	0.48
Ethyl butyrate	0.26	0.02	0.60
Ethyl hexanoate	0.18	0.02	0.50
Aliphatic alcohols			
Ethanol	716.50	256.00	1150.00
Methanol	85.25	26.00	121.00
Hexanol	0.40	0.14	0.87
1-propanol	0.38	0.32	0.44
2-methyl-1-butanol	0.34	0.34	0.34
n-octanol	0.30	0.13	0.70
Monoterpenes alcohols			
Linalool	0.84	0.16	1.96
α-terpineol	0.48	0.24	0.74
Terpinen-4-ol	0.38	0.15	0.70
Aldehydes & ketones			
Acetaldehyde	11.45	11.00	11.90
Nootkatone	0.60	0.50	0.70
Decanal	0.51	0.41	0.61
Octanal	0.43	0.01	0.78
Hexanal	0.17	0.02	0.40
β-sinensal	0.11	0.11	0.11

Notes and Sources:

(a) The mean, min and max values have been extracted from the following sources: Nisperos-Carriedo & Shaw (1990); Hernandez *et al.*, (1992); Moshonas & Shaw (1994); Moshonas & Shaw (1997); Maccarone *et al.*, (1998); Brat *et al.*, (2003); Kelebek & Selli (2011)

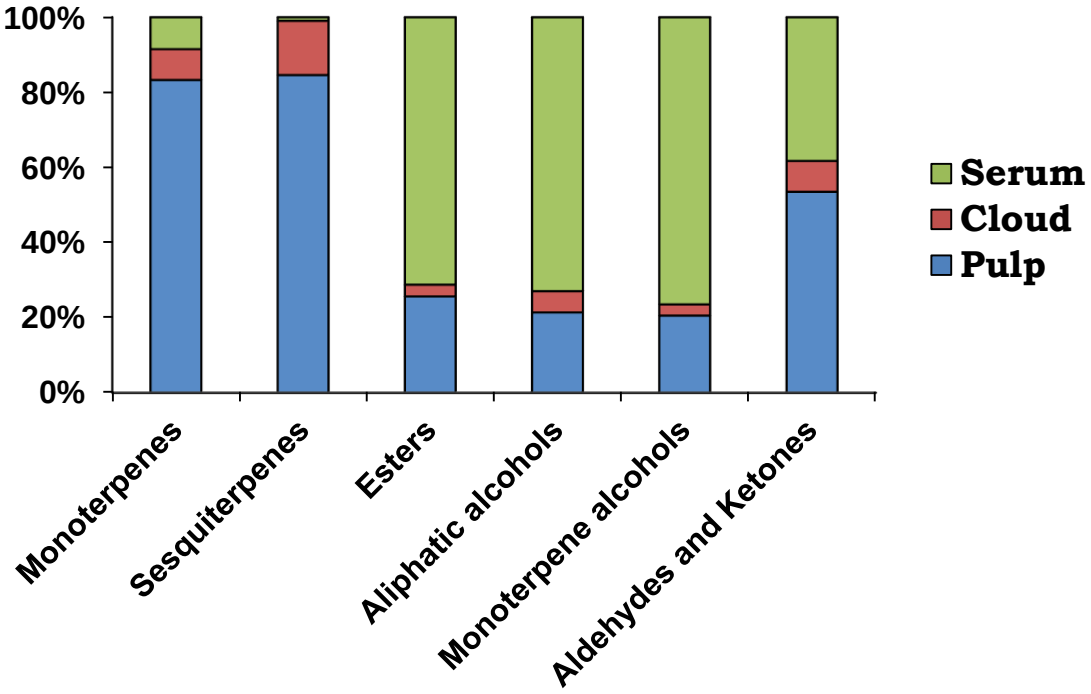


Figure 1.4: Distribution of volatile compounds in pulp, cloud and serum of OJ

Pulp and Cloud of OJ are the coarse and colloidal particles of the insoluble-phase of OJ respectively. Serum in the pale yellow aqueous phase of OJ containing the water-soluble compounds.

Source: Brat *et al.*, (2003)

Many studies have been conducted with the aim of investigating the antimicrobial activities of volatile compounds and essential oils of oranges (Bisignano & Saija, 2002). Nevertheless, in most cases, the concentrations used in these studies are much higher than the levels naturally found in OJ. For instance Fisher & Phillips (2006) reported the minimum inhibitory concentration (MIC) of orange peel oil (> 95% limonene) for *E. coli* O157:H7 to be 1% (v/v). This is significantly greater than the optimal level of peel oil in OJ (0.015–0.025%; Sandhu & Minhas, 2007). In a recent study, Espina *et al.*, (2013) reported the MIC of limonene for *E. coli* strains BJ4 and O157:H7 to be 0.5% (v/v). They also investigated the mechanisms of bactericidal action of 0.02% limonene against *E. coli* BJ4 at pH 4.0. They showed that limonene is capable of modifying the protein fraction of the outer membrane. They postulated that this could increase the permeability of the membrane to lipophilic compounds such as limonene. In turn, this results in greater interaction of the limonene with components of the cytoplasmic membrane leading to inactivation of the bacterial cell. In another study, Di Pasqua *et al.*, (2007) suggested that the limonene-induced increase in permeability of the outer membrane of *E. coli* O157:H7 could result in greater access of desaturase enzymes to principal fatty acid of the membrane (particularly the C6:0 and C16:0). Nevertheless, the concentration used in the latter study was 2.72% *d*-limonene, which is approximately 160 times the maximum concentration of limonene in freshly squeezed OJ.

Antimicrobial effects of other minor volatile compounds in OJ have also been investigated. In general, it has been shown that compared to terpenes (e.g. limonene, valencene and α-pinene), aldehydes and alcohols such as octanal, linalool and terpinen-4-ol exhibit stronger antimicrobial activity against *E. coli* (Liu *et al.*, 2012; Inouye *et al.*, 2001). However, Caccioni *et al.*, (1998) proposed that due to synergic action of various antimicrobials, a holistic approach needs to be taken in evaluating the antimicrobial activity of orange volatile compounds.

Phenolic Compounds

These compounds consist of a wide range of secondary plant metabolites, which in case of oranges can be divided to two main groups: flavonoids and phenolic acids (Table 1.6). The main phenolic compound in OJ is hesperidin (glycoside of hesperetin) which is present in the endocarp section of the fruit, mostly in soluble form inside the juice sacs. However, upon extraction of OJ, it commonly forms insoluble crystals mainly due to its low solubility in acidic condition, as well as the nucleation around pectin and membrane particles which are released during the extraction step. This consequently results in precipitation of hesperidin crystals in OJ (Gattuso *et al.*, 2007, Gil-Izquierdo *et al.*, 2001; Ellerbee, 2009).

Due to their alleged therapeutic properties, many studies have been conducted with the aim of studying the antioxidant and antimicrobial properties of OJ phenolic compounds, particularly hesperidin (Benavente-García *et al.*, 1997;

Table 1.6: The concentration of phenolic compounds in OJ ($mg.L^{-1}$)

Compound	Type of OJ	
	Fresh[(a)(b)]	Commercial[(a)(c)]
Flavanones		
Hesperidin	187.13 ± 39.71	375.00 ± 192.00
Narirutin	37.96 ± 10.37	59.00 ± 16.00
Didymin	10.63 ± 3.07	18.90 ± 5.60
Eriocitrin	3.10 ± 1.80	21.30 ± 30.10
Naringin	1.59 ± 0.10	9.50 ± 3.20
Neohesperidin	1.20 ± 0.04	—
Flavones		
6,8-di-C-Glu-Apigenin	44.79 ± 20.80	41.60 ± 13.70
Poncirin	10.40 ± 7.80	—
Neoeriocitrin	5.90 ± 0.00	—
6,8-di-C-Glu-Diosmetin	3.50 ± 1.40	—
Diosmin	0.90 ± 0.00	34.60 ± 19.20
Neodiosmin	0.80 ± 0.00	—
Isorhoifolin	0.70 ± 0.00	—
Rhoifolin	0.50 ± 0.00	—
Polymethoxyflavones		
Naringenin	—	8.00 ± 0.00
Sinensetin	3.70 ± 0.00	2.40 ± 0.90
Nobiletin	3.30 ± 1.90	2.60 ± 0.70
Heptamethoxyflavone	0.80 ± 0.60	0.50 ± 0.30
Quercetogetin	—	0.40 ± 0.10
Isoscutellarein	—	0.50 ± 0.40
Tangeretin	0.40 ± 0.40	0.40 ± 0.20
Taxifolin	0.30 ± 0.00	—
Acacetin	0.30 ± 0.00	—
Hydroxybenzoic acids		
Gallic acid	3.17 ± 0.11	N/A
Protocatechuic acid	0.73 ± 0.03	N/A
p-Hydroxybenzoic acid	0.93 ± 0.01	N/A
Vanillic acid	2.93 ± 0.05	N/A
Hydroxycinnamic acids		
Ferulic acid	23.56 ± 0.83	N/A
Sinapic acid	18.20 ± 0.54	N/A
Caffeic acid	10.97 ± 0.19	N/A
Chlorogenic acid	8.88 ± 0.32	N/A
p-Coumaric acid	4.20 ± 0.13	N/A

Notes and Sources:

(a) Average ± Standard Deviation

(b) Sources: Gattuso *et al.*, (2007), Kelebek *et al.*, (2009), Kelebek & Selli (2011). The reported standard deviation is the average of pooled value of all three studies (squared root of the squared sum)

(c) Sources: Data from Gattuso *et al.*, (2007)

Tripoli *et al.*, 2007; Milenkovic *et al.*, 2011). Yi *et al.*, (2008) reported the MIC of hesperidin against both *E. coli* and *Salmonella*, the main foodborne pathogens associated with acidic fruit juices (apple juice and OJ respectively) to be 800 ppm. Although this is greater than the mean concentration generally present in OJ, it is still within the ranges reported by Gorinstein *et al.*, (2006).

In addition to their antimicrobial activity, OJ flavonoids have also been shown to possess antioxidant activity. For instance, the antioxidant activity of hesperidin has been found to match the antioxidant activity of ascorbic acid (both 1 mM Trolox based on Trolox-equivalent antioxidant capacity or TEAC; Rice-Evans *et al.*, 1997). However, it is important to note that, due to the low solubility and precipitation of hesperidin in acidic conditions, its bioavailability (the concentration available for biological activity) is generally low (Ross & Kasum, 2002; Gil-Izquierdo *et al.*, 2001).

1.2.3 Production of OJ

The main problem with describing the stages involved in the production of OJ is that no single method of production exists. This is primarily because OJ is not only prepared in large scale by fruit juice manufacturers but also in smaller scale by medium or small size businesses (SMEs) such as catering units or simply by individual consumers at home. Nevertheless, the generic flow diagram shown in [Figure 1.5] is an attempt to illustrate the principle stages involved in the production of both pasteurized and unpasteurized OJ. The processing stages of OJ have been described in detail by Veldhuis (1971),

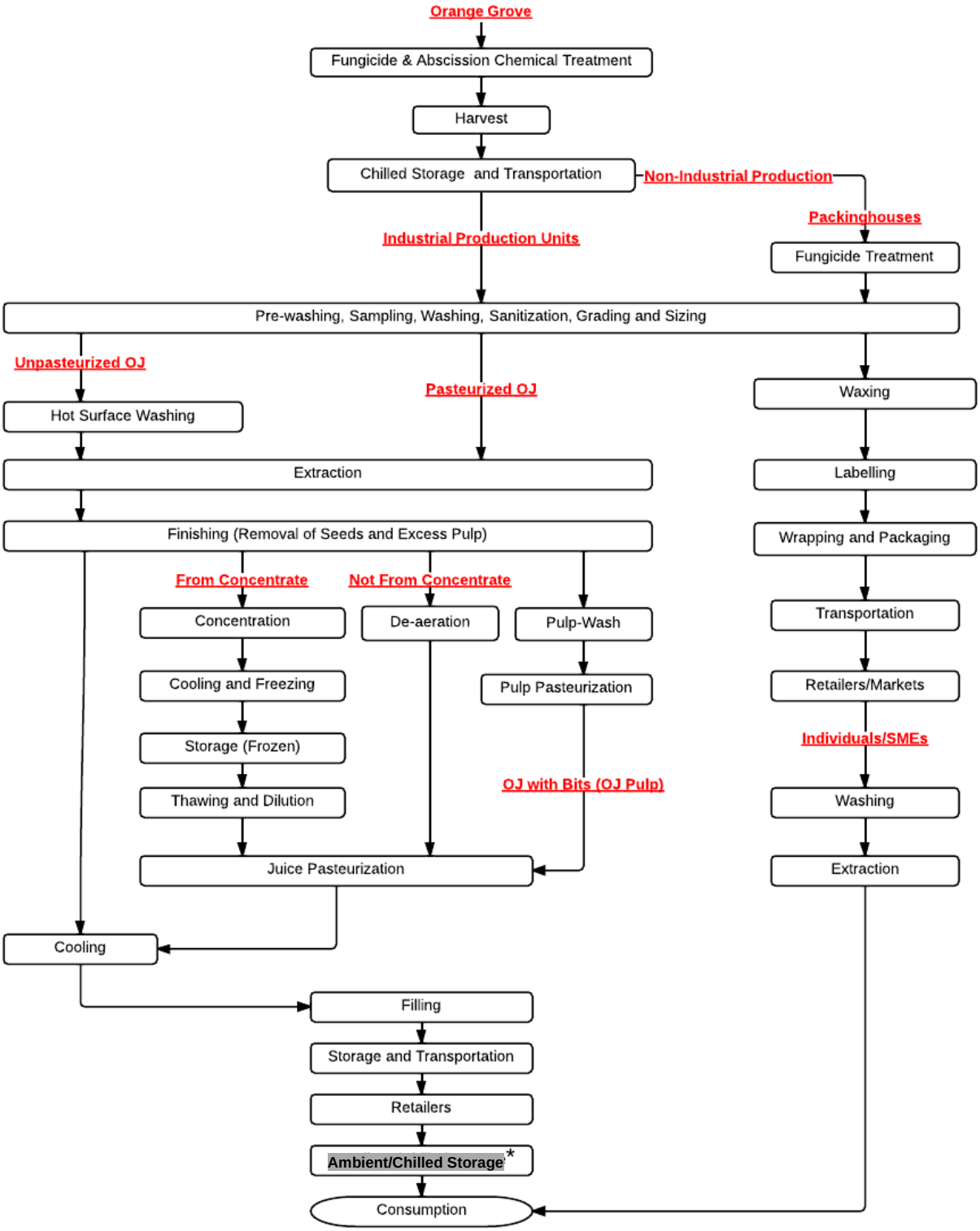


Figure 1.5: General flow-chart of OJ production

Notes and Sources:
*Chilled or ambient storage when pasteurized at 80 °C or beyond 90 °C respectively.
- Adapted from: Veldhuis (1971); Salunkhe & Desai (1984); Schmidt *et al.*, (1997); Crupi & Rispoli (2002) and Kimball *et al.*, (2005) and Di Giacomo (2002)

Salunkhe & Desai (1984), Schmidt *et al.*, (1997), Crupi & Rispoli (2002); McLellan & Padilla-Zakour (2004) and Kimball *et al.*, (2004). What follows is an extracted summary of these works.

Pre-harvest and Harvest

Orange fruits are treated with various fungicides such as Benomyl and also abscission chemicals (mainly methyl Jasmonate, coronatine or abscisic acid) before harvest. Harvesting of ripe fruits is performed either manually by fruit pickers or mechanically with the help of harvesting machines. Following the harvest, fruits are transported to the production units or packinghouses for OJ production or fresh produce packaging respectively.

Industrial Production

In OJ plants, prior to extraction, the fruit goes through sampling, washing and grading stages. The main chemicals used for washing and sanitization of oranges are non-ionic detergents, sanitizers such as chlorinated water or sodium ortho-phenylphenol (SOPP). The latter chemical acts not only as a sanitizer and detergent, but also as an effective fungicide against postharvest decay. The fruits are then rinsed with clean water in order to remove the detergent from the surface of the fruit. With regard to the temperature of washing water, studies have shown that positive temperature difference between the surface of the fruit (ambient temperature) and the rinsing water (4 °C) can result in infiltration of microorganisms into the fruit. As a result the temperature of the washing water is generally maintained at 10 °C higher than

that of the surface of the fruit (Parish *et al.*, 2003). In case of the oranges used for the production of unpasteurized juice, they are subjected to several stages of washing. An additional washing stage with hot steam/water is also common in production of unpasteurized OJ in order to maximise the microbial reduction.

Following the grading and sizing stages, fruits enter the extractor. The main types of extractors used in juice industry are Food Machinery Corporation (FMC) and Brown extractors. FMC extractors can be used for simultaneous extraction of OJ and peel oil, whereas in Brown extractors peel oil is extracted either before or after fruit juice extraction depending on the type of Brown extractor used. Following the extraction, seeds and pulp are removed using finishers and centrifuges. In case of the unpasteurized OJ, the extracted juice is bottled immediately after the finishing stage.

With regard to pasteurized OJ, the next important stage is de-oiling and de-aeration. Raw OJ contains high level of peel oil which not only makes the juice bitter but also decreases its shelf-life at low temperatures. Excess oxygen introduced into the juice during the extraction and finishing stages can also increase the risk of spoilage. One of the most common types of equipment used for simultaneous de-aeration and de-oiling is spinning cone columns. It consists of a series of rotating inverted cones inside a vertical stainless steel cylinder. The juice enters the column under a vacuum at the top and flows down the cylinder due to gravity. At the same time, water vapour is introduced into the equipment from the bottom. Rotation and vacuum, leads to the

generation of a thin turbulent film on the upper surface of the cones which helps with removing more than 90% and 95% of excess oil and oxygen respectively.

For the production of pasteurized OJ with bits (fruit juice particles), the remaining pulp is pasteurized subsequent to the washing stage and added back to the orange extract, prior to fruit juice pasteurization. The OJ is then pasteurized at 80 °C or beyond 90 °C for 15–30 seconds in order to produce chilled stored and ambient temperature stable OJ respectively. The product is then cooled immediately to 4 ºC, packed and stored chilled until the time of consumption.

Non-Industrial Production

In packinghouses, oranges go through similar cleaning, washing and sanitizations steps to those described above. However, in order to increase the appearance and also the shelf-life of the fruits, oranges are subjected to extra preparation steps such as fungicide application, waxing and colour-adding. The most common waxes used for waxing oranges are shellac, polyethylene, carnauba waxes and ammoniated wood resins. Oranges are usually sprayed with alkaline waxes (pH 8.2–11.6) and subsequently dried at high temperatures (50–55 °C) for 2–3 min (Pao *et al.,* 1999)

Oranges which after pre-grading and final grading do not meet the criteria for fresh fruit market/supermarkets are sent to OJ plants for OJ processing.

Acceptable fruits are transported to fresh fruit markets and retailers and subsequently purchased by SMEs or the public. In these cases, the OJ is generally prepared by squeezing the oranges using medium or small size squeezers respectively and served shortly after preparation.

1.2.4 Composition- and Process-induced Microbial Stress Response

As previously mentioned (see Section [1.1]), among fruit juices, apple juice but not OJ has mainly been associated with *E. coli* outbreaks. It has been suggested that is mainly due to the use of dropped apples contaminated with animal faeces and subsequent infiltration of *E. coli* into the damaged apples (Lee & Kang, 2005). In case of oranges, however, the damage occurs mainly during harvesting by tearing the portion of the peel around the oranges' stem end as well as split and puncture of the fruits during mechanical harvesting. Puncturing of the fruit could also occur during the transport stage by firm oranges with attached stems (Almed *et al.,* 1973).

Although the difference between the principle routes of contamination for apples and oranges could explain the possible reason behind the lower reported cases of OJ-associated *E. coli* outbreaks, infiltration of microbes into oranges has also been reported. Walderhaug *et al.,* (1999) demonstrated that the infiltration of *E. coli* into oranges through the stem scar can occur at uptake frequency of 3% and at a level of 0.001 to 0.0001% of the challenge levels.

Depending on the type of OJ, orange fruits are subjected to different food

processing stages. It is well known that various production steps such as the sanitization can provoke microbial stress responses. In addition, the intrinsic physical and chemical characteristics of foods, such as pH and acid composition can also cause bacterial stress. Depending on the severity of these stresses, the growth arrest, loss of viability and even lethal injury of microorganisms can occur (Yousef & Courtney, 2003). Therefore, it could be suggested that the *E. coli* present on the surface of (or infiltrated into contaminated orange fruits could also be subjected to stress conditions throughout the production chain (Table 1.7). As a result, it is important to first learn more about the mechanisms of the stress responses of this microorganism and the likely effects that they could have on the viability and physiological responses of the pathogens in OJ.

1.3 Microbial Stress Responses

In microbiology, stress is defined as a sub-lethal treatment or a deleterious factor which can potentially have an adverse effect on the growth or survival of the microorganism (Yousef & Courtney, 2003; Wesche *et al.*, 2009). These stresses include, but are not limited to, various intracellular and environmental factors such as non-optimal pH, temperature, oxygen and nutrient availability, oxidative state and osmolarity (Wesche *et al.*, 2009). Often, a distinction is made between the "stress" which is a gradual deviation from the optimal condition and the "shock" which refers to a sudden drastic change (Yousef & Courtney, 2003).

Table 1.7: The main stresses encountered by microorganisms during OJ production

Processing Step / Stress	Osmotic	Oxidative	Starvation	Metal ion	Chemical	Cold	Heat	Acid	Alkaline
Harvest									
Pre-harvest			✓		✓	✓	✓		
Pre-harvest fungicide treatment					✓				
Industrial Production									
Transportation				✓					
Unloading & Storage			✓			✓			
Washing					✓				
Sanitization		✓			✓				
Hot Surface Cleaning							✓		
Extraction				✓				✓	
De-aeration and De-oiling			✓						
Freezing	✓		✓			✓			
Chilled Storage						✓			
Packinghouse									
Waxing									✓
De-greening					✓				
Post-harvest fungicide					✓				

Adapted from Yousef & Courtney (2003)

Each microorganism has a certain threshold for tolerating stress which, if exceeded, could potentially lead to cell injury in case of moderate stress or cell death if the stress is more severe. Cell injury and death could also occur as a result of inadequate or delayed response to the stress (Yousef & Courtney, 2003). On the other hand, at stress levels below the threshold, an adaptive response is induced which could lead to increased resistance of the cell to stresses. In general, two types of microbial adaptive response are observed: (a) adaptation or an increased resistance (i.e., higher threshold level) to the same type of stress and (b) cross-protection which is an increased resistance to a stress different from the one the cell initially encountered. Therefore, subjection to sub-lethal stress can result in greater resistance to either the same or a different type of stress. In simple words, in case of bacteria, as Boor (2006) put it: *"what doesn't kill them can make them stronger"*.

A good example of bacterial stress response was reported by Ryu & Beuchat (1998). They demonstrated that compared to non-stressed *E. coli*, acid-adapted cells displayed a greater acid resistance in an acidified medium with lower pH (pH 3.9). They also showed that acid adaptation resulted in cross-protection by increasing the heat resistance (D-value) of the acid-adapted cell in orange and apple juices.

The most important implication of these bacterial stress responses, from the food safety point of view, is the greater chance of survival of the stressed cells in food. It has been suggested that there is an inverse correlation between the acid

resistance of a pathogen and its infectious dose or ID (Richard & Foster, 2003). For instance, while the highly acid resistant EHEC and *Shigella spp.* have an ID of 10–10^4 CFU, the infectious dose of the mildly acid resistant *Salmonella* is generally in excess of 10^5–10^6 organisms (Cliver, 1990). In the case of OJ contaminated with *E. coli* which is the focus of this study, the bacteria are subjected to pH of around 3.2–3.6 before entering the stomach. This could potentially increase the tolerance of the cells to an even lower pH of the gastric juice (pH 1.5–2.5) through the phenomenon of acid adaptation. Furthermore, it is likely that the *E. coli* in contaminated OJ could have been subjected to various stresses throughout its production chain resulting in cross protection of *E. coli* to acidic condition. Moreover, unlike solid foods in which the bacteria are surrounded and partially protected by the food from acidic condition of the stomach, *E. coli* present in OJ comes into direct contact with the low pH of the gastric juice (Law, 2000). Therefore it is important to investigate the mechanisms of the stress responses in *E. coli*.

1.3.1 General Stress Response

When *E. coli* is subjected to different stresses such as starvation, low pH, cold, heat and high osmolarity it undergoes a diverse physiological and morphological changes in order to adapt to the new condition. Alternative sigma factor σ^s (RpoS or σ^{38}) is a subunit of RNA polymerase which plays an important role in the general stress response of the bacteria (Hengge-Aronis, 2000). RpoS is considered to be the master regulator of the stress responses, responsible for regulating the transcription of more than 220 genes. These include genes which

are involved in oxidative stress (*katE, katG*, *dps*, *oxyR* and *acnA*), acid stress (*gadB* and *gadC*), and cold shock (*gyrB*) responses of *E. coli* (Hengge-Aronis, 2002).

Depending on the type of stress, the cellular level of RpoS is regulated in three levels of gene transcription, *rpoS* mRNA translation and proteolysis of RpoS. For instance, gradual shift from exponential-phase (log-phase) to stationary-phase results in expression of *rpoS*, whereas the cold shock increases the translation of *rpoS* mRNA. On the other hand, acid shock and carbon starvation which could pose a more serious threat to viability of the cell induce the rapid inhibition of RpoS proteolysis (Hengge-Aronis, 2000).

1.3.2 Acid Stress Response Systems

Before reaching the intestine and causing disease, pathogenic *E. coli* needs to pass through the highly acidic environment of the stomach (pH ca. 1.5-2.5) and survive this condition while staying in the stomach for approximately 2 hours (Foster, 2004, Richard & Foster, 2003). It has been shown that in common with other enteric pathogenic bacteria such as *Salmonella* and *Shigella* spp., *E. coli* employs various acid stress response mechanisms in order to survive these harsh acidic conditions.

The mechanisms employed by *E. coli* have been shown to be affected by many factors such as the growth phase, pre-stress conditions for instance the pH and nutrient availability during the growth and also the presence of amino acids

during the stress. These systems have been extensively reviewed by Castanie-Cornet *et al.*, (1999), Richard & Foster (2003), Foster (2004) and Zhao & Houry (2010). In general, three primary mechanisms have been described for acid resistance of stationary-phase *E. coli*: oxidative AR1, glutamine-dependent AR2 and arginine-dependent AR3, each conferring different levels of protection against acids. For instance, while AR2 is the most effective system capable of protecting the cells at pH 2 or less, AR1 and AR3 only protect the cells at pH values of greater than 2.5 (Audia *et al.*, 2001). In case of exponentially growing *E. coli*, the acid tolerance response (ATR) and acid habituation (AH) systems are shown to play an important role in increasing the survival of *E. coli* to acidic conditions (Lin *et al.*, 1995; Rowbury & Goodson, 1998; Šeputienė *et al.*, 2006).

Oxidative Acid Resistance System (AR1)

Oxidative AR or AR1 is an acid-induced glucose-repressed system and requires RpoS, Cyclic adenosine monophosphate (cAMP) and cAMP receptor protein (CRP) as its regulators (Castanie-Cornet *et al.*, 1999, Richard & Foster, 2003). The exact acid resistance genes controlled by these regulators and the mechanisms of AR1 system are not known. Nevertheless, it has been suggested that AR1 is a part of the RpoS-regulated general stress response. This system also requires F_O/F_1 proton-translocating ATPase; however, its exact role in AR1 is also not clear. It has been suggested that the ATPase could potentially increase the resistance to acidic condition by either generating ATP fuel for AR1 or extruding protons from the cell. In addition, it was shown that HdeA, a member of the RpoS regulon, can play an important part in acetate-induced

AR1 (Richard & Foster, 2003). Gajiwala & Burley (2000) suggested that HdeA acts similar to chaperones by preventing the aggregation of acid-induced denatured proteins. It is believed that in acidic conditions, the homodimer proteins of HdeA dissociate to their monomers and subsequently bind to acid-denatured proteins within the periplasmic space of the bacteria.

Amino Acid-based Acid resistance (AR2 and AR3)

The other two systems of AR2 and AR3 are amino acid-dependent systems and require the presence of extracellular exogenous glutamate and arginine respectively. The principle behind both the AR2 and AR3 systems is making use of pyridoxal phosphate (PLP)-containing decarboxylase enzymes for replacing the α-carboxyl groups of amino acids with protons. These enzymes remove the intracellular protons by converting the glutamate and arginine amino acids to positively charged compounds of γ-amino butyric acid (GABA) and agmatine respectively. These systems also rely on antiporters which concomitantly import the amino acids into the cell and expel the positively charged compounds from the cell (Foster, 2004).

Glutamate-dependent AR system: AR2 system consists of two isoenzymes, GadA and GadB located on two separate operons. It has been shown that while double mutation of *gadA* and *gadB* will block the system, mutation of one of these genes does not have a significant effect on the performance of the system (Richard & Foster, 2003). Another essential component of this system, GadC or γ-amino butyric acid antiporter is encoded by *gadC* which has the same

promoter as *gadB* and therefore located within the *gadBC* operon (Richard & Foster, 2003).

Glutamate has three ionizable groups of α-carbonyl, carbonyl group of the side chain or R-group and amino group with pK_a of 2.30, 4.28 and 9.67 respectively. Therefore, in a highly acidic environment, for instance, pH of 2–2.5, depending on the pH, the glutamate has between 2 to 3 fully protonated groups, hence having a net charge of +1 to 0 respectively. When acid stressed *E. coli* with internal pH of between 4 and 5 enters this environment, the glutamate is imported into the cell with the help of GadC antiporter, which is located on the inner membrane of the cell. GadC has a similar internal pH to the cell, and therefore, the passage of glutamate through GadC results in deprotonation of the α-carbonyl group. This proton is then released into the periplasmic space of the cell, avoiding additional intracellular proton stress. Once inside the cell, with the help of GadA or GadB isoenzymes the glutamate accepts a proton and is decarboxylated to GABA, resulting in release of CO_2. GABA which has similar net charge to the original glutamate that entered is then expelled from the cell. GABA which has not been fully protonated (net charge of 0) is also capable of accepting an extra proton outside the cell, further decreasing the proton gradient across the cell membrane (Richard & Foster, 2003).

Arginine-dependent AR system: As mentioned above, the principle mechanisms involved in AR3 system are similar to AR2. The system is based on intracellular consumption of a proton during the decarboxylation of arginine

with the aid of an arginine decarboxylase (AdiA) and its conversion to agmatine. Again, similar to AR2, an antiporter which in this case is the arginine-agmatine antiporter (AdiC) expels the protonated agmatine outside the cell (Gong *et al.*, 2003). Arginine has three ionizable groups, α-carbonyl, amino group of the side chain or R-group and amino group of the α-carbon with pK_a of 2.1, 7.1 and 9.2 respectively. As a result, unlike glutamate which had a maximum of two proton-accepting sites, in acidic conditions only the α-carbonyl of arginine can participate as a proton acceptor. This also means that the final product of this reaction or agmatine cannot participate in extracellular proton reduction (Richard & Foster, 2003).

Log-Phase Acid Tolerance Response and Acid Habituation

In addition to stationary-phase AR mechanism, log-phase cells of *E. coli* can also adapt to acidic conditions. In general, two mechanisms have been described for acid resistance of cell in log-phase: Acid tolerance response (ATR) and acid habituation (AH) (Lund & Eklund, 2000). Both AR and ATR systems can be induced in cells grown in high phosphate containing medium whereas AH requires the growth in medium with low phosphate content (Lin *et al.*, (1995). Lin *et al.*, (1995) showed that when exponentially grown *E. coli* were adapted to pH of 4.3–5.8 for 20–90 min, they could tolerate the pH condition of 3.3. They attributed the observed ATR to the production of unspecified acid shock proteins (Heyde & Portalier, 1990). Rowbury & Goodson (1998, 1999) reported that log-phase *E. coli* grown at pH 7 produce a heat-stable protein which can act as a precursor for AH by sensing the acidic condition. Upon acid

stress, this protein is converted to an extracellular induction component (EIC) which induces the AH response in *E. coli*. Furthermore, Brown *et al.*, (1997) demonstrated that upon acid habituation, cells convert their monounsaturated fatty acids to cyclopropane which have been suggested to decrease the permeability of the membrane to protons.

1.3.3 Acid Resistance Mechanisms in Fruit Juices

Price *et al.*, (2004) undertook a study, with the aim of understanding which AR mechanism contributes to the survival of acid-adapted *E. coli* O157:H7 in apple cider (pH 3.5). In this study, they acid-adapted the cells by growing them in Luria-Bertani (LB) which had been either acidified with 100 mM of morpholineethanesulfonic acid (MES, pH 5.5) or supplemented with 0.4% glucose. The presence of glucose in the latter represses the AR1 system but supports the amino acid-dependent systems of AR2 and AR3. The stationary-phase cells were then diluted in commercial apple cider and their survival at 25 °C was studied for three days. The results showed a significantly lower viability for cells which had been acid-adapted with glucose. They also demonstrated that whereas the viability of *gadC*- and *adiA*-mutant cells (lacking the AR2 and AR3 systems respectively) were not affected in apple cider, there was a dramatic decrease in the viability of *rpoS*-mutant cells. Consequently, they concluded that AR1 is the only AR mechanism necessary for the survival of *E. coli* in apple cider.

Although *E. coli* cells are capable of responding to acid stress regardless of their

growth phase, compared to log-phase cells, the acid stress responses of stationary-phase cells are more effective in protecting the cells in acidic conditions (Small *et al.,* 1994; Arnold & Kasper, 1995; Chang & Cronan Jr., 1999; Price *et al.,* 2004). This issue is discussed in more details later in sections 3.3.2 and 3.4.1.

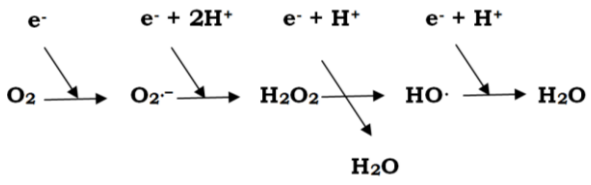
1.3.4 Reactive Oxygen Species

Molecular oxygen (O_2) is a stable molecule with two unpaired electrons and is generally a weak electron donor due to parallel spin of the electrons and thermodynamic reasons. However, interaction with metals or organic radicals can result in the reduction of molecular oxygen and the formation of reactive oxygen species (ROS). These compounds can adversely affect the bacteria by damaging the nucleic acids (DNA and RNA), proteins and lipids. The imbalance between the concentration of ROS and their inactivation by the defence mechanism of bacteria results in cellular oxidative stress (Farr & Kogoma, 1991).

Aerobic ROS Production

The four-step reduction of the molecular oxygen is shown in Equation 1.1 (Imlay, 2003).

Equation 1.1:



The production of O_2^- (superoxide) can occur during normal aerobic growth of the bacteria as a consequence of enzymatic autoxidation of dehydrogenases or non-enzymatic autoxidation of intracellular compounds such as ubiquinols and flavins. O_2^- subsequently goes through spontaneous and/or enzymatic dismutation, for example, by superoxide dismutase to form hydrogen peroxide (H_2O_2). In acidic conditions, it can also become protonated to form a much stronger radical of hydroperoxyl ($HOO^{\cdot}$). Both O_2^- and H_2O_2 compounds can damage the cell through oxidation of the iron sulphur (Fe:S) clusters of the enzymes. H_2O_2 can also cause damage by oxidizing the sulphur atoms of the cysteine and methionine residues in enzymes and also protein carbonylation. Nevertheless, due to anionic charge of O_2^- and the stability of oxygen bond in H_2O_2, their reactivity is low. On the other hand, hydroxyl radicals ($HO^{\cdot}$) can directly damage most cellular molecules including the DNA. The production of $HO^{\cdot}$ occurs as a result of the reaction of H_2O_2 with the free iron or Fe^{2+} during the Fenton reaction (Farr & Kogoma, 1991; Imlay, 2003). During the Fenton-mediated hydroxyl radical formation, intracellular ferric ions are first reduced to ferrous with the help of reductants such as O_2^-, $FADH_2$, cysteine and the iron-sulphur clusters. The ferrous ion subsequently reacts with H_2O_2, to form Fe^{3+}, hydroxide anion (OH^-) and $HO^{\cdot}$ radical. The Fe^{3+} then participates in another cycle of iron reduction whereas the $HO^{\cdot}$ attacks the DNA resulting in DNA damage (Imlay, 2003; Kohanski *et al.*, 2007).

The generation of ROS and the resultant stress response in *E. coli* could also

play an important role in acid stress response of *E. coli*. For instance, it has been shown that AR2 and AR3 acid resistance mechanisms in *E. coli* are capable of protecting the cells against oxidative stress in extreme acidic conditions (pH 2.5; Bearson *et al.,* 2009). Moreover, *rpoS* which is believed to be the main regulator of the AR1 acid resistance response in *E. coli* also plays an important role in protecting the cells against ROS-induced oxidative stress in *E. coli* (Hengge-Aronis, 1993 & 2002).

Furthermore, it has been demonstrated that the oxidative stress response in *E. coli* could lead to up-regulation of multiple stress response gene of *ycfr* which has also been shown to play an important role in acid resistance of *E. coli* in acidic conditions (Bergholz *et al.,* 2001; Maurer *et al.,* 2005; Zheng *et al.,* 2007). The roles of *ycfR* and *rpoS* in stress response of *E. coli* have been discussed in more details in section 5.4.3.

Sanitizer-Induced ROS Production

In addition to aerobic production of ROS, exposure to extracellular ROS in food or during the food production stages (e.g., washing fruit surfaces with sanitizers such as H_2O_2) can also lead to DNA damage (Bergholz *et al.*, 2009). For instance, Demple and Halbrook (1983) demonstrated that exposure to low concentration of H_2O_2 (5 mM) is capable of inducing oxidative DNA damage in *E. coli*. Furthermore, subjection of the cells to lower concentration of H_2O_2 (0.2 to 1 μM) significantly increased their resistance to 5 mM H_2O_2 indicating a resistance mechanism to sub-lethal concentrations of H_2O_2. It has also been

demonstrated that subjection of *E. coli* to acidic condition of a model apple juice (Bergholz *et al.*, 2009) or high hydrostatic pressure processing condition (Aertsen *et al.*, 2005) can result in activation of major oxidative stress and ROS response genes.

1.3.5 SOS Response

As mentioned above, normal aerobic growth of bacteria as well as acidic condition and subjection to sanitizers (for instance H_2O_2 and hypochlorous acid) can result in DNA damage. Since this could lead to further mutagenesis and subsequently the death of the cell, bacteria employ various regulatory networks in order to respond to their DNA damage. Those involved in DNA damage repair response of *E. coli* have been extensively reviewed by Walker (1984).

The most important of these mechanisms is the SOS response which is regulated by *recA* and *lexA* (reviewed by Cox, 2007). In summary, under normal, non-stressed conditions LexA acts as the repressor of *recA* and more than 20 other SOS-response related genes. However, upon DNA damage and the formation of single-stranded DNA (ssDNA), RecA which under non-stressed conditions is expressed at basal level, binds to the ssDNA resulting in the formation of activated RecA nucleoprotein complexes. The activated RecA then cleaves the LexA protein (at its Ala^{84}-Gly^{85} bond) resulting in its auto-digestion. The inactivation of the repressor gives rise to the expression of *recA* and other SOS response-related genes (Walker, 1984; Walker *et al.*, 2000).

It has also been shown that RecA has a significant role in pathogenicity of EHEC by up-regulating the genes responsible for production of STs (Fuchs *et al.*, 1999). Moreover, as stated above, in addition to responding to DNA damage, RecA can participate in cold shock response of *E. coli*.

1.3.6 Viability and Culturability

One of the most interesting aspects of the bacterial stress response, for instance to starvation, acid stress, cold shock and oxidative stress is the induction of "viable but non-culturable" (VBNC) state. Various pathogenic microorganisms have been shown to be capable of undergoing VBNC state, including *Salmonella*, *Shigella* and *E. coli* (Oliver, 2005)

The VBNC phenomenon has been extensively discussed by Kell *et al.*, (1998); Rowan (2004) and Oliver, (2005; 2010). In general, VBNC cells are morphologically intact as well as metabolically active (alive), but unable to grow and form colonies on growth media (Oliver, 2005; Kell *et al.*, 1998). A possible reason suggested for lack of culturability of sub-lethally stressed cells on common rich media is the high toxicity of nutrient rich media for VBNC cells (Rowan, 2004). According to Bloomfield *et al.*, (1998) stress conditions can arrest the growth of the cells without affecting their metabolic activity (de-coupling of growth and metabolism). As a result, when these cells are grown on rich medium, they generate lethal concentrations of free superoxide radicals. However, due to a lack of the necessary coping mechanism and adaptive

responses such as protein production, they are incapable of reducing the toxic level of ROS. This subsequently initiates a cell suicide process, leading to cell death. Whether or not VBNC cells can resuscitate is a highly debated subject. For instance, it has been shown that supplementation of VBNC *E. coli* with amino acids and radical scavengers such as sodium pyruvate and catalase can result in resuscitation of VBNC cells (Mizunoe *et al.*, 1999; Pinto *et al.*, 2011). Moreover, Boaretti *et al.*, (2003) and Asakura *et al.*, (2005) demonstrated that the expression of *rpoS* could improve the survival of VBNC cells as well as their subsequent culturability. However, Arana *et al.*, (2007) suggested that *E. coli* is unable to resuscitate from VBNC state, and the observed increase in other resuscitation studies could have been due to the re-growth of culturable cells. They hypothesized that the VBNC state is simply an adaptive stage of *E. coli* during which the organic substances of non-culturable cells are used by the surviving cells, so the population can benefit as a whole.

The presence of VBNC *E. coli* in various food and drinks such as lettuce (Dinu & Bach, 2011), spinach (Dinu & Bach, 2013), parsley (Lang *et al.*, 2004), salmon roe (Makino *et al.*, 2000), milk (Gunasekera *et al.*, 2002), drinking water (Liu *et al.*, 2008) and grapefruit juice (Nicolò *et al.*, 2011) has been reported. In case of the latter, there was no difference between the numbers of culturable (grown on plate count agar) and viable (assessed using viability dyes) *E. coli* in samples incubated at 4 °C. However, after 24 hours there was a dramatic 6 $\log_{10}$ increase in the number of VBNC cells. Further incubation for 24 h, resulted in total loss of viability and culturability. Resuscitation was observed for samples

after 24 h but not 48 h.

It is important to note the suggestion that VBNC *E. coli* is capable of retaining some of its virulence factors. For instance, Liu *et al.*, (2010) reported that VBNC *E. coli* O157:H7 was capable of expressing both Vero toxin genes of *stx1* and *stx2*. However, this does not necessarily mean that VBNC EHEC retain their pathogenicity as many other virulence factors such *eae*, *tir*, *espA* and *espB* are also involved in causing foodborne diseases (Li *et al.,* 2000).
Moreover, Makino *et al.*, (2000) suggested that VBNC *E. coli* O157:H7 could have contributed to a salmon roe-associated outbreak in 1998. They hypothesized that the induction of VBNC state could have been the main reason for under-estimation of the real infectious dose of the pathogen by investigators of the outbreak.

1.3.7 Detection of VBNC Cells

Based on what was described above, it is important to use appropriate techniques in order to determine the presence of VBNC *E. coli*. Plate-culturing which is the most common technique used in food industry for evaluating the microbiological quality of the foods, is incapable of detecting these potentially pathogenic cells. In recent years, various procedures have been employed for detecting VBNC cells. These include quantitative real-time PCR (qRT-PCR) (Brandt & Podivinsky, 2008), the application of viability dyes (Breeuwer & Abee, 2000), studying the expression of genes (by means of a green fluorescent protein or GFP indicator) as well as flow cytometry (FCM) (Gunasekera *et al.*,

2002; Davey & Kell, 1996). It has been shown that the application of qRT-PCR for detection of VBNC in environmental samples could lead to both under- and over-estimation due to interferences of the matrix (Artz *et al.*, 2006). Therefore, viability staining and FCM seems to be a more suitable tool for detecting VBNC cells.

1.4 Flow Cytometry (FCM)

1.4.1 Introduction to FCM

The most common techniques used in food microbiology for detection, enumeration and viability assessment of food-borne pathogens are viable plate count method. This technique mainly relies on using selective or non-selective media (De Boer & Beumer, 1999). For instance, in case of *E. coli*, MacConkey agar and tryptone soy agar are commonly used as selective and non-selective media respectively (Rocelle *et al.*, 1995). However these techniques are not only time-consuming but also incapable of providing a true picture of the physiological state of the microbial population. For instance, some food-borne pathogens including *E. coli* can enter a VBNC state in response to a wide range of chemical and environmental stresses. These cells are viable but unable to form colonies on growth media. More importantly, they could potentially recover and cause disease when the growth condition becomes suitable (Oliver, 2010). Therefore, it is of utmost importance to employ a technique which is capable of detecting these cells.

In recent years, various novel immunological- and biosensor-based methods

such as bioluminescence and fluorescence microscopy have been successfully used for detection of food-borne pathogens within food matrices (Velusamy *et al.*, 2010), however the majority of these techniques suffer from lack of specificity and are incapable of comprehensive characterization of the physiological state of the cells. For instance, fluorescence microscopy, which has been commonly used for evaluating the viability of the cells and also in cell counting studies, suffers from low reproducibility as well as being subjective to the population observed (Comas-Riu & Rius, 2009). By contrast, FCM can be used not only for enumeration and detection of microorganisms within a food matrix, but also for simultaneous objective study of the morphological or physiological state of the cells at the single cell level. This includes investigating the cell size and granularity, DNA content, intracellular pH (pH_i), membrane integrity and potential, enzymatic, metabolic and respiratory activities, heterogeneity of the microbial population as well as cell counting (Ueckert *et al.*, 1995). Most importantly, when FCM is used along with plate counting, this technique is effectively capable of calculating the number of VBNC cells by subtracting the number of culturable cells from the total viable cell count (Khan *et al.*, 2010). Before discussing the applications of FCM in food microbiology it is important to describe the principles behind this technique.

1.4.2 Principles of FCM

As inferred from its name, FCM is a technique which is principally used for characterizing cells which have been suspended in a fluid while they pass through the system. The principles of FCM have been discussed exhaustively by

Shapiro (2003) and hence will be described here in summary only. A flow cytometer consists of four main units: fluidics, optics, electronics and software [Figure 1.6]. The main purpose of the fluidics unit is to transfer cells through the flow cell where cells are observed as well as hydrodynamic focusing of the cells in order to introduce single cells to the laser beam. When cells pass through the laser, they scatter and emit light, which are collected by the optics unit and introduced to the detectors. This results in generation of photoelectrons. The electronics unit then converts the generated analogue voltage to a digital output (channel number) which is used by appropriate software for analyzing the characteristics of the cells.

FLUIDICS

The hydrodynamic focusing of the cells is generally achieved using a fluid called sheath; though the application of microcapillary systems has also been reported. In sheath-based flow cytometers, which were used for this study, the flow cell is similar to a conical nozzle consisting of a conical tapered region linked to a rectangular flat-sided quartz cuvette [Figure 1.7]. A stream of fluid containing the cells is injected into the centre of a sheath stream at the top of a conical tapered region resulting in co-axial laminar flow of the two streams. The sheath fluid acts as the focusing fluid, forcing the cells into the centre of the narrow core stream, ideally with a diameter close to the diameter of the cells. This is called hydrodynamic focusing and is achieved by regulating the velocity of the sheath using vacuum or constant volume peristaltic pumps. Hydrodynamic focusing of the cells is done in order to ensure that not only

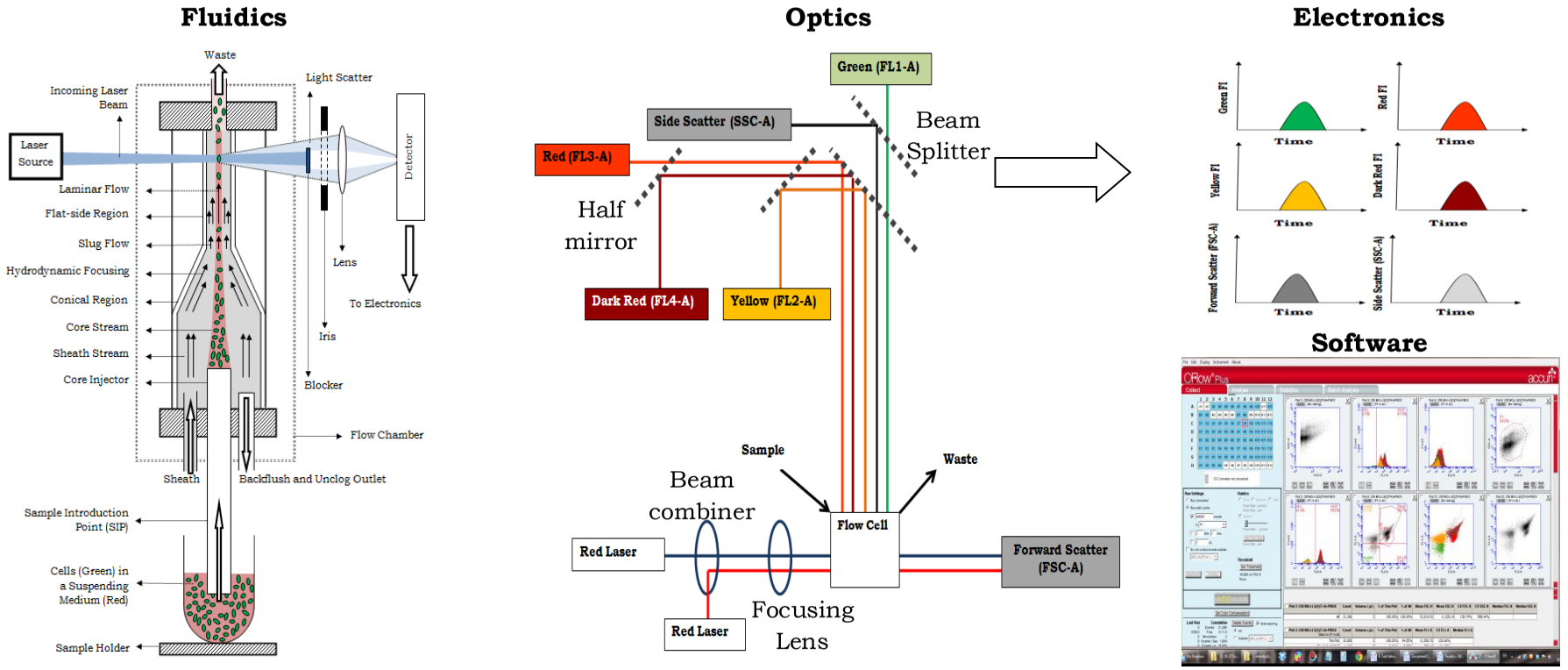


Figure 1.6: Schematic illustration of a flow cytometer similar to the one used in this study

The main purpose of the fluidics unit is to transfer cells through the flow cell where cells are observed as well as hydrodynamic focusing of the cells in order to introduce single cells to the laser beam. When cells pass through the laser, they scatter and emit light, which are collected by the optics unit and introduced to the detectors. This results in generation of photoelectrons. The electronics unit then converts the generated analogue voltage to a digital output (channel number) which is used by appropriate software for analyzing the characteristics of the cells.

Adapted from: BD Biosciences (2013) (http://www.bdbiosciences.com/research/multicolor); ; Shapiro (2003)

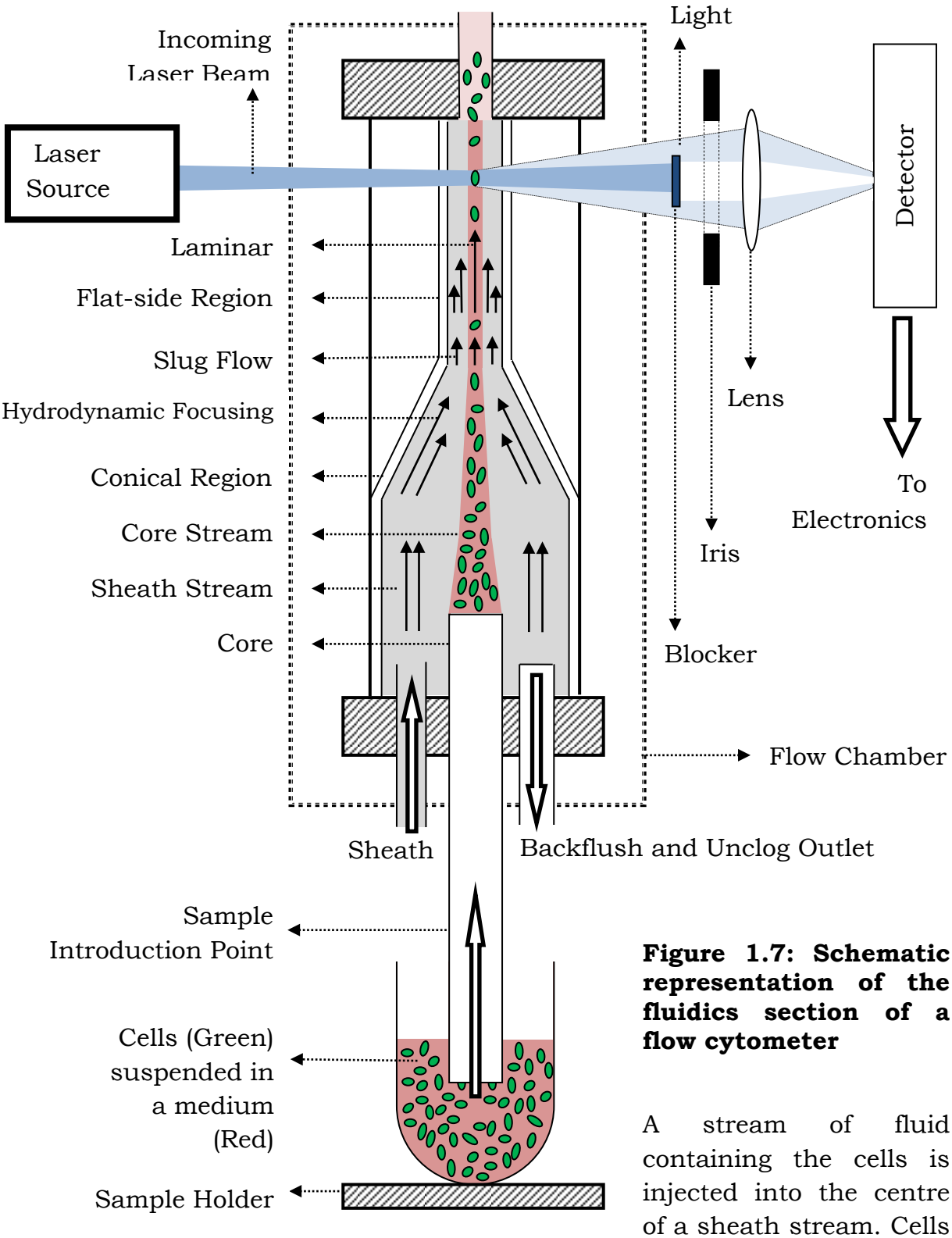


Figure 1.7: Schematic representation of the fluidics section of a flow cytometer

A stream of fluid containing the cells is injected into the centre of a sheath stream. Cells are hydrodynamically focused before passing through the laser.

Adapted from:
Shapiro (2003)

single cells are introduced to the laser but also these cells are subjected to relatively uniform maximal intensity of the light beam.

Optics

The purposes of the optics section of flow cytometers are to illuminate the cells with light from an appropriate light source, collecting the scattered and emitted lights from the cells, and directing them to appropriate detectors in order to measure the physical parameters of interest such as light scatter and fluorescence.

Light Sources: Throughout the history of the FCM, various types of light source have been used, such as Arc lamps, quartz-halogen lamps and light emitting diodes (LEDs). The main disadvantage of these light sources is the need for using various light excitation filters and dichroic mirrors for defining the desired excitation and emission wavelengths. In contrast to the aforementioned extended lights, lasers are powerful point light sources which not only benefit from being monochromatic but also have greater light intensity. For this reason, nowadays, lasers are the most common types of light source used in optical system of flow cytometers. Depending on the type of laser, for instance, gas lasers (e.g., argon, krypton, or helium-neon), solid state and semiconductor lasers or diode lasers (e.g., red diode gallium indium phosphide), various excitation wavelengths can be achieved ranging from 275 nm to 800 nm. Nonetheless, the most common excitation wavelength used in flow cytometers is 488 nm (blue-green laser) followed by 633-640 nm (red laser).

Light Scatters: Once cells are subjected to light, excluding the amount which is absorbed by the cells, the remaining is scattered in all directions. However in FCM, scattered light is detected in two ways: small angle or forward scatter (FSC) which is detected along the axis of the light source; and, large angle or side scatter (SSC) which is collected perpendicular to the light beam. The measurement of scatter in these two directions is mainly because of their significance in characterizing the morphological state of the cells. FSC is commonly used as a rough indicator of the cell size, and for uniform spherical particles the intensity of FSC is almost proportionate to their size. Strictly speaking, the intensity of FSC depends not only on the cell size but also the refractive index of the cells and/or suspending medium, extinction (i.e., absorbed light), the light wavelength and also the angle at which the light was collected (0.5-5°). SSC on the other hand, has been shown to be a good indicator of the cell granularity and surface roughness.

Fluorescence: The other important optical parameter measured with the majority of flow cytometers is fluorescence. In case of fluorescent cells (either naturally fluorescent or stained with various fluorophores), depending on the type of fluorophore and the light wavelength, the light absorbed by the cell can results in excitation of the fluorophore and therefore emission of fluorescence. The fluorescence intensity (FI) could then be used for understanding various physiological parameters of the cell (e.g., viability, gene expression, internal pH, etc.). Using dichroic mirrors and filters, the emitted fluorescence is directed to

several detectors, each collecting light at a specified range of wavelengths.

Detectors: Depending on the intensity of the generated signal (number of photons), two types of detector are used in flow cytometers: Silicon photodiodes (generally for strong signals of forward scatter only); or photomultiplier tubes (PMTs) for both side scatter and fluorescence which are by and large weak signals. PMTs are vacuum tubes which are capable of binary logarithmic increase of the weak signals received by the anode using a chain of electrodes (dynodes). Both detectors generate photoelectrons, hence electric current upon receiving photons; however, the PMTs require external power, unlike most photodiodes. The number of photoelectrons released from a PMT depends on the number of dynodes and the voltage applied to the PMT which could either be fixed (e.g., in BD Accuri C6 flow cytometer used in this study) or adjusted by the operator.

It is important to note that, the background light received by the detectors from the light beam, optical components or fluid stream can create a signal even when no cell is present in the sheath. In other words, flow cytometers have an inherent detection limit (threshold level). As a result, in FCM studies, it is important to set the threshold limit before analyzing the samples and collecting cells in order to eliminate the excess background signal.

ELECTRONICS

When a particle passes through the light beam, it creates an electric current or

signal with a distinctive peak height, width and area (integral). The geometry of the peak depends not only on the properties of the particle (e.g., size and/or fluorescent contents) but also the speed of sheath stream and the geometry of the light beam. For instance, in case of the fluorescent measurements, when the diameter of the light beam is larger than the particle size, the peak height is relatively proportionate to the total fluorescence content of the cell. However when the beam is narrower than the cell diameter (slit-scanning), peak width and height, are proportionate to the size and fluorescent density of the cells respectively, whereas the area indicates the total fluorescence.

In order to facilitate the analysis of these parameters with FCM software, the analogue voltage values are first converted into a digital number called channel number, using an analogue-to-digital converter (ADC). Due to binary nature of ADCs, an n-bit resolution ADC has 2^n channels. The flow cytometer used in this study contained 24-bit resolution ADCs, capable of generating 16,777,216 channels. The digital data are then analysed using a FCM software.

SOFTWARE

Data Plotting: Various software packages are available for displaying and analyzing the digitised data. Data can either be plotted linearly or logarithmically, however this mainly depends on the parameter of interest and the values obtained for it. For instance, in case of measuring the FI, cell size and granularity which are the subjects of this study, logarithmic presentation of data, makes it easier to distinguish between the positive (for instance

fluorescent cells) and negative (non-fluorescent) cells. The data are generally displayed in three formats: histograms (generally for single-parameter display of data) or dot plots and density plots for multi-parameter plots. [Figure 1.8] shows an example of all three types of graphs for displaying the data for a sample of OJ, containing a mixture of *E. coli* K-12 and natural OJ flora yeast.

Gating: After displaying the data in a plot and identifying the population of interest (e.g., cells), the next step is to define this population by creating a region which is called the gate. In addition to defining the population, gating also facilitates the exclusion of the interfering populations, particulates and noise from the analysis of the sample. The gating can be performed either based on a single parameter, such as FSC, SSC or FI, or a combination of these parameters (multi-parameter gating). Examples of different types of gating region such as shapes (polygons, rectangles), quadrants and markers (horizontal and vertical) have been have been shown in [Figure 1.9].

1.4.3 Applications of FCM in Food Microbiology

History and Obstacles

In 1947, Gucker *et al.*, (1947) reported the use of a photoelectronic counter for detection of viable spores of *Bacillus globigii* (now known as *B. atrophaeus;* Fritze & Pukall, 2001). This is widely considered to be the first application of a flow cytometer for the detection of microorganisms. However, in spite of the fact that the potential of FCM for microbial studies has been known for more than 60 years, the widespread utilization of this technique in microbial studies is

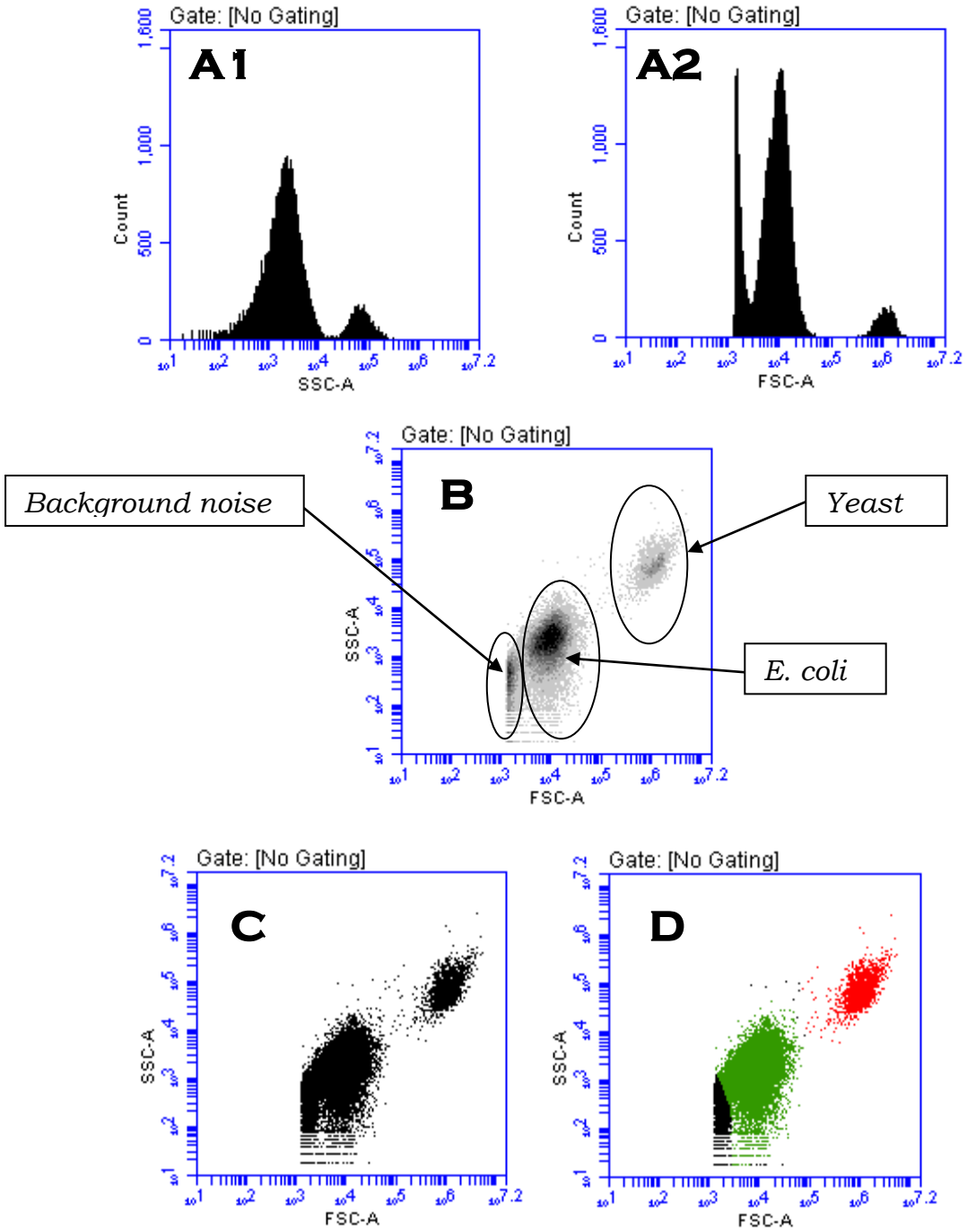


Figure 1.8: Three types of plots for showing one or two parameters

Plots shown here are for a single sample of OJ inoculated with *E. coli* (Green population) and yeast (Red population).

(A1 and A2) Histogram for SSC-A and FSC-A respectively;
(B) Black and white density plot of FSC-A (histogram A2) versus SSC-A (histogram A1);
(C) Black and white dot of FSC-A versus SSC-A. Each dot represents a single cell or noise event.
(D) Coloured dot plot dot of FSC-A versus SSC-A

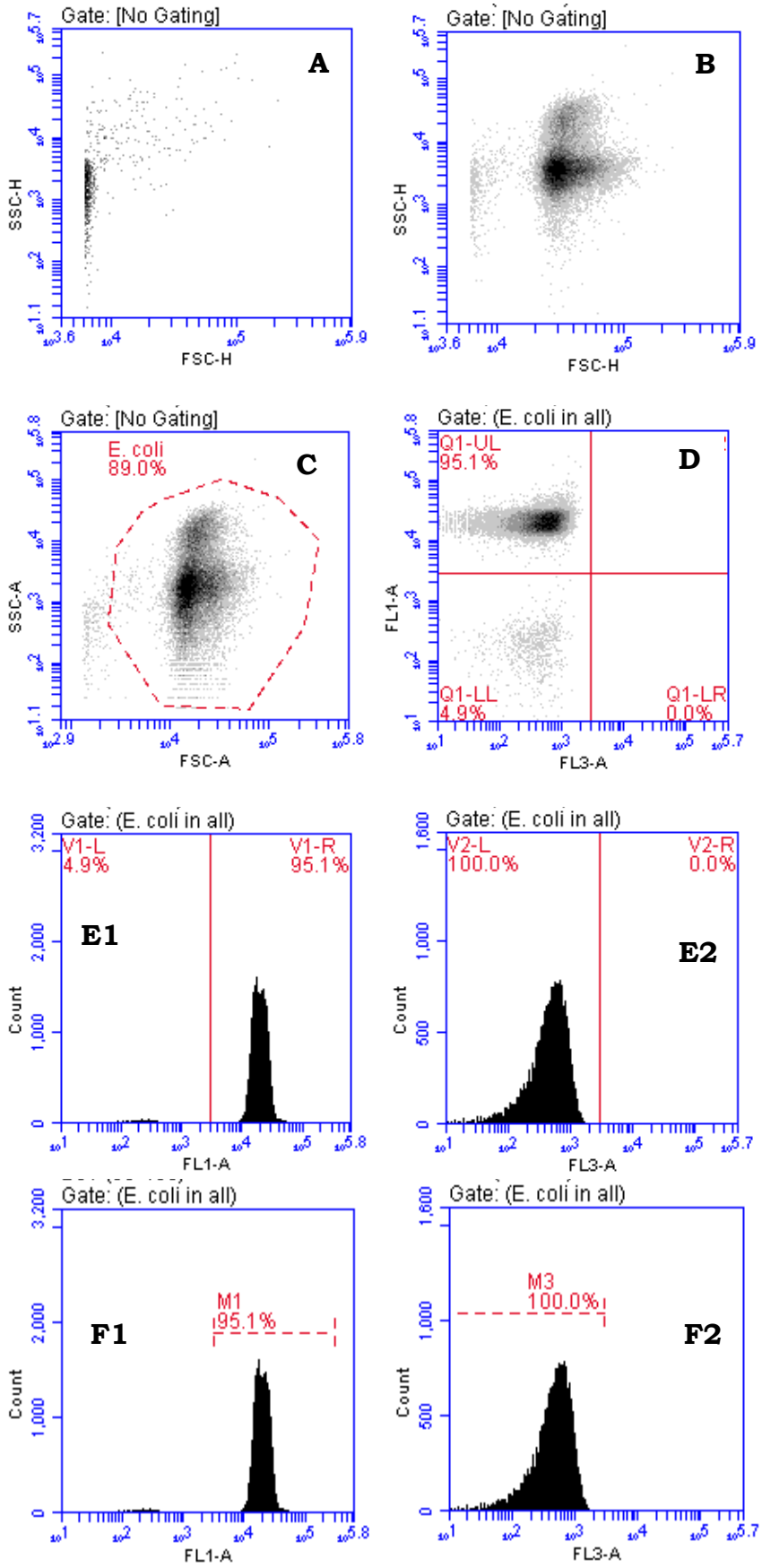


Figure 1.9: An example of a gating strategy

(A) Distilled Water is run in order to detect the noise floor or threshold. (B) Cells (here GFP+ *E. coli* SCC1) are run on the flow and identified based on their characteristic FSC-H and SSC-H. (C) The population of interest are gated (here with a polygon) (D) Here the percentage of GFP+ and GFP- cells is calculated using a quadrant. These values can also be calculated using vertical (E1 and E2) or horizontal (F1 and F2) markers.

quite new. This has been mainly due to the high cost of FCM instruments as well as various other hindrances such as the complex nature of the flow cytometers and the need for expertise for operating these instruments.

In addition, various technical limitations such as lack of effective staining techniques or software for data handling limited the widespread use of flow cytometers. However, in recent years major advances in fluorescence staining and FCM software as well as the introduction of several user-friendly flow cytometers have increased the attractiveness of this technique (Steen, 2000, Comas-Riu & Rius, 2009). Furthermore, in FCM detection of food-borne microorganisms within a food matrix, the cells of interest are generally detected based on their FSC and SSC light intensities. However, the small size of bacteria which is generally close to the intrinsic detection limit of the instrument as well as the presence of suspended food particulates could make it difficult to discriminate between the cells of interest and other signals.

In order to overcome this obstacle, various physical, enzymatic and chemical treatment of food samples have been utilized in order to reduce the level of interferences by removing the food particles, proteins or lipids. However, these techniques are not always effective. Nowadays, the applications of appropriate fluorescent dyes (mainly antibody-linked) along with the multiple gating strategies have been shown to be effective in increasing the sensitivity and detection of limit of the instrument (Comas-Riu & Rius, 2009; Wilkes *et al.*, 2012). The characteristics and method of action of the fluorophores used for

FCM studying of food-borne microorganisms have been described by Comas-Riu and Rius (2009). The current study involved the utilization of multiple fluorophores specifically propidium iodide (PI), Bis-(1,3-dibutylbarbituricacid) trimethine oxonol (BOX), SYTO®62, as well as green fluorescent protein (GFP). The properties and mode of action of these fluorophores have been described in more detail in subsequent chapters.

The first study to apply FCM for the purpose of detecting the presence of a microorganism (*L. monocytogenes*) within a food matrix (i.e., milk) was conducted by Donnelly and Baigent (1986). In this study, cells were stained with fluorescein isothiocyanate-labelled (FITC-labelled) *Listeria* antibodies in order to identify the cells of interest based on their fluorescence. Cells were also stained with PI in order to identify the dead cells. They also showed that plotting the FSC versus SSC resulted in successful discrimination of cells from noise particles.

The application of FCM for detection of *E. coli* in food was first reported by Patchett *et al.*, (1991). The flow cytometer used in this study was Argus 100 Flow Cytometer which used a mercury arc lamp combined with a filter for exciting the fluorophore at 436 nm (Davey, 1994). In this study two nucleic acid binding fluorescent dyes (ethidium bromide and mithramycin) were used for detecting the cells within three food matrices: pâté, braising steak and pasteurized skimmed milk. In case of the pâté and steak, they reported detection limit to be 10^5–10^6 *E. coli* cells per gram of food. However, they were

not able to detect the cells in milk despite treatment of the sample with chemicals (in this case benzalkonium chloride) in order to disrupt the lipid and reduce the background noise.

During the past three decades, many studies have reported the use of FCM for detection, enumeration and characterization of the food-borne microorganisms, within food matrices, including fruit juices. A comprehensive list of these studies can be found in Table 1.8. However, to the best of the author's knowledge, this work is the first study to apply FCM for time-point (static) and real-time (kinetic) investigation of the behaviour of a food-borne pathogen in OJ and a model OJ (MOJ) system.

1.5 Aims and Objectives

The main aim of this study is the investigation of the viability of *E. coli* in OJ under various conditions by using flow cytometric techniques and, where possible, to compare the results with those obtained using traditional plate counting methods.

In order to achieve this goal, the study will focus on the main pre-production (composition of orange juice), production (filtration and washing stages) and post-production (storage) stages of OJ production which were considered to have the potential to affect the physiology of *E. coli* in OJ. The specific objectives are as follows:

Table 1.8: Application of FCM for detection of microorganisms within food matrix (excluding water and alcoholic beverages)

Reference (Year)	Food (Fruit juices in Bold)	Microorganism(s)	Fluorophore(s)	Instrument
Donnelly and Baigent (1986)	Milk (raw)	*L. monocytogenes*	Fluorescein isothiocyanate (FITC), Propidium iodide (PI)	Ortho 50-H/H Dual Laser Cytofluorogra
Donnelly *et al.*, (1988)	Milk (raw)	*L. monocytogenes*	FITC	
Dumain *et al.*, (1990)	Fromage frais	Yeasts and Moulds		ChemFlow AutoSystem
Patchett *et al.*, (1991)	Milk (pasteurized skimmed), Pate, Braising steak	*E. coli*	Ethidium bromide (EB), Mithramycin	Skatron Argus 100
Pettipher (1991)	**Orange juice** (long life, unsweetened), Orange carbonate, Lemonade, **Tomato juice** (ready to eat without salt)	*Saccharomyces cerevisiae, Zygosaccharomyces bailii*	Not specified (Proprietary)	ChemFlow AutoSystem
Bankes *et al.*, (1991)	Dairy and **fruit-based products**	Yeasts		ChemFlow AutoSystem
Laplace-Builhé *et al.*, (1993)	Cheese (cottage), Yoghurt, Buttermilk, Spinach (raw, Blanched, frozen)	*S. cerevisiae, Byssochlamys nivea, Spinach natural bacterial flora, Lactobacillus acidophilus*	Not specified (patented, non-immunofluorescence technique for detection of dead cells only)	ChemFlow AutoSystem
Pinder & McClelland (1994)	Milk (full fat)	*E. coli, S.* Typhimurium, *Salmonella* Montevideo,	FITC	
McClelland & Pinder (1994)	Milk (pasteurized full fat, semi-skimmed, UHT), Egg (white, yolk, whole)	*S.* Typhimurium, *E. coli*	FITC	Proprietary
Seo *et al.*, (1998)	Milk (raw), Ground beef, **Apple juice**	*E. coli* O157:H7	FITC	Coulter Elite Analyser
Iannelli *et al.*, (1998)	Milk (raw milk of cow or water buffalo)	*S. aureus, Brucella abortus*	FITC	BD FACScan

Continued

Goodridge *et al.*, (1999)	Milk (raw), Ground beef,	*E. coli O157:H7*	YOYO-1 stained bacteriophage	Coulter Elite
Suhren *et al.*, (2000)	Milk (raw)	*Natural flora (total count)*	EB	Proprietary (Bactoscan)
Gunasekera *et al.*, (2000)	Milk (raw, UHT)	*E. coli, Staphylococcus aureus*	SYTO BC, PI	BD FACSCalibur
Bunthof & Abee (2002)	Milk, (semi-skimmed pasteurized), Yogurt, Probiotics (yogurt drink, powder, and starters)	*Lactobacillus plantarum*	Carboxy fluorescein diacetate (cFDA), TOTO-1, SYTO 9, PI	BD FACSCalibur
Gunasekera *et al.*, (2003)	Milk	*E. coli, S. aureus, Pseudomonas fluorescens,*	SYTO BC and 9, PI, Cyanoditolyl tetrazolium chloride (CTC), Bis-1,3-dibutylbarbituric acid trimethine oxonol (BOX)	BD FACSCalibur
Holm & Jespersen (2003)	Milk (raw)	*E. coli, S. aureus, Bacillus cereus, Lactobacillus lactis, Pseudomonas spp. Streptococcus dysgalactiae, Enterobacter cloacae, Klebsiella oxytoca, Micrococcus luteus, Streptococcus spp.*	Oregon Green—conjugated wheat germ agglutinin (WGA), Hexidium iodide (HI)	Partec PAS IIIi
Yamaguchi *et al.*, (2003)	Milk (raw), Ground beef, **Apple juice**,	*E. coli O157:H7, E. coli K-12*	CTC, FITC	Beckman Coulter Epics ELITE
Holm *et al.*, (2004)	Milk (raw)	*E. coli, S. aureus, B. cereus, L. lactis, Pseudomonas spp., Streptococcus uberis*	WGA, HI	BD FACScan
Sheehan *et al.*, (2005)	Cheddar cheese (Cheese starter broth, cheese juice)	*L. lactis*	SYTO9, PI	BD LSR
Lahtinen *et al.*, (2006)	Milk (sterile skimmed)	*Bifidobacterium longum, Bifidobacterium lactis, Lactobacillus acidophilus*	SYTO9, PI, cFDA,	BD FACSCalibur

Continued				
Owens *et al.*, (2009)	Spinach, Jalapeño, Ground beef, Bagged salad, Salami, Cookie dough, Hot dogs, Nut meat, Beef (muscle)	*E. coli O157:H7*	Proprietary reagents (fluorescein-conjugated antibodies)	LITMUS RAPID-B 9013
Leach *et al.*, (2010)	Spinach	*E. coli O157:H7*	Antibody-coupled microbeads	Luminex BioPlex 200
Kim *et al.*, (2010)	**Apple juice**, Milk (2% fat), Spinach, Pepper, Tomato, Alfalfa sprouts, Lettuce, Chicken (carcass wash), Ground beef	*E. coli O157:H7, S.* Typhimurium*, C. jejuni, L. monocytogenes*	Antibody-coupled microbeads	Luminex 100
Wilkes *et al.*, (2012)	**Fruit juices (apple and pear)**, Spinach, Sauces (horseradish, tartar, cheese), Baby foods (bananas, peas, carrots, beef/gravy), Milk (pasteurized 2% fat), Ice cream, Chocolate, Mayonnaise, Peanut butter	*E. coli O157:H7*	Proprietary reagents (fluorescein-conjugated antibodies)	LITMUS RAPID-B 9013
Langerhuus *et al.*, (2013)	Milk (raw)	*Staphylococcus spp. (including S. aureus), Streptococcus spp., Enterococcus spp., E. coli, Klebsiella pneumoniae, Serratia marcescens, Pseudomonas aeruginosa*	WGA, FITC, Acridine orange, allophycocyanin	BD FACS-Canto
De Lamo-Castellví *et al.*, (2013)	Milk (UHT skimmed)	*E. coli O157:H7*	SYTO 9, SYTO BC, PI	Beckman Coulter CyAn

1) To investigate the effects of the growth phase of the cells and incubation temperature on the physiology (viability and culturability) of *E. coli* in freshly squeezed unpasteurized OJ as well as a bespoke model orange juice (MOJ) system (Chapter 3).
2) To determine the effects of the main soluble components of OJ (i.e., sugars, organic acids, amino acids and ascorbic acids) on the physiology of *E. coli* in MOJ and OJ (Chapter 3).
3) To investigate the possible role of the insoluble components of OJ (i.e., pulp and cloud particles) on the physiology of *E. coli* (Chapter 4).
4) To study the possible role of the orange fruit sanitation stage on the physiology of *E. coli* in OJ (Chapter 5).

Chapter 2

Materials and Methods

2.1 Materials

Unless stated otherwise, all chemicals were from Sigma-Aldrich (Gillingham, UK), Oxoid (Basingstoke, UK), BDH Ltd. (Poole, UK), BD Biosciences (Oxford, UK) or Fisher Scientific (Loughborough, UK). Consumables were purchased from Greiner Bio-One (Stonehouse, UK), Sarstedt (Leicester, UK) and Fisher Scientific.

2.2 Buffers and Solutions

Unless indicated otherwise, all buffers and solutions were prepared by dissolving the appropriate chemicals in double distilled water (ddH_2O) and autoclaving them at 121 °C and 15 psi for 15 min (with 10 min purging time) or filter-sterilized using a 0.22 µm syringe filter (Millipore).

2.2.1 Diluents

Dulbecco's Phosphate Buffer Saline (PBS)

PBS was used as the primary isotonic solution for diluting or rinsing the cells. It was prepared by dissolving commercial PBS tablets (Oxoid) in ddH_2O (final concentration of NaCl 8.0 $g.L^{-1}$, KCl 0.2 $g.L^{-1}$, Na_2HPO_4 1.15 $g.L^{-1}$ and KH_2PO_4 0.2 $g.L^{-1}$, pH 7.3) according to manufacturer's instructions.

Maximum Recovery Diluent (MRD)

MRD was exclusively used for decimal serial dilution of the samples prior to their plating on solid growth media due to its optimal osmolarity for recovery of stressed cells (Jordan *et al.*, 1999). MRD was prepared by dissolving 8.5 $g.L^{-1}$ NaCl and 1.0 $g.L^{-1}$ peptone in ddH_2O (both Sigma; pH 7.0 ± 0.2) and was autoclaved before use.

2.2.2 Viability Dyes for Flow Cytometric (FCM) Studies

Propidium Iodide (PI)

PI is a red fluorescent viability dye which binds to the DNA of dead cells but is excluded from cells with an intact membrane. The working solution of PI (Sigma) was prepared by dissolving 4 mg of PI powder in 20 mL of Milli-Q de-ionized H_2O (300 µM). The solution was filter-sterilized, divided into 1 mL aliquots, stored at 4 °C and used within six months of preparation.

Bis-Oxonol (BOX)

The anionic dye bis-(1,3-dibutylbarbituric acid)trimethine oxonol [$DiBAC_4(3)$] also known as Bis-oxonol or BOX was used in this study for detecting cells with depolarized membrane (i.e. injured or dead). Stock solutions (19.36 mM) were prepared by dissolving 25 mg BOX (Sigma) in 2.5 mL dimethyl sulfoxide (Sigma). It was then divided into 100 µL aliquots and stored at -18 °C until use. In order to prepare the working solution of 19.36 µM, the stock solution was defrosted from which 5 µL was added to 4,795 µL of sterile PBS and 200 µL of filter-sterilized 0.1 M EDTA. Working solutions were stored at 4 °C for up to 1 month. BOX was not filter-sterilized.

SYTO-62

The cell-permeant nucleic acid fluorescent dye of SYTO62 was used in order to differentiate between cells and the background noise (i.e. particulates) based on the fluorescence of the cells at 675 ± 25 nm. The stock solution of SYTO-62 (5 µM) was prepared by mixing 1 µL of 5 mM SYTO-62 (Molecular Probes, Leiden, Netherlands) with 999 µL of Tris buffer solution and stored at -18 °C until use. The Tris buffer solution contained 50 mM Tris (Sigma-Aldrich) and 1 mM EDTA in ddH_2O (pH 7.5).

2.2.3 Sanitizers

Chlorine-Based Sanitizers

Sodium hypochlorite (NaOCl) containing 10–15% available chlorine (Sigma) was utilized for preparing chlorine-based sanitizing solutions. The mid-range value of 12.5% available chlorine was used for calculating the concentration of NaOCl needed for preparation of the sanitizers. The solutions containing 50, 100 or 200 and 250 ppm available chlorine were prepared by diluting 0.2, 0.4, 0.8, 1 and 1.25 mL of NaOCl in ddH_2O to the total volume of 5 mL and performing two decimal serial dilution (1:100) just before use. In order to prepare the "acidified chlorine" and "surfactant-containing chlorine" solutions, citric acid (BDH Ltd.) and Tween-80 (Sigma) were used as acidulant and surfactant respectively. With regard to acidified chlorine solution (200 ppm available chlorine + 2% citric acid), the solution with 250 ppm available chlorine was gently added to a 10%

(w/v) citric acid solution in the ratio of 4:1 (v/v). For surfactant-containing chlorine solution, 250 ppm chlorine solution was mixed with 500 ppm Tween-80 in the ratio of 4:1 (v/v) in order to achieve a solution containing 200 ppm available chlorine and 100 ppm Tween-80. The solution was not filter-sterilized

Hydrogen Peroxide (H_2O_2)

A 30% (w/w) solution of H_2O_2 (Sigma) in H_2O with density of 1.11 $g.mL^{-1}$ was used for preparing 1%, 2.5% or 5% (all w/w) H_2O_2 sanitizing solutions. This was achieved by diluting the 30% H_2O_2 in ddH_2O in the ratio of 1:32.19, 1:12.21 and 1:5.55 respectively. Solutions were prepared just before use and not sterilized.

Organic Acids

2% and 4% (w/v) solutions of citric acid were prepared respectively by dissolving 2 g and 4 g of citric acid (BDH Ltd., Poole, UK) in ddH_2O and adjusting the volume to 100 mL. An 85% (w/w) in H_2O lactic acid (Sigma) with density of 1.206 $g.mL^{-1}$ was diluted in ddH_2O in the ratio of 1:24.42 in order to prepare a 4 % (w/w) solution. The 4% (w/v) acetic acid solution was prepared by diluting 3.813 mL of glacial acetic acid (Fisher Scientific, density: 1.049 $g.mL^{-1}$) in ddH_2O and adjusting the volume to 100 mL. With regard to pH adjusted acetic acid-containing solutions (pH 3–7), 3.813 mL of glacial acetic acid was diluted in ddH_2O before adjusting the pH with 1 M NaOH and adjusting the volume to 100 mL. All solutions were prepared on the day of the experiment and filter-sterilized before use.

2.3 Orange Juice (OJ)

Freshly squeezed unpasteurized OJ with "bits" was purchased from a local retailer. According to the product label it contained 9.3 g sugars, 0.71 g protein and 46 mg ascorbic acid per 100 mL OJ. The OJ was stored at 4 °C until its expiry date and used within 24 h once opened.

2.3.1 Clarification of OJ

Various protocols have been reported in the literature for separation of OJ pulp and cloud particles from the OJ serum (Mizrahi & Berk, 1970, Corredig et al., 2001; Brat et al., 2003; Ackerley & Wicker, 2003). Based on the aforementioned studies and preliminary experiments, the following protocols were developed for the purpose of OJ clarification.

Removal of Pulp

In order to obtain pulp-free OJ, approximately 300 mL of OJ was transferred to a 500 mL 69×160 mm polypropylene centrifuge bottle (Beckman Coulter Inc., High Wycombe, UK) and centrifuged with a Beckman J2-21 centrifuge. The speed, centrifugation time, and the minimum and maximum temperature settings used were 17,696 g, 40 min (excluding the acceleration and deceleration times), 2°C and 8°C respectively. The temperature compensation of -2 °C was also applied according to the manufacturer's instruction.

Removal of Cloud Particles

The supernatant of the centrifuged pulp-free OJ was filtered through 11, 8, 1.6, 1.2 or 0.7 µm sterile filter papers (Whatman, Maidstone, UK) or the 0.22 µm syringe filter (Millipore) in order to remove different components of the cloud. Filter papers were first placed in a 25 mm acetal-resin syringe disk filter holder (Pall Corporation, Port Washington, USA) through which OJ was filtered using a plastic 50 mL syringe (Sarstedt). With regard to 0.7 and 0.22 µm-filtered OJ samples, due to blockage of the filter with large particles of the centrifuged OJ, the filtrate of 1.2 µm filtration was used instead of the former.

2.3.2 Supplementation of Centrifuged OJ with Pulp

Following the centrifugation, in order to obtain OJ with known pulp content, the supernatant was supplemented with 5% or 10% (w/w) pulp (the pellet).

2.4 Model Orange Juice (MOJ)

2.4.1 Composition

MOJ solutions containing different concentrations of the major components of OJ were prepared. The MOJ consisted of different concentration of sucrose, glucose (both BDH Ltd.), fructose (Sigma), citric acid (BDH Ltd.), malic acid, ascorbic acid, L-proline, L-arginine, L-aspartic acid, L-asparagine, L-glutamic acid, L-serine, L-alanine, γ-aminobutyric acid (all Sigma) and potassium citrate monohydrate (Fluka). The exact compositions and/or concentrations of various MOJ solutions used in this study and their physico-chemical characteristics have been described in the following chapter. Regardless of the composition, all solutions were prepared on the day of the experiment by dissolving the required

amount of each compound in Milli-Q de-ionized H_2O and filter-sterilized using a 0.22 µm syringe filter (Millipore). MOJ solutions were prepared not more than 30 min prior to the start of the experiment and kept at 4 °C until use.

2.4.2 Physico-chemical Analysis of OJ and MOJ

Moisture Content of Pulp

The moisture/dry mass content of the OJ pulp was determined by placing approximately 10 g of the pulp in porcelain evaporation dishes and placing them in a 90 °C incubator until no change in their weight was observed.

OJ Cloud Particles Size Distribution

The size distribution of cloud particles in OJ was measured by laser diffraction using a Malvern Mastersizer 2000 equipped with a Malvern Hydro 2000SM particle size analyzer (Malvern, UK). The refractive indices of cloud particles and dispersed phase and the absorption index of cloud particles were set at 1.73, 1.33 and 0.1 respectively as described by Corredig *et al.*, (2001). A mixture of 10 mL filtered OJ and 100 mL de-ionized H_2O was stirred at 2,500 rpm when passed through the optical cell. In total ten measurements were recorded per sample for particles of between 0.02 µm and 2 mm.

pH (MOJ and OJ)

A Hanna HI 9321 bench pH meter (Hanna Instruments) was utilized for measuring the pH of OJ and MOJ. The pH measurements were performed for samples after equilibration at room temperature. Before each measurement, the

pH meter was calibrated using standard buffer solutions with pH of 4, 7 and 10.

Optical Density (OD) of OJ

The OD of the filtered OJ samples (pre- and post-autoclaving) was measured with a UVIKON 922 spectrophotometer (UVIKON, UK) at 600 nm (Klavons *et al.*, 1991). OJ was autoclaved in order to investigate the filtration-dependant presence of heat-sensitive cloud particles (see Chapter 4).

Osmolality (MOJ and OJ)

The osmolality of the MOJ and OJ (both filtered with a 0.22 µm syringe filter) was measured using a Wescor 5500 vapour pressure osmometer (Wescor, Logan, USA). Prior to the experiments, the osmometer was cleaned and calibrated with 100, 290 and 1,000 $mmol.kg^{-1}$ standard calibration solutions (Wescor) according to the manufacturer's instructions. The osmolality measurements were performed by placing a 6.5-mm paper sample disc in the central depression of the sample holder and loading 2 µL of the sample onto the disk using a pipette before initiating the measurement. The osmolality of each sample was measured at least ten times and repeated at least three times.

2.5 Bacterial Methods

2.5.1 Bacterial Strains and Plasmids

For this study, two *E. coli* K-12 strains were used: (a) MG1655 (Wild type F^- λ^- *ilvG- rfb-50 rph-1*) as the non-fluorescent strain, and (b) SCC1 as the fluorescent

strain. The latter is a genetically manipulated MG1655 containing a chromosomal insertion of $P_{A1/04/03}$-*gfp*mut3* promoter which drives the constitutive expression of green fluorescence protein or GFP (Miao *et al.*, 2009). The strains were kindly provided by Dr Vizcaino Caston and Mr Christopher Wyre respectively (School of Chemical Engineering, University of Birmingham).

2.5.2 Growth Media

Liquid Growth Media

Throughout this study, double concentrated Lennox broth (2×LB; Lennox, 1955) was used for the bacterial culture. It was prepared by dissolving 20 $g.L^{-1}$ tryptone-peptone, 10 $g.L^{-1}$ yeast extract (both Difco) and 10 $g.L^{-1}$ NaCl (Sigma) in ddH_2O.

Solid Growth Media

Solid growth medium used in the study included nutrient agar, MacConkey agar (MAC) and eosin-methylene blue agar (EMBA) (all Oxoid). Based on the preliminary experiments, nutrient agar was chosen as the primary non-selective medium in culturability studies for recovering cells from samples on solid media. The study involved comparing the rate of recovery of *E. coli* immediately (0 h) and 3 h post-inoculation in MOJ (4 °C and 37 °C) on the most commonly used non-selective (tryptic soy agar and nutrient agar) and selective (MacConkey agar) growth media for *E. coli* (see Appendix 1 for more details). Nutrient agar, MAC and EMBA were prepared according to manufacturer's instructions by dissolving respectively 28 g, 52 g and 37.5 g in 1 L ddH_2O. They

were then sterilized by autoclaving and allowed to cool down to reach around 60 °C before pouring 20–25 mL of molten media into sterile 92×16 mm Petri dishes (Sarstedt). Plates were kept at 4°C, used within one month and dried for 20–30 min at 60 °C before use in order to remove the excess moisture.

2.5.3 Glycerol Stock Preparation and Isolation of Pure Cultures

For each strain, glycerol stocks were prepared by growing an overnight (18 h) culture of a single colony in 20 mL of 2×LB in a 250 mL Erlenmeyer flask at 37 °C with aeration (150 rpm). Culture was then diluted (1:500) in fresh medium and grown again in the same condition until it reached mid-exponential-phase ($OD_{(650\ nm)} \approx 0.5$). It was then mixed with 80% sterile glycerol (Sigma-Aldrich) in the ratio of 4:1 and stored at -80°C until use. In order to isolate cells from glycerol stocks, an inoculum of bacteria from the frozen stock was streaked onto a MAC agar plate using a sterile inoculating loop, and grown at 37 °C for 24 hours to obtain single colonies. Subsequently, 2 single colonies were re-streaked on a separate nutrient agar plate and grown for 24 h. Finally, plates were stored at 4 °C and used within a two week period.

2.5.4 Growth Conditions (Pre-cultures and Cultures)

Unless stated otherwise, a single colony was picked from the surface of the re-streaked agar plate and dispersed in 20 mL of 2×LB. Cells were grown overnight (18 h) in a 250 mL Erlenmeyer flask at 37 °C with aeration (150 rpm) in a shaker incubator. Subsequently, 50 µL of the overnight culture was added to 50 mL of fresh medium in a 500 mL Erlenmeyer flask (1:1,000 dilution) and

allowed to grow at the same condition until it reached the desired growth phase. The $OD_{(650\ nm)}$ of the culture was measured with a spectrophotometer.

2.5.5 Harvest and Inoculation of *E. coli*

Unless stated otherwise, 3×10^9 *E. coli* cells were transferred to 50 mL plastic centrifuge tubes and centrifuged at 3,256 g for 10 min in a Jouan C4.22 centrifuge (Jouan, Saint-Mazaire, France). Subsequently, the supernatant was disposed and the pellet dispersed in 50 µL of PBS by vortexing. The suspension of cells in PBS was then aspirated with a pipette and added to 15 mL of the sample (e.g., OJ, MOJ, PBS, etc.) in a 25 mL universal bottle to achieve a final concentration of 2×10^8 cells.mL^{-1}. Cells were dispersed in the medium by vortexing the bottle for 12 seconds before taking samples. When appropriate, cultures were also transferred to 2 mL microcentrifuge tubes, which were centrifuged in a 5418 Eppendorf centrifuge (Eppendorf, Hamburg, Germany) and centrifuged for 5 min at 16,873 g.

2.6 Cell Physiology Analyses

2.6.1 Culturability

In order to determine the number of culturable cells, each sample was serially (decimal) diluted in MRD by addition of 0.5 mL of the sample to 4.5 mL of MRD in test tubes. Then, 100 µL of the appropriate dilutions were plated on separate agar plates and incubated upside down at 37 °C for 48 h. As recommended by Jordan *et al.*, (1999), colony counts were performed twice: once after 20 h; and again after 48 h in order to identify the number of stressed cells with delayed

recovery. The values reported throughout this body of work are the colony counts after 48 h of incubation. Plates with colony forming unit (CFU) counts of between 30 and 300 were chosen. The $\log_{10}$ CFU.mL^{-1} was calculated using the following equation:

Equation 2.1: $$CFU.mL^{-1} = \frac{CFU \times 10^{(number\ of\ decimal\ dilutions)}}{Samples\ Volume\ Plated_{(mL)}}$$

2.6.2 Flow Cytometric (FCM) Studies

The Accuri BD C6 flow cytometer (BD Accuri, Oxford) was used throughout this study in order to investigate the viability and light scatter properties of *E. coli* cells as well as cell counting. The equipment was cleaned before and after analysis and its performance was validated according to the manufacturer's instructions. De-ionized Milli-Q H_2O and PBS used for diluting the samples were filter-sterilized with 0.22 µm syringe filter before use. Unless stated otherwise, analysis of samples with FCM consisted of the following stages:

Setting up the Instrument and Validation

Before analyzing the samples, it was important to determine the threshold (detection limit) in order to differentiate between the background noise and the signal. In order to achieve this, a flow tube (Sarstdet) containing 1 mL of de-ionized H_2O was placed at sample injection point (SIP) and analyzed at fast settings (fluidics rate 66 µL.min^{-1}). The minimum forward scatter peak height channel number (FSC-H) resulting in less than 10 events.µL^{-1} was chosen as

the threshold which ranged between 6,000 and 20,000 (mean 10,000) depending on the performance of the instrument on the day of experiment.

Identification of *E. coli* Cells and Gating

Once the threshold was set, the fluidics rate was reduced to 13 µL.min^{-1} (slow setting). Depending on the type of sample and/or the number of *E. coli* cells present in the sample, they were either analyzed without dilution or diluted in 0.5 mL of filter-sterilized PBS before analysis. The principle behind dilution was to achieve a cell suspension containing between 5×10^6 and 1×10^7 cells.mL^{-1}. This ensured the optimal data resolution could be obtained by collecting between 1,000 and 2,500 events.s^{-1} at 13 µL.min^{-1}. Following the dilution, the sample was placed on the SIP and analyzed on the FCM. The cells were detected based on their typical light scatter parameters (forward and side scatters peak area, FSC-A and SSC-A respectively) on a density plot as shown previously in [Figure 1.9]. Next, cells were defined by drawing a polygonal gating region around the cells within the FSC-A/SSC-A density plot. Subsequently, the FCM was set to collect the data for 20,000 events within the defined gate.

Staining Protocol

In addition to light scatter parameters of the cells (i.e., FSC-A and SSC-A), their fluorescence intensity (FI) was also measured. The C6 consisted of four fluorescence emission detectors, three of which were used in this study: green (FL1-A: 530 ± 15 nm), red (FL3-A: > 670 nm) both for excitation with 488 nm blue laser, and red (FL4-A: 675 ± 12.5 nm) for excitation with 640 nm red laser.

FL1-A was used for GFP^+ cells or cells stained with BOX. FL3-A and FL4-A were used for measuring the FI of cells stained with PI or SYTO-62 respectively. Samples were stained with PI and/or BOX by diluting the stock solution in the sample in the ratio of 1:50 and 1:100 in order to achieve final concentration of 5.89 µM and 191.64 nM respectively. SYTO-62-was added to the sample in the ratio of 1:100 (final concentration of 49.51 nM SYTO-62 in the sample) and was only used for differentiating the cells from the background noise when the threshold was larger than 20,000 on FSC-H.

Live/Dead Controls

For each experiment, alive and dead cells were stained with both PI and BOX in order to identify the quadrant gating regions of the density plots for healthy (BOX^-/PI^-), injured (BOX^+/PI^-) and dead (BOX^+/PI^+) cells. Dead cells were prepared either by using 100% ethanol or heat. For preparing ethanol-killed cells, 1 mL of the culture was centrifuged and the pellet dispersed in 50 µL PBS by vortexing. Then, 950 µL of 100% ethanol (Fisher Scientific) was added to the cells and left at room temperature for 10 min before analysis. With regard to heat-killed cells, 1 mL of culture in a microcentrifuge tube was heated at 110 °C for 5 min and allowed to cool down. Alive and dead cells were then stained with PI and BOX and analyzed on the FCM.

Colour Compensation

Each fluorophore has a particular emission spectrum. As a result, when two or more fluorophores with overlapping spectra are used concurrently – as was the

case in this study – the fluorescence emissions could be measured in more than one detector. This phenomenon and the process to rectify this problem are called "spillover" and "colour compensation" respectively (BD, 2009). The colour compensation was performed for each fluorophore and its primary detector by first plotting a density plot of the primary detector versus each non-primary detector and then measuring the median FI of the negative (non-fluorescent) and positive (fluorescent) cells in non-primary detectors. When the latter was larger than the former, an arbitrary percentage of the FI of the primary detector was subtracted from the affected non-primary FI in order to remove the spillover. The values used for rectifying the spillover are shown in [Table 2.1].

Table 2.1: The correction factors used for FCM colour compensation

	Correction factors (%) for non-primary detector			
	FL1-A	**FL2-A**	**FL3-A**	**FL4-A**
Primary Detector and Fluorophore				
FL1-A				
BOX		4.22	0.52	0.00
GFP		3.95	0.45	0.00
FL3-A				
PI	0.07	12.57		0.07
FL4-A				
SYTO-62			7.83	

FCM Data Analysis

The FCM data were analyzed using C6 CFlow (Version 1.0.264.15). The parameters reported by the software and used in this study included the median FSC-A, SSC-A, FL1-A, FL3-A, FL4-A, run time (s) and the percentage of cells with different viability states. The CFlow-generated sample volumes were

non-negative integers, reported once approximately every 4.6 seconds when 13 µL.min^{-1} fluidics rate was used. Considering the settings used in this study (20,000 cells, data rate 1000 - 2500 events.s^{-1}), the CFlow-reported events.µL^{-1} (calculated by dividing the total number of events by the integer volume value) was not found to be accurate. As a result, a calibration curve of volume (13 µL) versus time (60 s) was performed three times in order to determine the appropriate equation for better estimation of the volume (trendline of XY plots, R^2 = 1.00).

Equation 2.2: $$Volume_{(\mu L)} = \left[0.2199 \times \left(Time_{(s)}\right)\right] - 0.0088$$

Knowing the volume, it was possible to calculate the cell concentration using the following equation:

Equation 2.3: $$Cell\ concentration\ _{(cells.mL^{-1})} = \left(\frac{20{,}000_{(cells)}}{Volume_{(\mu L)}}\right) \times 1{,}000$$

The values reported by the software were transferred into Microsoft Excel where statistical analysis of the data was performed.

2.6.3 Biofilm Formation

15 mL of PBS, MOJ or 1.2 µm-filtered OJ was transferred into a glass universal bottle and inoculated with 3×10^9 cells of late stationary (24 h culture) *E. coli* K-12 SCC1. Samples were stored at 4 °C for 13 days without aeration. An empty universal bottle was chosen as the control. On day 13, the PBS, MOJ or OJ was

disposed, bottles were washed once with 3 mL of sterile ddH_2O, and were stained with 2 mL of 0.1% solution of crystal violet (Basic Violet 3 dissolved in ddH_2O; Aldrich). Samples were incubated at room temperature for 15 minutes after which the solution was disposed and the bottles were washed three times with ddH_2O. The stain was subsequently dissolved in 2 mL of 100% ethanol (Fisher Scientific) and its absorbance was measured after 1.5 h at 570 nm using a spectrophotometer.

2.7 Data Analysis and Software

Microsoft® Office Excel 2007 was utilized for performing all the parametric tests (t-test and ANOVA) and regression analysis (calculating the coefficient of determination). With regard to logarithmic data and total cell counts ($CFU.mL^{-1}$ and $cells.mL^{-1}$), the values were normalized by calculating the $\log_{10}$ prior to statistical analysis of the data. Where the aim of the test was to analyse the change in trends over time, the values were standardized before performing the test. VassarStats on-line statistical software (Richard Lowry, PhD; http://vassarstats.net/) was also utilized for performing non-parametric Mann-Whitney and Wilcoxon Signed-Ranks tests. C Comp Calculator™ software (BD Biosciences) was also used for calculating the correction factors of the colour compensation. CurTiPot software (Version 3.6.1.; © Prof Ivano G.R. Gutz downloadable from: http://www2.iq.usp.br/docente/gutz/Curtipot_.html) was used for calculating the percentage of undissociated and dissociated organic acids in sanitizing solutions.

Chapter 3

The Effects of Orange Juice Composition on the Physiology of *E. coli* in Orange Juice

3.1 Introduction

Orange juice (OJ) contains a wide range of compounds, mainly sugars (sucrose, fructose and glucose) and organic acids (citric and malic acids) as well as minerals (chiefly potassium), various amino acids and ascorbic acid (see Section 1.2.2).Some of these compounds have been shown to play an important role in growth and/or survival of *E. coli*. For instance, glucose and fructose could be utilized by *E. coli* as a carbon source for metabolism (Anderson & Wood, 1969; Fraenkel, 1968; Ferenci & Kornberg, 1973). On the other hand, citric and malic acids have been shown to exhibit antimicrobial activity against *E. coli* (Raybaudi-Massilia *et al.*, 2009; Eswaranandam *et al.*, 2004; Bjornsdottir *et al.*, 2006). With regard to amino acids present in OJ, arginine and glutamate are the major components of the acid resistance mechanisms in *E. coli* (Richard & Foster, 2004). Moreover, it has been suggested that proline – the most abundant amino acid in OJ – could improve the survival of *E. coli* in an acidic model apple juice (Reinders *et al.*, 2001). In addition, ascorbic acid has been shown to act not only as an antioxidant but also an antimicrobial against *E. coli* particularly in combination with organic acid (Padayatty *et al.*, 2003; Tajkarimi & Ibrahim, 2011).

Consequently, it was hypothesized that changes in major components of OJ (e.g., seasonal, cultivar, processing-induced, etc.) could influence the physiological response of *E. coli* in OJ. In order to test this hypothesis, it was decided to use a model solution [Table 3.1] instead of a real OJ. The main principle behind using a model OJ (MOJ) was to investigate the role of each component independent of the biological variability in the composition of real fruit juice (Reinders *et al.*, 2001). This also made it possible to create MOJ solutions with known sugar or acid compositions mimicking the seasonal and/or cultivar variability of the OJ (Villamiel *et al.*, 1998; Kelebek & Selli, 2011). Furthermore, using a model system eliminated the need for chemical analysis of the OJ composition. The MOJ used in this study consisted of only the main sugars, organic acids and minerals of OJ (Robards & Antolovich, 1995).

3.2 General Protocol (FCM Analysis and Plate Count)

In this study, the *E. coli* K-12 strain MG1655 was used due to its suitability as a surrogate strain for *E. coli* O157:H7 in food microbiological studies (Valdramidis *et al.*, 2007). Moreover, its metabolism, complete genome sequence and gene regulation is known and well characterized (Feist *et al.*, 2007; Blattner *et al.*, 1997). A single colony of *E. coli* was inoculated in 2×LB and was allowed to grow for 18 h at 37 °C. The overnight culture was subsequently diluted (1:1,000) in fresh medium and was grown until it reached the appropriate optical density (OD). The OD of the culture was measured at 650 nm using a spectrophotometer. Cells were subsequently harvested by centrifugation,

Table 3.1: The composition and characteristics of the MOJ

	MOJ ($g.L^{-1}$)
Sugars	**85**
Sucrose	45
Glucose	20
Fructose	20
Organic Acids	**11.5**
Citric Acid	9.5
Malic Acid	2
Buffering Agent	**5.02**
Potassium Citrate	5.02
pH*	3.23 ± 0.01
Osmolality ($mOsmol.kg^{-1}$)**	488 ± 15
	OJ (Filtered with 1.2 µm filter paper)
pH*	3.25 ± 0.05
Osmolality ($mOsmol.kg^{-1}$)**	497 ± 2

* : Mean ± standard deviation of 3 samples
**: Mean ± standard deviation of 3 samples (10 measurements per sample)

washed and dispersed in PBS. Then, 50 µL of cell suspension containing 3×10^9 *E. coli* cells was inoculated into either of 15 mL of MOJ or OJ which had been brought to 4 °C or 22.5 °C before use. MOJ and OJ were respectively filtered with 0.22 µm polyethersulfone syringe filter and 1.2 µm filter paper before use. OJ filtration was performed in order to prevent the blockage of the flow cytometer with rag fragments, while retaining chromoplastids and hesperidin compounds naturally present in the OJ (see Section 4.2.2 & Figure 4.2).

Following inoculation, samples were incubated at 4 °C or 22.5 °C and the physiological state of the cells such as their viability, culturability and morphology was studied immediately after inoculation and 4, 24 and 48 h later. For viability studies, samples were first diluted in PBS (approximately 1:20) and then stained with 191.64 nM bis-oxonol [Bis-(1,3-Dibutylbarbituric Acid) Trimethine Oxonol, $DiBAC_4(3)$], henceforth referred to as BOX and 5.89 µM propidium iodide (PI). BOX stains the lipophilic sites of the cells with depolarized membranes (injured or dead cells) whereas PI binds to the DNA of dead cells. As a results, cells which were not stained with BOX or PI (BOX^-/PI^-) were considered as healthy. On the other hand BOX^+/PI^- and BOX^+/PI^+ cells were considered as injured and dead respectively. Samples were subsequently analyzed using a flow cytometer (see Section 2.6.2 for the protocol). For culturability experiments, samples were serially (decimal) diluted in maximum recovery diluent (MRD) and plated on nutrient agar plates in order to determine the number of culturable cells.

3.3 Results

3.3.1 Growth Curve

The aim of this experiment was to investigate the effects of growth phase of *E. coli* and/or the samples' incubation temperature on the viability and/or culturability of *E. coli* in MOJ and OJ. Before conducting the experiment, it was important to obtain a growth curve of the *E. coli* in order to identify the growth phases of the cells [Figure 3.1].

Based on the results shown in [Figure 3.1(A)], *E. coli* cells in cultures with OD of 0.5, 0.75 and 1 were considered to be in mid-exponential, late-exponential and late-exponential/early-stationary growth phases respectively. On the other hand, cultures with OD of 2, 4 and 6 were shown to contain early stationary, stationary and late-stationary phase cells. By plotting the $OD_{(650\ nm)}$ versus $CFU.mL^{-1}$ and applying the linear regression [Figure 3.1(B)], it was possible to predict the approximate concentration of the cells present in the culture. This information was then used in order to calculate the cell density of the inoculum and the volume of the culture needed for inoculation of MOJ or OJ samples with *E. coli*.

3.3.2 Growth phase and Incubation Temperature

Percentage Healthy cells (FCM) in MOJ

[Figures 3.2(A) and 3.2(B)] show the effects of cell growth phase and incubation temperature on the percentage of healthy cells (BOX^+/PI^-) in MOJ samples at 4 °C and 22.5 °C respectively. Regardless of the growth phase or incubation

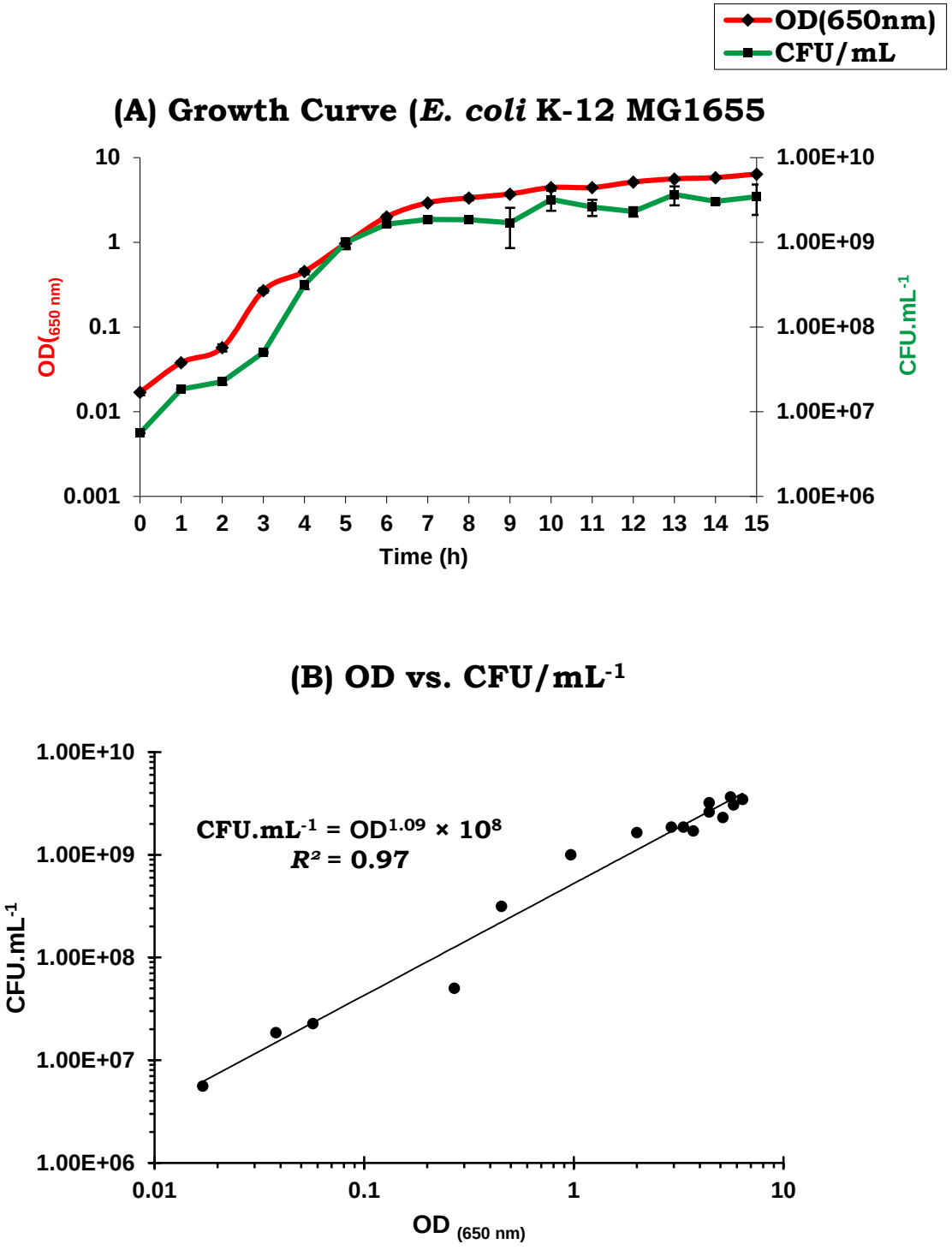


Figure 3.1: The growth curve of *E. coli* K-12 MG1655 in 2×LB at 37 °C

(A) The number of colonies grown on the plates was used in order to calculate the CFU.mL^{-1} of culturable cells. In addition, optical density (OD) of the culture was measured at 650 nm at each time-point using a spectrophotometer.

(B) Correlation between the $OD_{(650\ nm)}$ and CFU.mL^{-1}

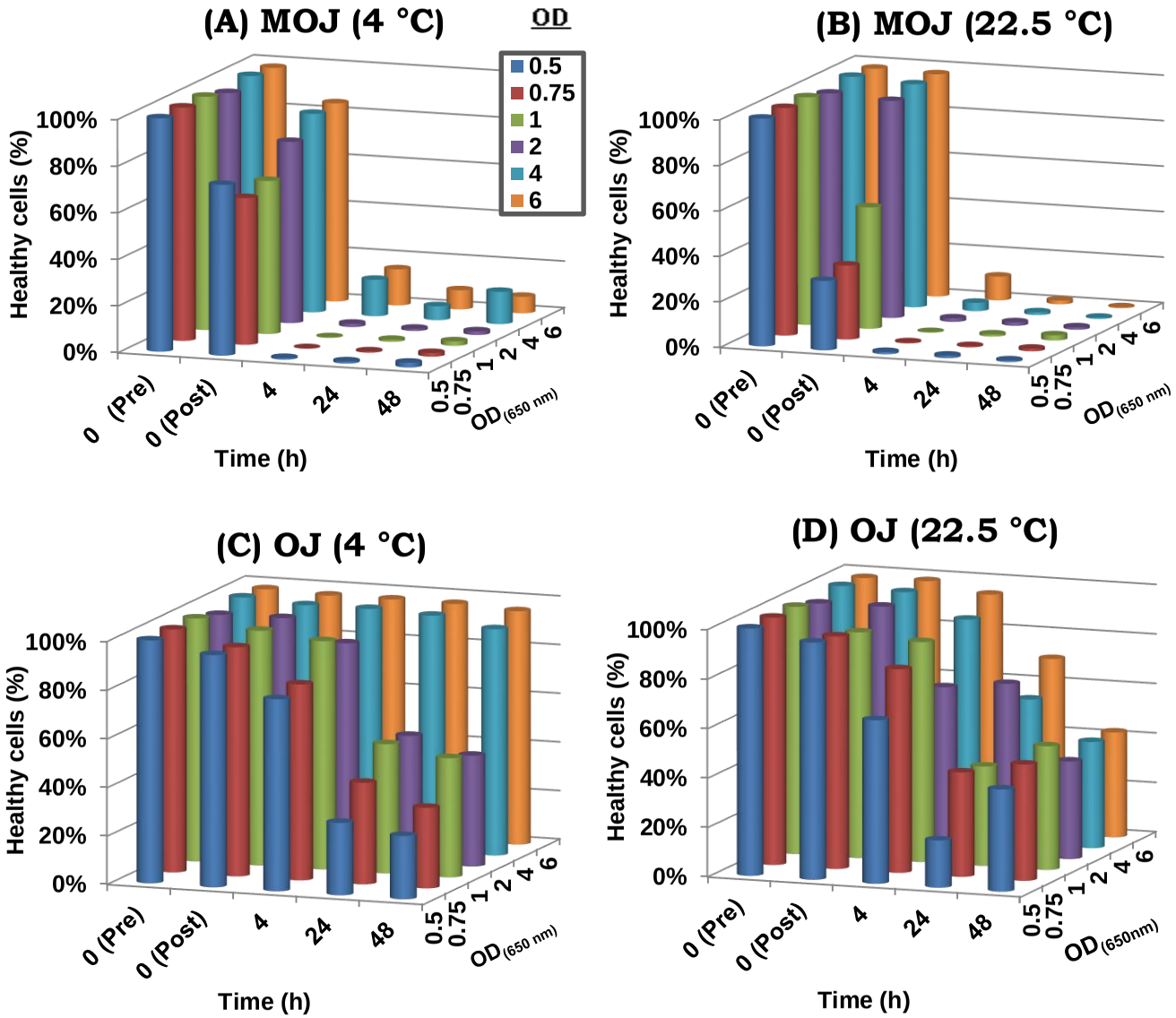


Figure 3.2: The effects of cell growth phase (exponential and stationary) and temperature (4 °C and 22.5 °C) on the viability of *E. coli* K-12 MG1655 in orange juice (OJ) and model orange juice solution (MOJ)

Figures show the mean percentage of healthy (BOX^-/PI^-) cells at times 0 h (pre- and post-inoculation in OJ or MOJ), 4 h, 24 h and 48 h post-inoculation. A single colony of *E. coli* K-12 MG1655 was inoculated in 2×LB and grown at 37 °C for 18 h with aeration (150 rpm). The overnight culture was subsequently diluted (1:1,000) in fresh medium and allowed to grow to reach optical density ($OD_{650\ nm}$) of 0.5, 0.75, 1, 2, 4 or 6. Cells were harvested by centrifugation (14000 rpm, 5 minutes) and the supernatant was disposed. The pellet was first dispersed in 50 µL of PBS and then added to 15 mL of OJ (filtered with 1.2 µm filter paper) or MOJ (Table 3.1). Samples were incubated at 4 °C or 22.5 °C. At different time-points, samples were diluted in PBS and stained with propidium iodide (PI) and bis-oxonol (BOX) and analyzed with a flow cytometer. Each experiment was repeated at least twice with duplicates. Data are the mean of a representative experiment.

temperature, there was a dramatic decrease in the viability of *E. coli* in MOJ during the first 4 h of the study. In case of the 4 °C MOJ samples [Figure 3.2(A)], more than 96% the cells were healthy (BOX^-/PI^-) before their inoculation into MOJ. However, immediately after their inoculation (time 0 h post-inoculation), there was between 13% and 39% decrease in the healthy population of *E. coli* depending on the growth phase of the cells. In agreement with the literature (Chung *et al.*, 2006; Benjamin & Datta, 1995; Arnold & Kaspar, 1995), stationary-phase cells were found to be more resistant to the acidic condition of MOJ than log-phase cells. The reduction in the number of healthy cells at time 0 h post-inoculation, for log-phase cells (OD 0.5, 0.75 or 1) was between 26% and 39%. This reduction in case of early-stationary (OD 2), stationary (OD 4) or late-stationary (OD 6) phase cells was found to be 19% ($p < 0.05$), 14% and 13% (both $p < 0.01$) respectively (p-values compared to log-phase cells). By time 4 h post-inoculation, there was a further dramatic decrease of 61% to 78% in the population of healthy cells after which the healthy population remained relatively constant in all samples regardless of the growth phase of the cells. Nevertheless, still at time 48 h, a significantly greater percentage of healthy cells was observed in MOJ samples containing stationary (OD 4) or late-stationary-phase (OD 6) cells compared to other MOJ samples ($p < 0.0001$ and $p < 0.001$ respectively). The greater resistance of the stationary-phase cells to acidic condition of the MOJ was believed to be mainly due to induction of general stress response in stationary phase cells (Richard & Foster, 2004) and the low pH-induced inhibition of RpoS proteolysis (Hengge-Aronis, 2002).

A similar trend in viability of *E. coli* was observed in MOJ samples at 22.5 °C [Figure 3.2(B)]. For instance, at time 4 h post-inoculation, the mean percentage of healthy cells in samples containing log-phase cells (OD 0.5, 0.75, 1) was significantly lower than the values found in early–stationary (OD 2), stationary (OD 4) or late-stationary-phase cells (OD 6) ($p < 0.05$, $p < 0.001$ and $p < 0.0001$ respectively). By time 48 h, in average, there was between 95% and 99% reduction in the healthy population of *E. coli* across all MOJ samples. At this time point, no significant difference was observed between the percentage of healthy cells in MOJ samples at 4 °C and 22.5 °C regardless of the growth phase of the cells. The reduced number of healthy cells in MOJ samples at 22.5 °C was presumed to be due to combined effects of the MOJ-induced growth arrest and the greater metabolic activity of the cells at this temperature leading to generation of reactive oxygen species (ROS), hence oxidative stress (Bloomfield *et al.*, 1998).

However, it is important to note that at time 0 h post-inoculation, compared to 4 °C, the growth-phase dependant percentage of healthy cells in MOJ at 22.5 °C was significantly different. With regard to MOJ samples containing exponential-phase cells (OD 0.5 and 0.75), a significantly lower number of healthy cells was observed at 22.5 °C compared to 4 °C ($p < 0.05$). Conversely, in samples that had been inoculated with stationary-phase cells (OD 2–6) the rate of reduction in healthy population immediately after inoculation in MOJ was significantly lower at 22.5 °C compared to 4 °C ($p < 0.01$). No significant difference was observed between the results obtained at 4 °C and 22.5 °C in MOJ samples

containing samples with late-log/early-stationary-phase (OD 1) cells. The reason behind this is not clear, however this could be due to the differences between the chemical composition and/or the flexibility of the membrane of exponential and stationary-phase cells (DiRusso & Nyström, 1998, De Mendoza & Cronan, 1983).

Percentage Healthy cells (FCM) in OJ

Compared to MOJ samples, there was a significantly greater number of healthy cells in OJ samples throughout the experiment both at 4 °C and 22.5 °C ([Figures 3.2(C)] & (D)] respectively). Nevertheless, similar to what was observed in case of MOJ samples, transition from log-phase to stationary-phase as well as incubation at 4 °C increased the number of healthy cells in OJ. At 4 °C, during the 48 h course of the experiment there was only 5% and 2% reduction in the mean percentage of healthy cells in OJ samples inoculated with stationary-phase (OD 4) or late-stationary-phase (OD 6) cells respectively. On the other hand, this reduction in case of samples containing log-phase (OD 0.5 and 0.75) and late-log/early-stationary-phase (OD 1 and 2) cells was 65% and 49% respectively (both $p < 0.0001$ compared to OD 4 and 6). No significant difference was observed between the rate of reduction in healthy population during 48 h incubation of OJ samples at 4 °C and 22.5 °C among samples containing log-phase or early-stationary-phase cells (OD 0.5–2). However, with regard to samples inoculated with stationary and/or late-stationary phase cells (OD 4 and 6), the percentage of healthy cells at time 48 h was significantly lower in samples incubated at 22.5 °C compared to 4 °C ($p < 0.0001$).

Culturability (Plate Count) in MOJ and OJ

The effects of growth phase and incubation temperature on the culturability of the cells has been shown in [Figure 3.3]. The trends of change in the culturability of the cells in MOJ and OJ were in agreement with the viability results. With regard to MOJ samples, the rate of reduction in culturability of the cells was significantly slower at 4 °C [Figure 3.3(A)] compared to 22.5 °C [Figure 3.3(B)] during the first 24 h of the experiment. By time 48 h post-inoculation, no significant difference was observed between the culturability of log-phase cells (OD 0.5–1) at 4 °C and 22.5 °C MOJ samples.

In general, compared to MOJ, the OJ appeared to exert a significantly lower adverse effect on culturability of *E. coli* both at 4 °C [Figure 3.3(C)] and 22.5 °C [Figure 3.3(D)] regardless of the growth phase of the cells. Nevertheless, similar to what was observed in MOJ samples, the rate of reduction in the number of culturable cells was significantly slower at 4 °C compared to 22.5 °C. Moreover, greater number of culturable cells was found in OJ samples inoculated with stationary-phase cells (OD 4 and 6) compared to samples containing early-stationary/log-phase cells (OD 0.5–2).

Viable but non-culturable (VBNC) Cells in MOJ and OJ

It is important to note that viable cells include not only the healthy cells (BOX^-/PI^-) but also the injured cells with depolarized membrane (BOX^+/PI^-). Using a flow cytometer it was possible to determine not only the percentage of each type

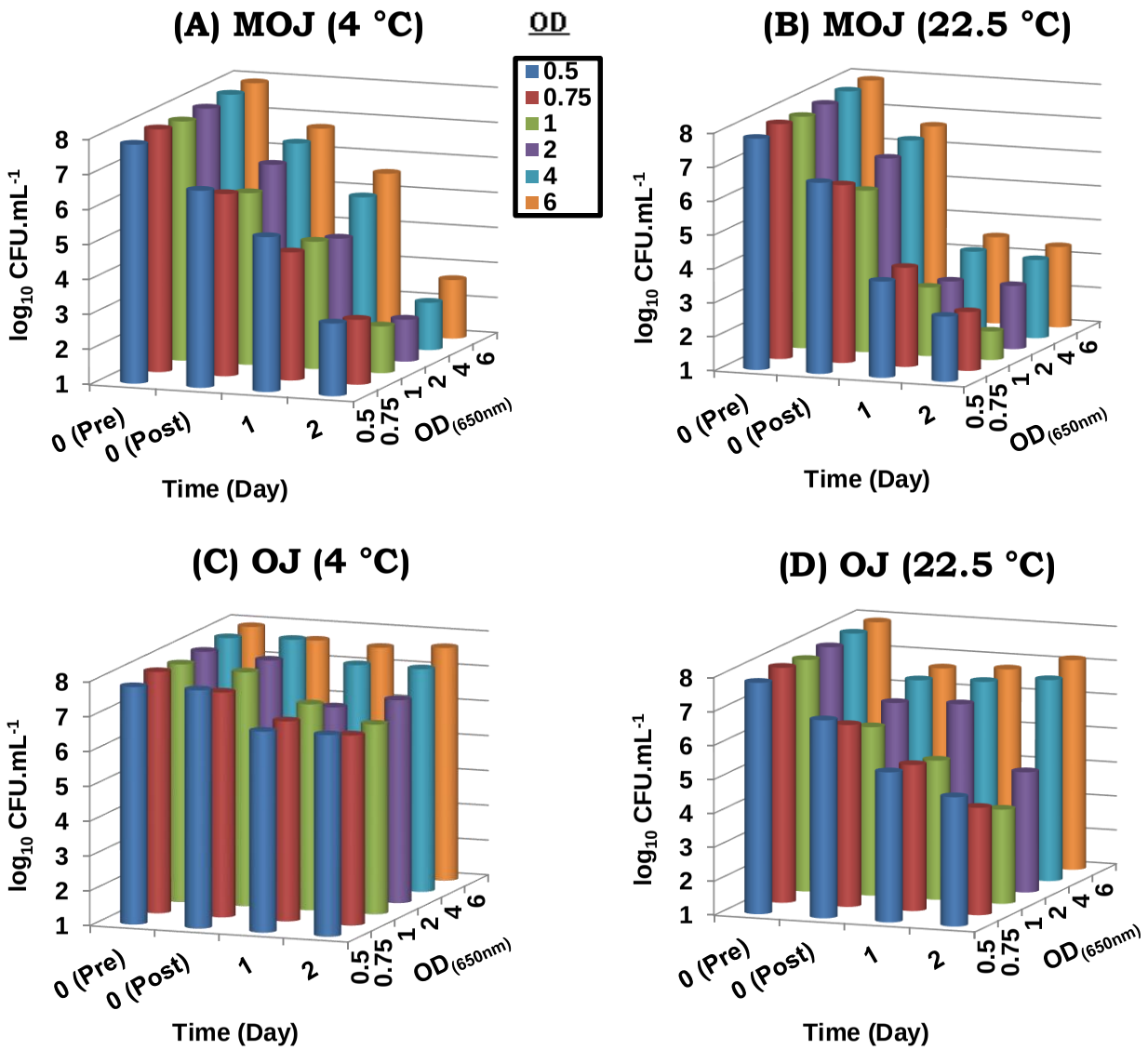


Figure 3.3: The effects of cell growth phase (exponential and stationary) and temperature (4 °C and 22.5 °C) on the culturability of *E. coli* K-12 MG1655 in orange juice (OJ) and a model orange juice solution (MOJ)

These figures show the mean $\log_{10}$ CFU.mL^{-1} for samples described in Figure 3.2. At different time points [0 h (pre- and post-inoculation in OJ or MOJ), day 1 and day 2 post-inoculation], samples were serially (decimal) diluted in maximum recovery diluent (MRD). Subsequently, 100 µL of the appropriate dilution was plated on nutrient agar plates and allowed to grow at 37 °C for 48 h.

of population (i.e. healthy, injured or dead), but also the number of cells per µL of a sample. By multiplying the percentage of each population by total number of cells per µL (FCM data) and the dilution factor of the sample used for FCM analysis it was possible to calculate the cells.μL^{-1} of the population of the interest within a sample. Further multiplication of this value by 1,000 provided the cells.mL^{-1} value.

[Figure 3.4] shows the change in $\log_{10}$ cells.mL^{-1} of culturable, healthy, injured and dead cells in MOJ and OJ samples inoculated with mid-log-phase (OD 0.5) *E. coli* cells during 48 h incubation at 4 °C or 22.5 °C. Inoculation of *E. coli* into OJ as well as MOJ led to an increase in the number of VBNC cells which consisted of not only the injured cells but also the healthy cells with intact polarized membrane. By day 2 of the study, 1.14 ± 0.14 and 3.05 ± 0.04 $\log_{10}$ cells.mL^{-1} *E. coli* cells were found to be VBNC in OJ samples incubated at 4 °C and 22.5 °C respectively ([Figures 3.4(A)] & (B)] respectively). During the same time period, no change in the total $\log_{10}$ number of viable cells was observed. Similarly, in case of MOJ samples, the viable population remained relatively constant; however, the rate of increase in VBNC population was significantly greater than what was observed in OJ samples, regardless of the incubation temperature used ($p < 0.01$ compared to corresponding OJ samples). By day 2 post-inoculation, 4.57 ± 0.32 and 4.79 ± 0.07 $\log_{10}$ cells.mL^{-1} were found to be VBNC in MOJ samples at 4 °C and 22.5 °C samples accounting for more than 60% $\log_{10}$ of total viable cells present in MOJ samples. ([Figures 3.4(C) & (D)] respectively)

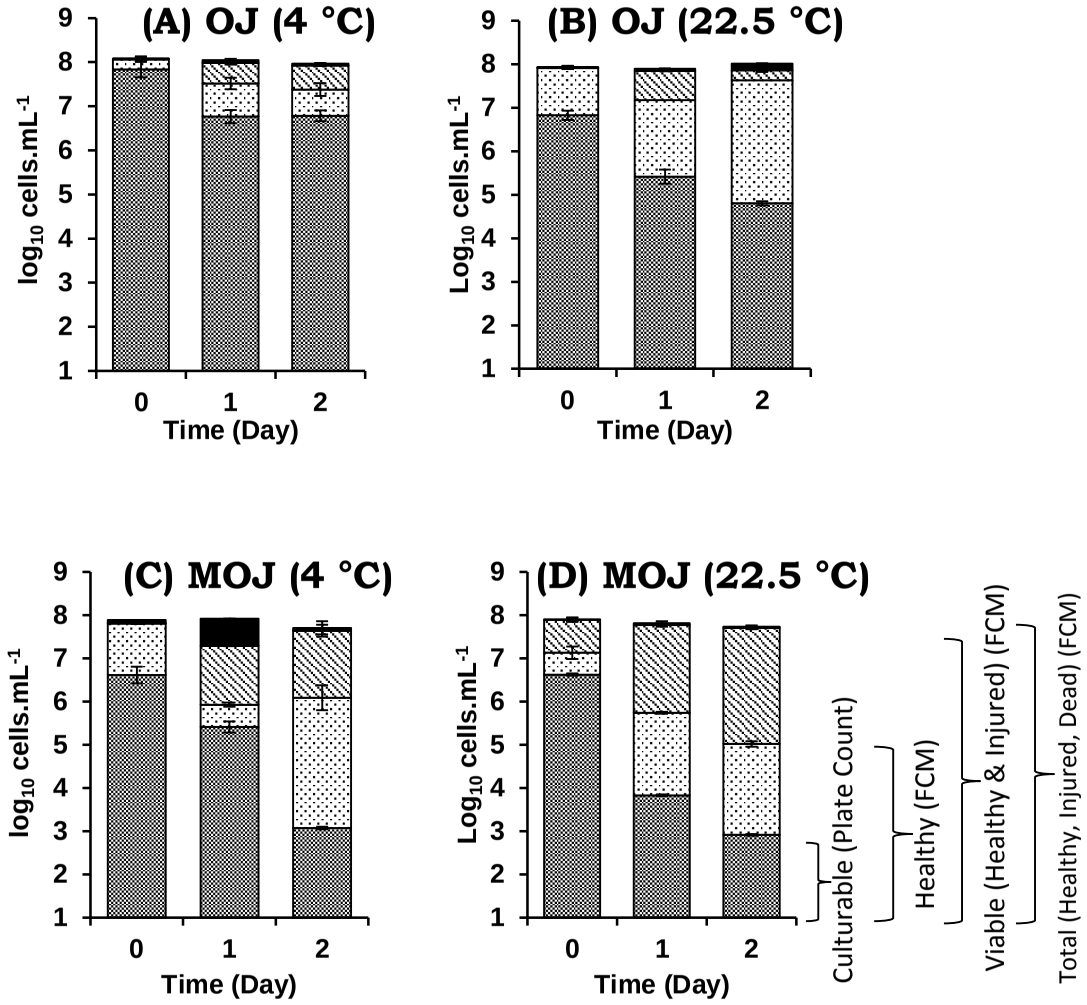


Figure 3.4: The viability and culturability of exponential phase *E. coli* K-12 MG1655 (OD_{650nm} 0.5) in orange juice (OJ) and model orange juice (MOJ) solution at 4 °C and 22.5 °C.

The log_{10} number of healthy, injured and dead cells as well as the culturable cells in OJ and MOJ samples containing mid-exponential (OD_{650nm} 0.5) *E. coli* K-12 MG1655. The data in these figures is based on the data already presented in Figure 3.2 and Figure 3.3. Cells which were stained with neither Bis-oxonol (BOX) or propidium iodide (PI) were considered healthy. Viable cells are the total number of PI^- cells and include not only the healthy cells but also cells with depolarized membrane (i.e., injured cells of BOX^+/PI^-). Total cell population includes both viable and non-viable (dead BOX^+/PI^+) cells. Viable but non-culturable cells (VBNC) were calculated by subtracting the log_{10} number of culturable cells (plate count) from log_{10} number of healthy or injured cells (both determined by FCM). Error bars are the ± standard deviation of the mean values.

In summary, it was shown that there was a significant discrepancy between the results obtained for the viability and/or culturability of *E. coli* in MOJ and OJ. This was despite the fact that the MOJ and OJ used in this experiment had relatively similar pH and osmolality [Table 3.1]. Moreover, although the chemical composition of the OJ was not analyzed, the MOJ contained the major components of the OJ within the ranges naturally found in freshly squeezed OJ used in this study. Therefore, it is reasonable to assume that other components present in OJ play an important role in physiology of *E. coli* in OJ (Nualkaekul & Charalampopoulos, 2011).

3.3.3 Sugars and Organic Acids

Sugars

In order to investigate the effects of change in sugar concentrations of OJ on the physiology of *E. coli*, mid-log-phase (OD 0.5) cells were inoculated in MOJ solutions containing low, medium and high sugar content [Table 3.2]. The sugar composition of MOJ was within the minimum, mean and maximum concentrations of sugar content of naturally found in OJ (see [Table 1.3]). The pH and osmolality of the MOJ solutions was measured using a pH meter and an osmometer respectively. With the exception of sugar profiles of the MOJ solutions, the method of the experiment was identical to the general protocol described above for growth phase experiments. In summary, following the inoculation of *E. coli* in MOJ solutions, samples were incubated at 4 °C for 24 h during which the viability and culturability of the *E. coli* was monitored at

Table 3.2: The composition and characteristics of the MOJ containing various sugar content

	Low Sugar ($g.L^{-1}$)	Medium Sugar ($g.L^{-1}$)	High Sugar ($g.L^{-1}$)
Sugars	**60**	**85**	**120**
Sucrose	30	45	60
Glucose	15	20	30
Fructose	15	20	30
Organic Acids	**11.5**	**11.5**	**11.5**
Citric Acid	9.5	9.5	9.5
Malic Acid	2	2	2
Buffering Agent	**5.02**	**5.02**	**5.02**
Potassium Citrate	5.02	5.02	5.02
pH*	3.21 ± 0.02	3.23 ± 0.01	3.20 ± 0.03
Osmolality ($mOsmol.kg^{-1}$)**	423 ± 18	488 ± 15	640 ± 15

Table 3.3: The composition and characteristics of the MOJ containing various acid content

	Low Acid ($g.L^{-1}$)	Medium Acid ($g.L^{-1}$)	High Acid ($g.L^{-1}$)
Sugars	**85**	**85**	**85**
Sucrose	45	45	45
Glucose	20	20	20
Fructose	20	20	20
Organic Acids	**6**	**11.5**	**18**
Citric Acid	5 (12.11)†	9.5 (21.89)	14 (33.51)
Malic Acid	1 (4.61)	2 (8.90)	4 (18.29)
Buffering Agent	**2.55**	**5.02**	**8.50**
Potassium Citrate	2.55	5.02	8.50
pH*	3.19 ± 0.02	3.23 ± 0.01	3.20 ± 0.03
Osmolality ($mOsmol.kg^{-1}$)**	426 ± 11	488 ± 15	594 ± 6
Potassium content ($g.L^{-1}$)	0.97	1.92	3.25
Molar Ratios			
Citric Acid/potassium citrate	3.13	3.02	2.63
Citric Acid/Malic Acid	3.49	3.32	2.44

* : Mean ± standard deviation of 3 samples
**: Mean ± standard deviation of 3 samples (10 measurements per sample)
† : Values inside the brackets show the concentration of undissociated acid in mM (calculated based on the Henderson-Hasselbalch equation).

different time-points by FCM and plate counting respectively.

No significant difference was observed between viability (e.g., FCM total viable or healthy cells) or culturability of *E. coli* in MOJ samples containing different sugar content [Figure 3.5]. Moreover, up until time 1.5 h post-inoculation, there was a close agreement between the $\log_{10}$ number of viable and culturable cells in all samples. However, by time 6 h post-incubation the difference between the $\log_{10}$ number of viable and culturable cells was widened reaching 1.48 ± 0.27 $\log_{10}$ cells.mL^{-1} (mean ± SD of all MOJ samples). During the subsequent 18 h incubation of the samples, a significantly slower rate of decrease in the viability and culturability of the cells was observed ($p < 0.01$ compared to time 0–6 h). Based on these results, it was hypothesized that while the increase in osmolality of the solutions most likely led to osmotic stress in *E. coli*, this stress was not sufficient to exert adverse effects on either of the viability or culturability of the cells.

Organic Acids

As previously mentioned (see Section 1.2.2), the main organic acids present in OJ are citric acid and malic acid. However, the concentration of these organic acids in OJ could vary widely depending on the harvest season, origin or the variety/cultivar of the orange fruit as of well as the OJ extraction techniques used.

In this study, the effects of various concentrations of organic acid on the

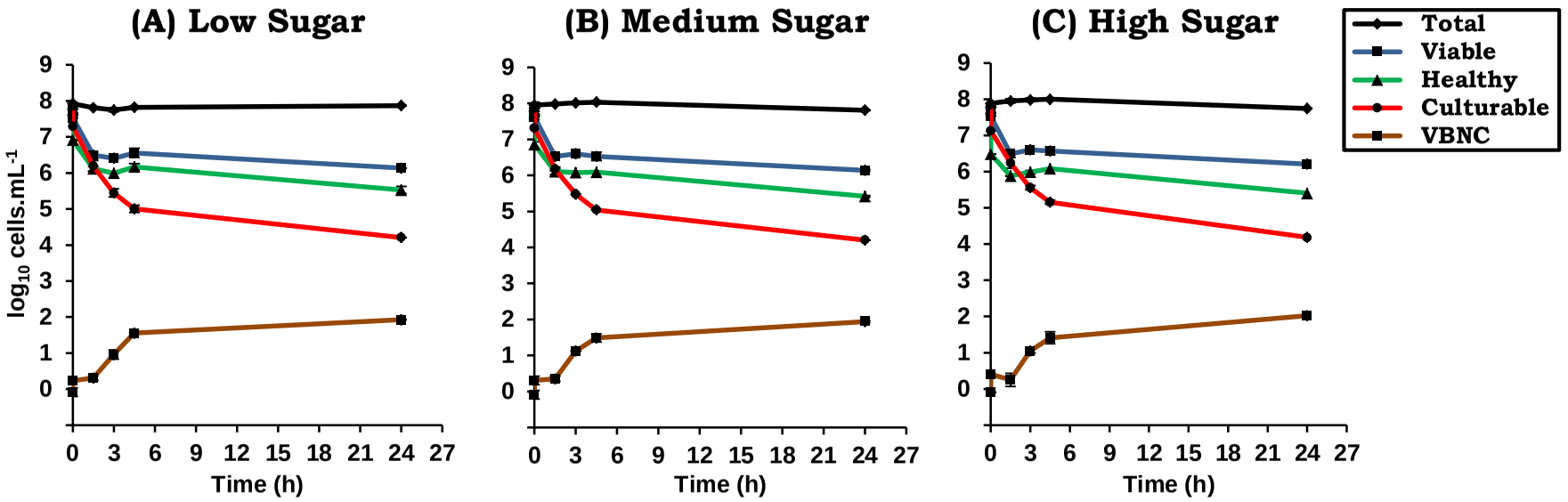


Figure 3.5: The effects of sugar content of MOJ on the viability and culturability of exponential phase *E. coli* K-12 MG1655 (OD_{650nm} 0.5) in MOJ incubated at 4 °C for 24 h

The method for this experiment was similar to what was described in Figure 3.2, however for this experiment, the exponential phase *E. coli* was inoculated in MOJ solutions containing low, medium or high sugar contents (Table 3.2). The $\log_{10}$ CFU.mL^{-1} of culturable cells was calculated based on the number of colonies grown on a nutrient agar plate after 48 h of incubation at 37 °C. Similar to the results shown in Figure 3.4, cells that were not stained with either of Bis-oxonol (BOX) or propidium iodide (PI) were considered healthy. Viable cells are the total number of PI$^-$ cells, which included not only the healthy cells but also those with depolarized membrane (i.e., injured cells of BOX$^+$/PI$^-$). Total cell population includes both viable and non-viable (dead BOX$^+$/PI$^+$) cells. The number of viable but non-culturable (VBNC) cells was calculated by subtracting the $\log_{10}$ number of culturable cells from viable cells. Error bars are the ± standard deviation of the mean values.

viability and culturability of *E. coli* in MOJ solutions were investigated. In order to achieve this aim, MOJ solutions containing various concentrations of organic acids were prepared [Table 3.3]. The pH of the MOJ solutions was kept relatively constant (pH 3.19–3.23) by changing the concentration of potassium citrate. Nevertheless in order to simulate the citrate buffer mixture of real OJ, the molar ratio of citric acid to potassium citrate (2.63–3.13) was kept close to 3:1 ratio previously reported by Lanford (1942) in real OJ. Moreover, the concentration of potassium in MOJ solutions (0.97–3.25 $g.L^{-1}$) and the molar ratio of citric acid to malic acid (2.63–3.13) were close to the range reported for real OJ (Saccani *et al.*, 1995; Robards & Antolovich, 1995). With the exception of the composition of MOJ solutions, the method used for this experiment was identical to what was described above for sugar experiments.

[Figure 3.6] shows the effect of organic acid concentration on the viability and culturability of *E. coli* in MOJ solutions containing low, medium or high organic acid content (henceforth referred to as low-acid, medium-acid and high-acid respectively). In general, changes in total organic acid concentration of MOJ (within the ranges naturally found in OJ) did not have any significant effect on the number of viable (PI^- as determined by FCM) during the course of the study. Nevertheless, statistically, the $\log_{10}$ reduction in number of healthy (BOX^-/PI^-) and culturable cells (as determined by CFU measurement) in high-acid MOJ samples during the same time period (1.86 + 0.02 and 3.67 + 0.17 $\log_{10}$ reduction respectively) was significantly greater than the level of reduction observed in low-acid samples (1.63 + 0.07 and 3.09 + 0.02 $\log_{10}$ respectively;

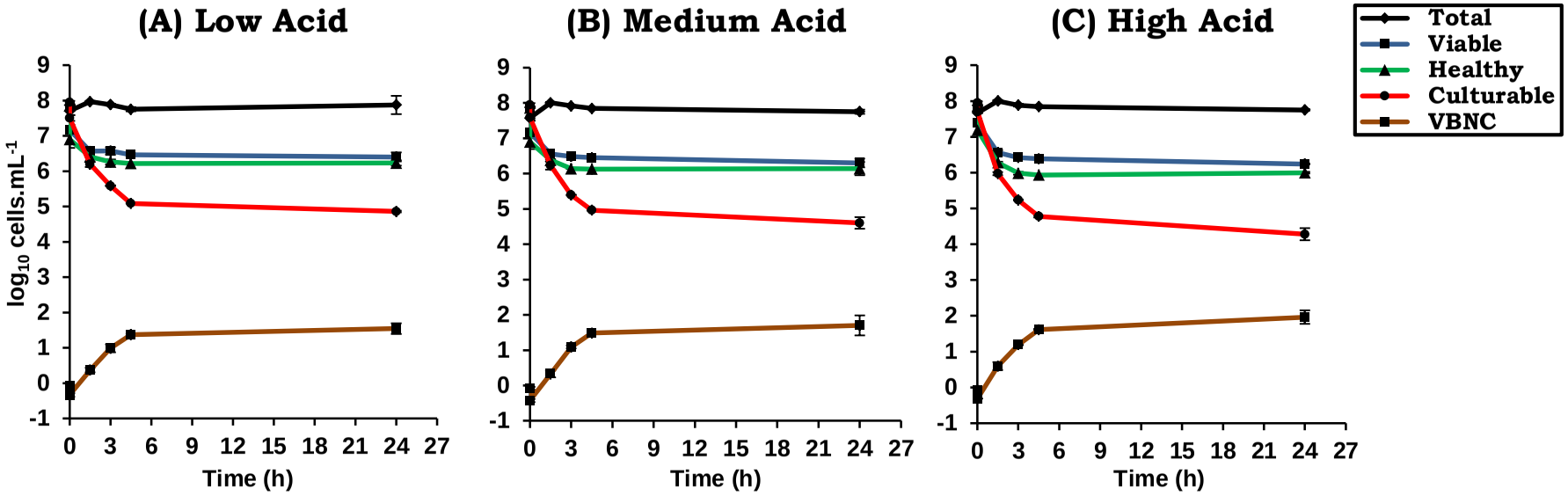


Figure 3.6: The effects of acid content of MOJ on the viability and culturability of exponential phase *E. coli* K-12 MG1655 (OD_{650nm} 0.5) in MOJ incubated at 4 °C for 24 h

The method of this experiment was similar to what was described in Figure 3.2, however, here exponential phase cells were added to MOJ solutions with different acid content (Table 3.3). Culturable cells were considered those that could form a colony on a nutrient agar plate after 48 h incubation at 37 °C. Similar to the results shown in Figure 3.5, cells that were not stained with either of Bis-oxonol (BOX) or propidium iodide (PI) were considered healthy. Viable cells are the total number of PI^- cells that include not only the healthy cells but also those with depolarized membrane (i.e., injured cells of BOX^+/PI^-). Total cell population includes both viable and non-viable (dead BOX^+/PI^+) cells. The number of viable but non-culturable (VBNC) cells was calculated by subtracting the $\log_{10}$ number of culturable cells from viable cells. Error bars are the ± standard deviation of the mean values.

both $p < 0.05$). The reason behind the small –nonetheless significant effect of 3-fold increase in organic acid concentration of MOJ on viability and/or culturability of *E. coli* was not clear. However this could have been due to different antimicrobial mechanisms of malic and citric acid at concentration used in this study. It has been shown that supplementation of a pH 3.2 solution with 5–10 mM fully protonated malic acid can have a protective effect on culturability of *E. coli* (Bjornsdottir *et al.*, 2006). Considering the concentration of undissociated malic acid present in low-acid and medium-acid MOJ (4.6 mM and 8.9 mM respectively; [Table 3.3]) it is reasonable to presume that a similar protective effect resulted in a significantly lower reduction in culturability of the cells in low-acid and medium-acid MOJ samples. Furthermore, it is also known that the hydrophilic organic acids of citric acid and malic acid act as an antimicrobial compound by chelating metal ions which are essential for the survival of microorganisms (Stratford & Eklund 2003; Brittain, 2001). Therefore it is also reasonable to postulate that the increase in organic concentration of MOJ led to a greater chelation of metal ions, hence greater reduction in the number of healthy and culturable cells.

Dead Sub-populations

An osmolality-dependant difference was observed between the green (BOX) fluorescence intensity (FI) of the dead cells in MOJ samples. [Figure 3.7(A)] shows the FCM density plots of green (BOX) versus red (PI) FI of 20,000 cells collected for a representative samples with low, medium or high sugar content at times 0 h and 24 h (both post-inoculation in MOJ). As it could be seen from

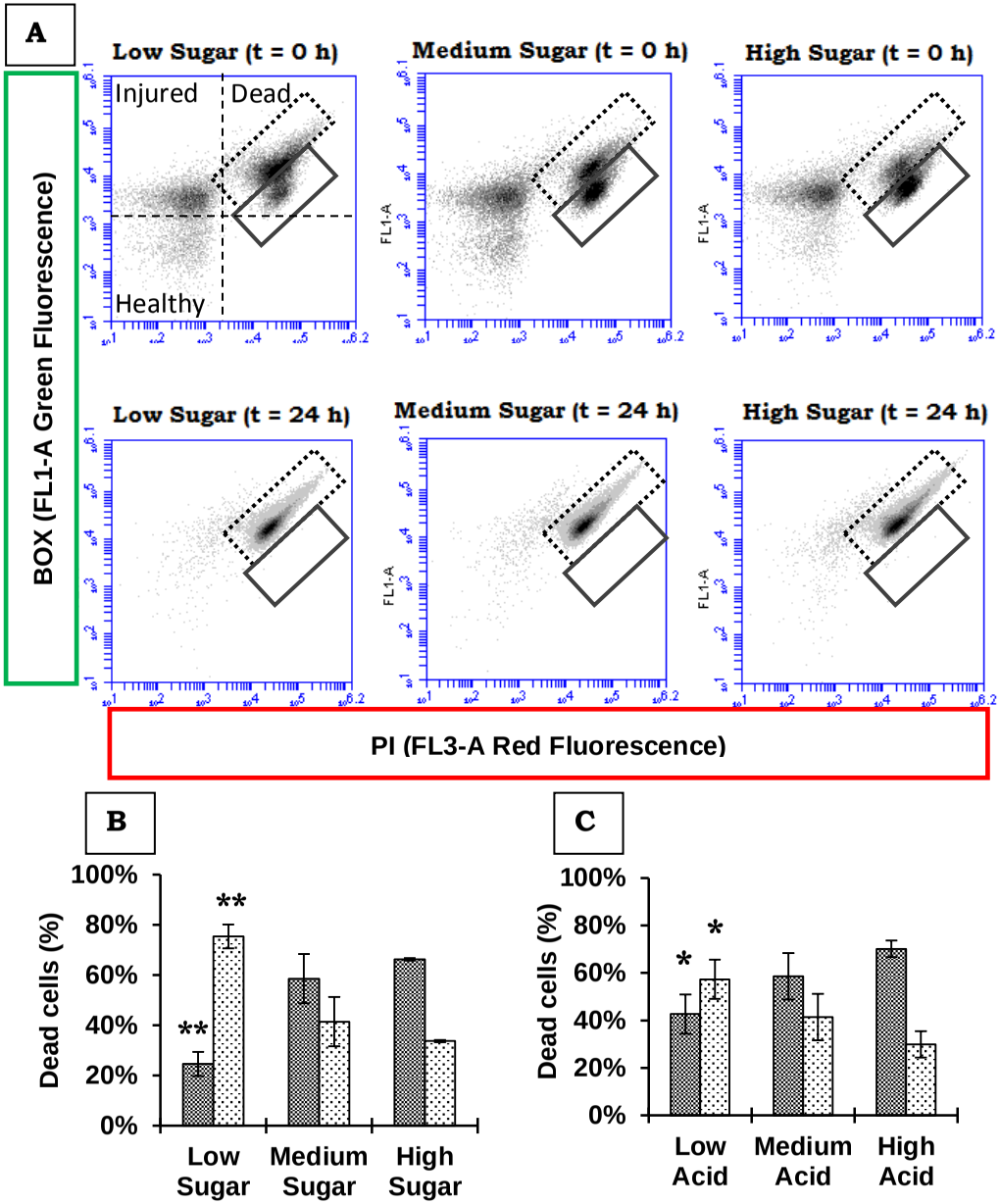


Figure 3.7: The effects of the MOJ sugar concentration on the optical properties of the dead cells

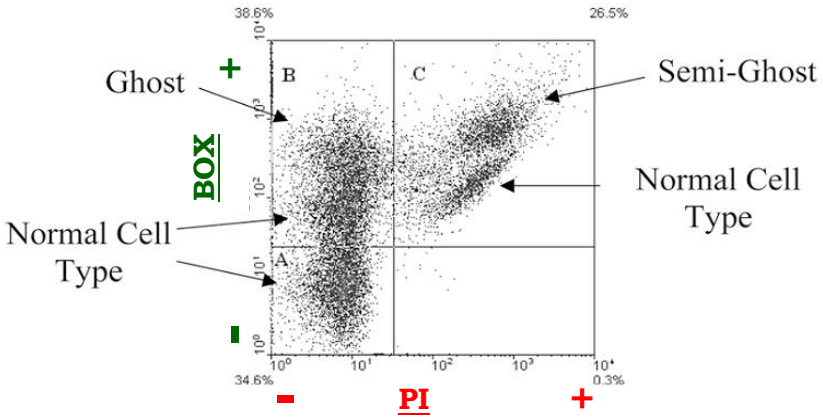
(A) The figure shows the green versus red fluorescent (FL1-A and FL3-A respectively) density plots for MOJ samples containing different sugar concentrations. Samples were stained with BOX and PI as previously described in Figure 3.2 and Figure 3.4. Solid and dotted boxes show two distinct dead sub-populations with similar red fluorescence intensity (FI) but different green FI. Each density plot shows the data for 20,000 cells analyzed by a flow cytometer for a representative sample.

(B) & (C) The percentage of each dead sub-population at time 0 h in MOJ solution containing different concentrations of sugars and organic acid respectively. Error bars are the ± standard deviation of the mean values. (**: $p < 0.05$ compared to MOJ with medium sugar or organic acid content)

this figure, in addition to typical healthy (BOX^-/PI^-) and injured cells (BOX^+/PI^-), two distinct dead sub-populations (both BOX^+/PI^+) were observed at time 0 h: (a) cells with high green FI (dashed box); and (b) cells low green FI (solid box). Similar results were also observed in MOJ samples containing various concentrations of organic acids (plots not shown). The percentage of each dead sub-populations at time 0 h post-inoculation for samples containing different concentration of sugars and acids has been shown in [Figures 3.7(B)] and (C)] respectively. In general, increasing the sugar or organic acid content and consequently the osmolality of the MOJ resulted in a greater decrease in the percentage of dead cells with high BOX FI. After 24 h, nearly all dead cells had high BOX FI regardless of the sugar content of MOJ.

The reason for the existence of two dead sub-populations is not clear. However, the pattern observed in this study for BOX FI of the dead cells was almost identical to the one reported by Lewis *et al.*, (2004) for *E. coli* cells during a batch fermentation process [Figure 3.8]. They postulated that in dead cells with low cytoplasmic content (possibly due to leakage of the cell content), there is a greater availability of lipophilic binding sites within the cells for BOX. Consequently, these cells – which they referred as "semi-ghost" cells – exhibit a greater BOX FI. Cells with lower BOX FI were considered as "normal" cells with normal cytoplasmic content.

Therefore, it could be proposed that the increase in osmolality of the MOJ affected the cellular membrane leading to a reduction in accessibility to the dye



Source: Lewis *et al.*, (2004)*

Figure 3.8: Normal, Semi-Ghost and Ghost *E. coli* cells*

The Figure shows the FCM density plots of green versus red fluorescence (i.e., BOX versus PI respectively) of *E. coli* MSD3735 batch fermentation. Cells that were either BOX^- or BOX^+ with small fluorescence intensity (FI) were considered normal. PI^+ cells with very high BOX^+ FI (semi-ghost) were presumed to contain low cytoplasmic content allowing the greater binding of BOX to lipophilic sites of the cells. PI^- cells with high BOX FI (ghost) were believed to be dead cells lacking DNA hence the absence of red fluorescence.

to lipophilic sites within the cells immediately after their inoculation in MOJ. By time 24 h however, most dead cells had low cytoplasmic content (possibly due to leakage) leading to a greater accessibility of BOX to the lipophilic sites of the membrane. Further work is needed to elucidate the nature of the dead sub-population.

3.3.4 Ascorbic Acid

In order to study the effects of ascorbic acid on the viability and culturability of *E. coli* in MOJ, the MOJ was supplemented with either of 0.5, 1, 5 or 10 $g.L^{-1}$ L-ascorbic acid. The pH and osmolality of MOJ with different concentration of ascorbic acid has been shown in [Table 3.4]. 0.5 $g.L^{-1}$ was chosen to mimic the typical concentration of ascorbic acid in real OJ. Commercial OJ brands supplemented with high concentrations of ascorbic acid (> 7 $g.L^{-1}$) have been reported by Zhang *et al.*, (1997). For that reason, 1, 5 and 10 $g.L^{-1}$ of ascorbic acid were chosen in order to imitate the OJ which has been artificially supplemented with two, ten and twenty times the levels of ascorbic acid naturally found in OJ. The results were compared to that of un-supplemented MOJ containing no ascorbic acid. The experimental method was identical to what was described above for sugar or organic acid experiments. However, samples were incubated for 11 days instead of 24 h in order to investigate the long-term survival of *E. coli* in MOJ.

Viability (FCM Studies)

[Figure 3.9(A)] shows the effects of increased concentrations of ascorbic acid on

Table 3.4: The composition and characteristics of the MOJ containing various ascorbic acid content

	($g.L^{-1}$)	pH*		Osmolality ($mOsmol.kg^{-1}$)**	
Sugars	**85**				
Sucrose	45				
Glucose	20				
Fructose	20				
Organic Acids	**11.5**				
Citric Acid	9.5				
Malic Acid	2				
Buffering Agent	**5.02**				
Potassium Citrate	5.02				
Ascorbic Acid	**0–10**				
	0	3.23 ± 0.01	A	488 ± 15	A***
	0.5 (2.84)†	3.24 ± 0.02	A	499 ± 21	AC
	1 (5.68)	3.22 ± 0.03	AB	508 ± 16	AD
	5 (28.39)	3.20 ± 0.03	AB	528 ± 18	BCD
	10 (56.78)	3.18 ± 0.02	B	550 ± 19	B

* : Mean ± standard deviation of 3 samples
** : Mean ± standard deviation of 3 samples (10 measurements per sample)
***: In each column values with similar letters are not statistically significant.
† : Values inside the brackets show the concentration of L-ascorbic acid in mM

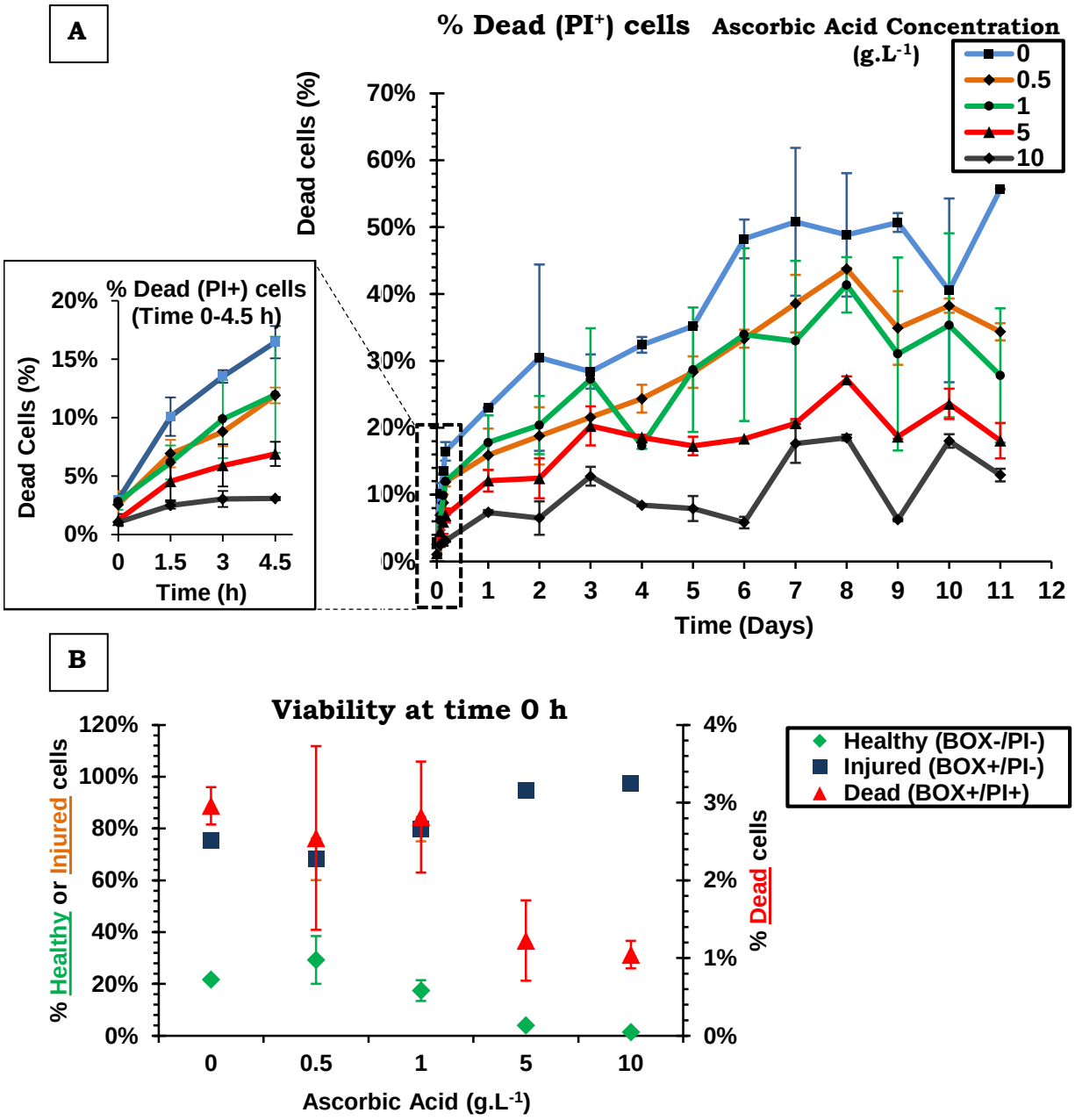


Figure 3.9: The effects of ascorbic acid content of MOJ on the viability of exponential-phase *E. coli* K-12 MG1655 (OD_{650nm} 0.5) in MOJ incubated at 4 °C for 11 days

(A) The method of this experiment was similar to what was described in Figure 3.2, however for this experiment, exponential phase cells were added to MOJ solutions containing different concentrations of ascorbic acid (Table 3.4). Cells, which were stained with both the Bis-oxonol (BOX) and propidium iodide (PI), were considered healthy (BOX+/PI+). Error bars are the ± standard deviation of the mean values.

(B) The Figure shows the effect of ascorbic acid concentration in MOJ on the percentage of healthy, injured and dead cells immediately after being inoculated in MOJ (time 0 h post-inoculation). Error bars are the ± standard deviation of the mean values.

the percentage of dead non-viable (BOX^+/PI^+) population in MOJ samples. In general, ascorbic acid appeared to have a protective effect against the acidic condition of MOJ in *E. coli*, reducing the size of dead population. Increasing the concentration of ascorbic acid from 0 $g.L^{-1}$ to 10 $g.L^{-1}$ led to a considerable reduction in the percentage of dead cells in MOJ during the 11-days course of the experiment. Compared to un-supplemented MOJ, addition of 0.5 $g.L^{-1}$ ascorbic acid caused a significant reduction in the dead population ($p < 0.05$). The results for MOJ samples supplemented with 1 $g.L^{-1}$ ascorbic acid did not differ from that of 0.5 $g.L^{-1}$ samples. However, compared to the latter, increasing the ascorbic acid concentration of MOJ to 5 or 10 $g.L^{-1}$ caused a significant decrease in the percentage of dead cells in MOJ ($p < 0.01$ and $p < 0.0001$ respectively).

However, it is important to note that ascorbic acid-dependent decrease in the number of dead cells in MOJ did not translate into greater percentage of healthy (BOX^-/PI^-) cells. [Figure 3.9(B)] shows the effect of different concentrations of ascorbic acid on the number of healthy, injured and dead cells immediately after their inoculation in MOJ (time 0 h). Compared to un-supplemented samples, supplementation of MOJ with 5 or 10 $g.L^{-1}$ ascorbic acid, caused a significant reduction in not only the number of dead cells but also healthy cells. Consequently, this meant that the observed increase in the percentage of viable cells was mainly due to an increase in the percentage of injured cells.

Culturability (Plate Count)

Supplementation of MOJ with 5 or 10 $g.L^{-1}$ ascorbic acid not only improved the viability of the cells in MOJ, but also their culturability [Figure 3.10(A)] ($p < 0.05$ and $p < 0.01$ respectively, both compared to un-supplemented MOJ). Compared to un-supplemented MOJ, the mean $\log_{10}$ $CFU.mL^{-1}$ of culturable cells in MOJ samples supplemented with 0.5 or 1 $g.L^{-1}$ was not significant. Therefore, it could be suggested that ascorbic acid is capable of improving the culturability of *E. coli* in MOJ. Nevertheless the concentrations of ascorbic acid required for achieving this, are significantly greater than those naturally found in real OJ.

VBNC Cells

As previously shown, un-supplemented MOJ was capable of inducing VBNC state in *E. coli*. The supplementation of MOJ with 0.5 or 1 $g.L^{-1}$ ascorbic acid had no clear effect on the VBNC population in MOJ [Figure 3.10(B)]. On the other hand, increasing the ascorbic acid concentration to 5 or 10 $g.L^{-1}$ significantly decreased the VBNC population in MOJ ($p < 0.05$ and $p < 0.01$ respectively, both compared to un-supplemented MOJ). The inverse relationship between the concentration of ascorbic acid and VBNC population could be attributed to the antioxidant activity of ascorbic acid. Oxidative stress and the formation of free radicals inside the cell have been suggested to as one of the primary causes of the induction of VBNC state in bacteria (Mizunoe *et al.*, 1999). Therefore, it is reasonable to hypothesize that the addition of ascorbic acid to the environment could improve the oxidative stress response of *E. coli*

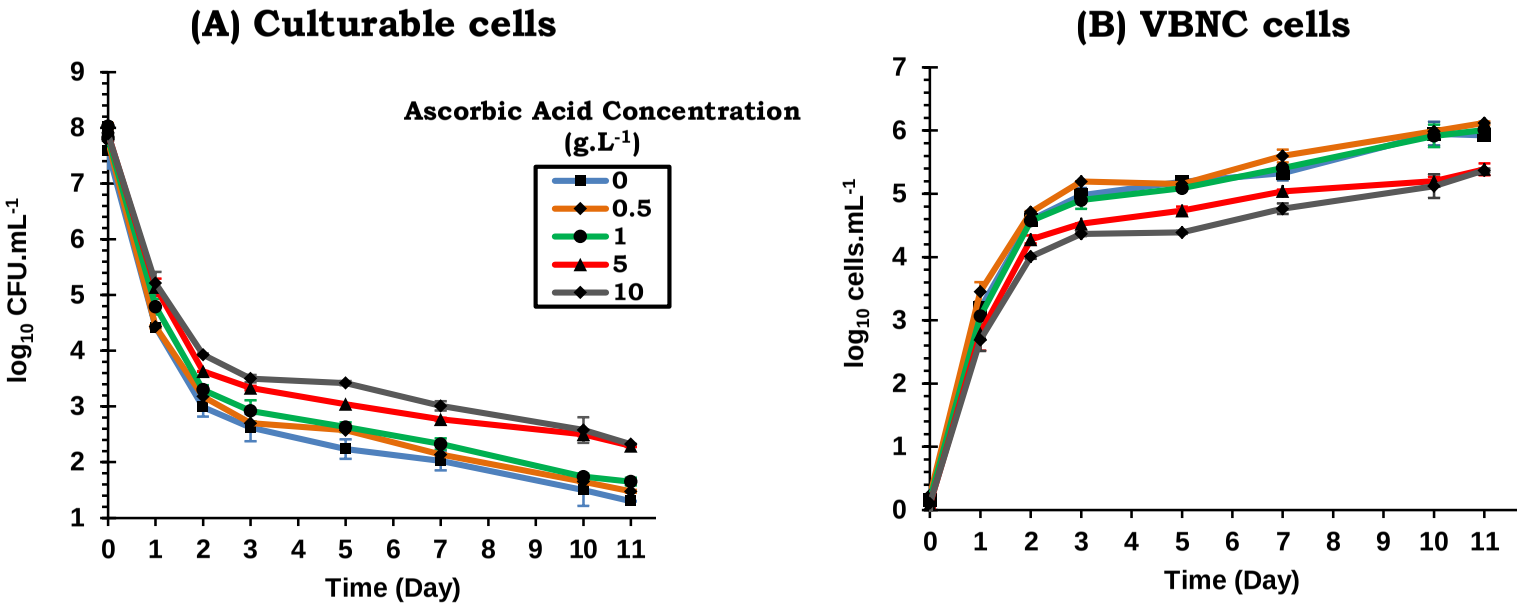


Figure 3.10: The effects of ascorbic acid content of MOJ on the culturability of exponential-phase *E. coli* K-12 MG1655 (OD_{650nm} 0.5) in MOJ incubated at 4 °C for 11 h

MOJ samples were serially (decimal) diluted in maximum recovery diluent (MRD) after which, 100 µL of the appropriate dilution was plated on nutrient agar plates and allowed to grow at 37 °C for 48 h. Culturable cells were considered those that could form a colony on a nutrient agar plate after 48 h incubation at 37 °C. Error bars are the ± standard deviation of the mean values.

and consequently reduce the induction of VBNC state in *E. coli*. This issue will be discussed in more detail later.

3.3.5 Amino Acids

The effect of amino acid supplementation of MOJ on the viability of *E. coli* was also investigated. For this study, a MOJ consisting of the top eight most abundant amino acids naturally present in OJ was prepared [Table 3.5]. For this purpose, the concentration of each amino acid was chosen based on the mean of values reported in the literature for freshly squeezed OJ (see [Table 1.4]). With the exception of the amino acid supplementation, the experimental method was identical to the general protocol described above for ascorbic acid experiment. In summary, log-phase *E. coli* (OD 0.5) was inoculated in MOJ with or without amino acids. Samples were incubated at 4 °C for 11 days and at different time-points, the viability and culturability of the cells was studied using FCM and plate counting respectively.

Viability (FCM Studies)

In agreement with the results described so far, inoculation of *E. coli* in MOJ led to dramatic decrease in viability of the cells regardless of the amino acid supplementation [Figure 3.11]. However, supplementation of MOJ with amino acids resulted in a significantly greater number of viable (healthy and injured) cells. For instance, in amino acid-free MOJ samples (control), only 5.04 ± 1.53% of the cells were found to be healthy (BOX^-/PI^-) at time 1.5 h post-inoculation. In amino acid-supplemented MOJ samples, however, this was nearly three-fold

Table 3.5: The composition and characteristics of the MOJ containing amino acids

	($g.L^{-1}$) (Amino Acid-Supplemented MOJ)
Sugars	**85**
Sucrose	45
Glucose	20
Fructose	20
Organic Acids	**11.5**
Citric Acid	9.5
Malic Acid	2
Buffering Agent	**5.02**
Potassium Citrate	5.02
Amino Acids	**2.455**
L-Proline	0.794
L-Arginine	0.564
L-Aspartic Acid	0.274
L-Asparagine	0.305
L-Glutamic Acid	0.145
L-Serine	0.128
L-Alanine	0.108
γ-Aminobutyric Acid	0.237
pH*	3.20 ± 0.04
Osmolality ($mOsmol.kg^{-1}$)**	513 ± 21

* : Mean ± standard deviation of 3 samples
**: Mean ± standard deviation of 3 samples (10 measurements per sample)

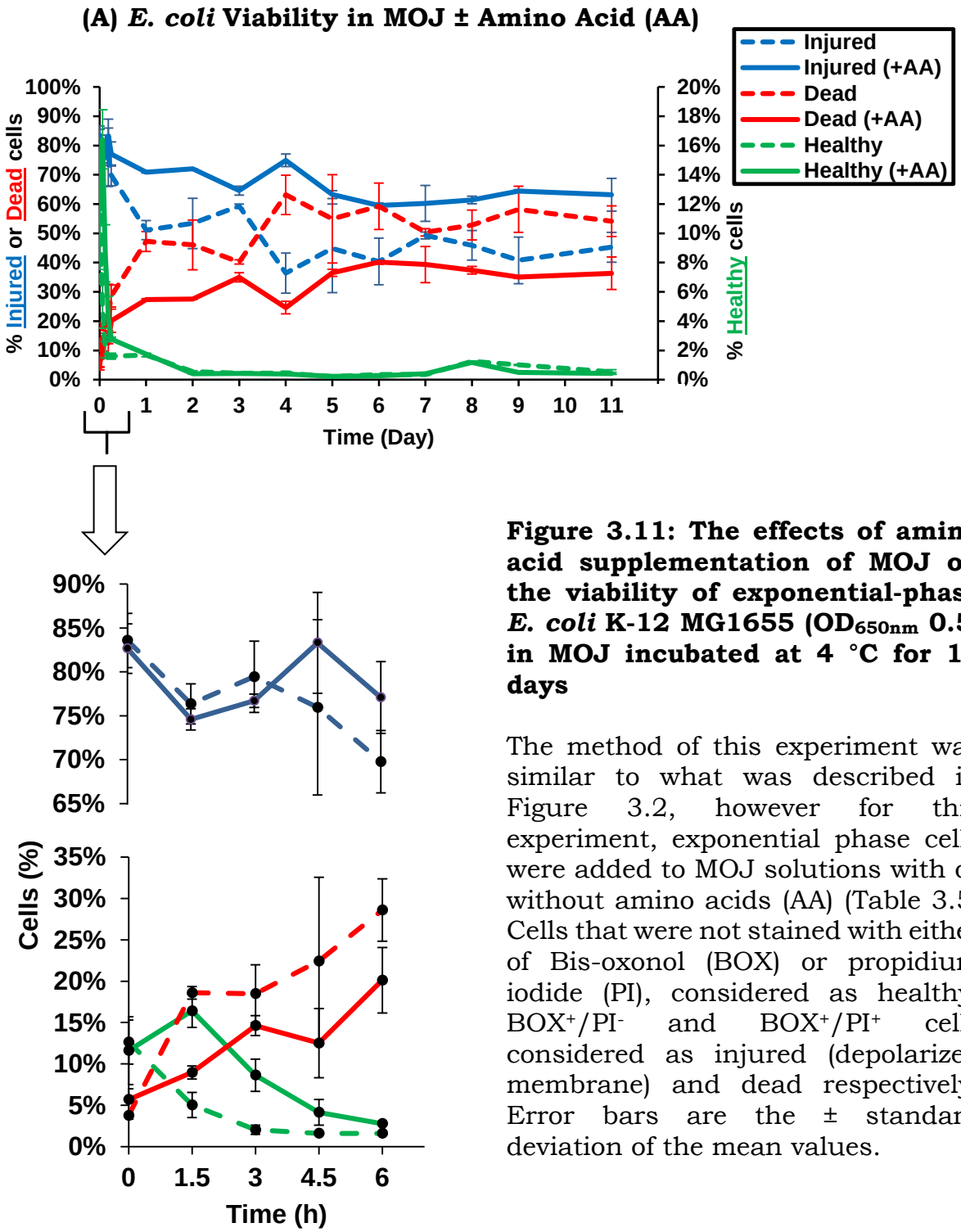


Figure 3.11: The effects of amino acid supplementation of MOJ on the viability of exponential-phase *E. coli* K-12 MG1655 (OD_{650nm} 0.5) in MOJ incubated at 4 °C for 11 days

The method of this experiment was similar to what was described in Figure 3.2, however for this experiment, exponential phase cells were added to MOJ solutions with or without amino acids (AA) (Table 3.5. Cells that were not stained with either of Bis-oxonol (BOX) or propidium iodide (PI), considered as healthy. BOX^+/PI^- and BOX^+/PI^+ cells considered as injured (depolarized membrane) and dead respectively. Error bars are the ± standard deviation of the mean values.

higher (16.42 ± 2.02%, $p < 0.05$). From time 4.5 h post-inoculation onwards, no significant difference was observed between the percentage of healthy cell in amino acid-free and -supplemented MOJ samples. Between day 1 and day 11 of the experiment, the percentage of healthy cells was less than 3% in all MOJ samples. Nevertheless, at this time-point, a significantly greater number of injured cells (viable BOX^+/PI^-) and lower number of dead cells (BOX^+/PI^+) was observed in MOJ samples supplemented with amino acids ($p < 0.05$). In other words, amino acid supplementation of MOJ led to a significantly greater percentage of viable cells consisting primarily of injured cells. However, no significant difference was observed between the results obtained at day 1 and day 11 of the experiment regardless of the amino acid content of MOJ samples. In other words, amino acid supplementation of MOJ was only effective in improving the viability of cells up to time 6 h post-inoculation (Figure 3.11(B)) after which the viability of *E. coli* in MOJ samples was similar regardless of the amino acid content of MOJ.

Culturability (Plate Count)

The supplementation of MOJ also led to an improvement in the culturability of *E. coli* subsequent to their inoculation in MOJ [Figure 3.12]. The greatest effect on loss of culturability was observed during the first 24 h of the experiment. At time 0 h post-inoculation no significant difference was observed between the culturability of the cells in MOJ regardless of the amino acid supplementation. However, at time 6 h post-inoculation, compared to un-supplemented samples, a significantly lower reduction in culturability of the cells was observed in

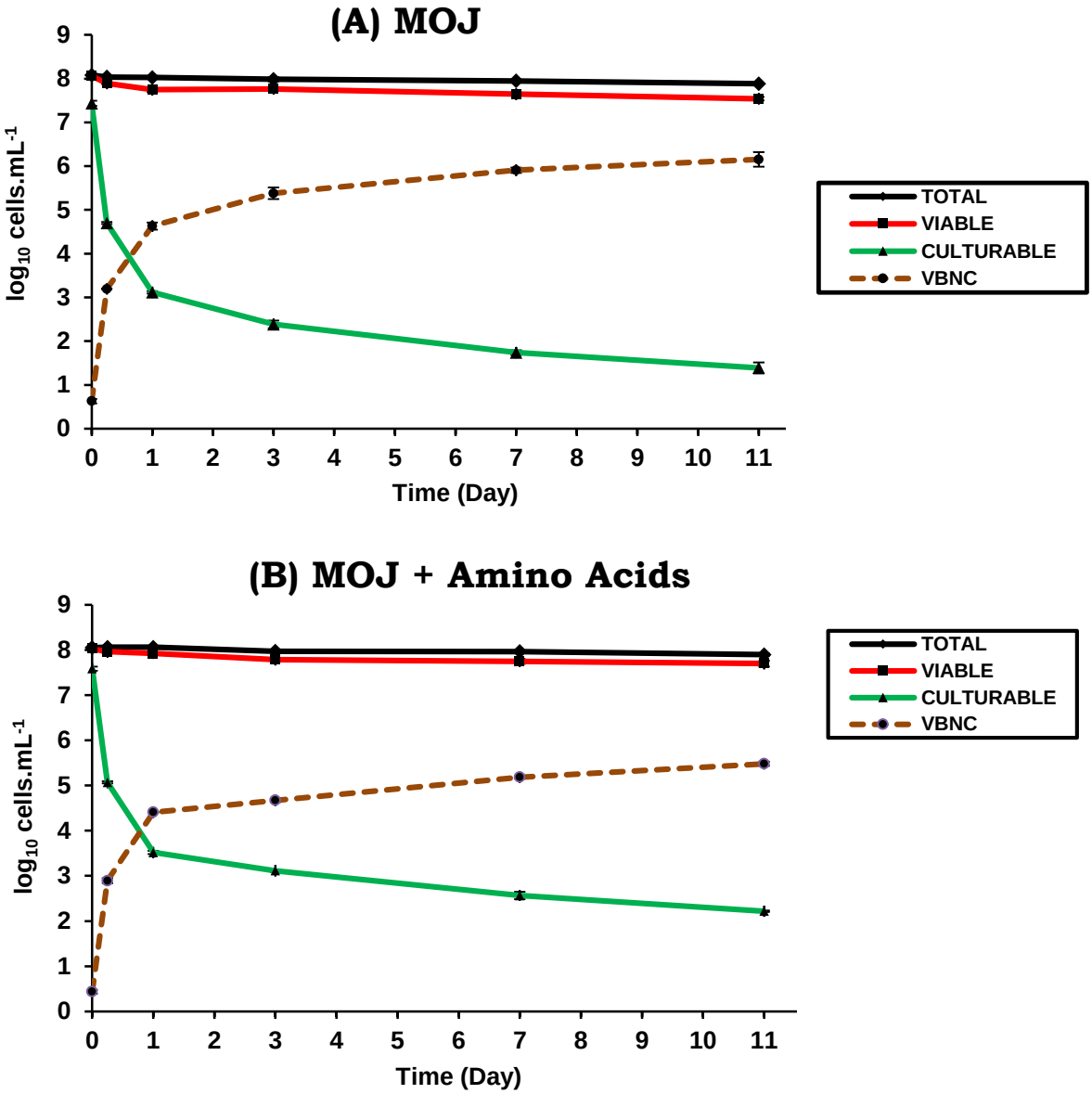


Figure 3.12: The effects of amino acid supplementation of MOJ on the viability and culturability of exponential-phase *E. coli* K-12 MG1655 (OD_{650nm} 0.5) in MOJ incubated at 4 °C for 11 days

Culturable cells were considered as those that could form a colony on a nutrient agar plate after 48 h incubation at 37 °C. Viable cells were the total number of PI negative cells, which included healthy cells as well as those with depolarized membrane (i.e., injured cells of BOX^+/PI^-). Total cell population includes both viable and non-viable (dead BOX^+/PI^+) cells. The number of viable but non-culturable (VBNC) cells was calculated by subtracting the $\log_{10}$ number of culturable cells from viable cells. Error bars are the ± standard deviation of the mean values.

samples which had been supplemented with amino acid ($p < 0.05$). The $\log_{10}$ reduction in the number of culturable cells between time 6 h and day 11 days post-inoculation in un-supplemented and amino acid-supplemented MOJ samples was not found to be statistically significant ($p = 0.05$). Nevertheless, compared to unsupplemented MOJ samples, the overall decrease in culturability of *E. coli* during 11 days incubation of the samples at 4 °C was significantly lower in amino acid-supplemented MOJ samples (6.05 ± 0.18 and 5.37 ± 0.07 respectively, $p < 0.05$).

VBNC Cells

During the 11-days course of the experiment, there was 0.55 ± 0.05 and 0.33 ± 0.07 $\log_{10}$ reduction in the number of viable cells in un-supplemented and supplemented samples respectively. As a result, the number of VBNC cells at each time-point closely resembled that of culturable cells which meant that the difference between the number of VBNC (brown dashed line in [Figure 3.12]) in amino acid-supplemented MOJ and the control samples during the course of the study was significant. For instance, at days 3, 7 and 11 post-inoculation, the VBNC population in amino acid-supplemented MOJ samples was respectively 0.71 ± 0.14, 0.72 ± 0.07 and 0.67 ± 0.17 lower compared to the control samples ($p < 0.05$, $p < 0.01$ and $p < 0.05$ respectively). The difference between the $\log_{10}$ number of viable cells in un-supplemented and supplemented samples was not statistically significant. Therefore, it could be suggested that amino acid supplementation exerts a greater beneficial effect on the culturability of the cells compared to their viability.

3.3.6. Amino Acids Supplementation of OJ

A crucially important criterion in using a model food system for predicting the behaviour of a microorganism in food is to validate the model system by comparing the model-derived observations by those obtained in real food (Silva *et al.*, 1999). Considering the apparent beneficial effects of amino acids on the viability and culturability of *E. coli* in MOJ, it was decided to examine whether the supplementation of OJ with amino acid could exert similar favourable effects on *E. coli*.

For this study, freshly squeezed OJ was supplemented with the same types and/or concentrations of amino acid which were used for supplementing the MOJ [Table 3.5]. The amino acid composition of OJ was not analysed and therefore, their concentration was presumed to be within the ranges reported in the literature for freshly squeezed OJ. Consequently, it was also assumed that the concentration of the top eight amino acids in supplemented OJ was twice that of un-supplemented OJ. Similar to previous experiments described above, mid-log-phase (OD 0.5) *E. coli* K-12 MG1655 cells were inoculated in OJ samples. Samples were subsequently stored at 4 °C for 11 days during which the viability and culturability of the cells were studied.

Viability (FCM Studies)

[Figure 3.13(A)] shows the change in the percentage of healthy cells in amino acid supplemented and un-supplemented OJ samples. In agreement with the

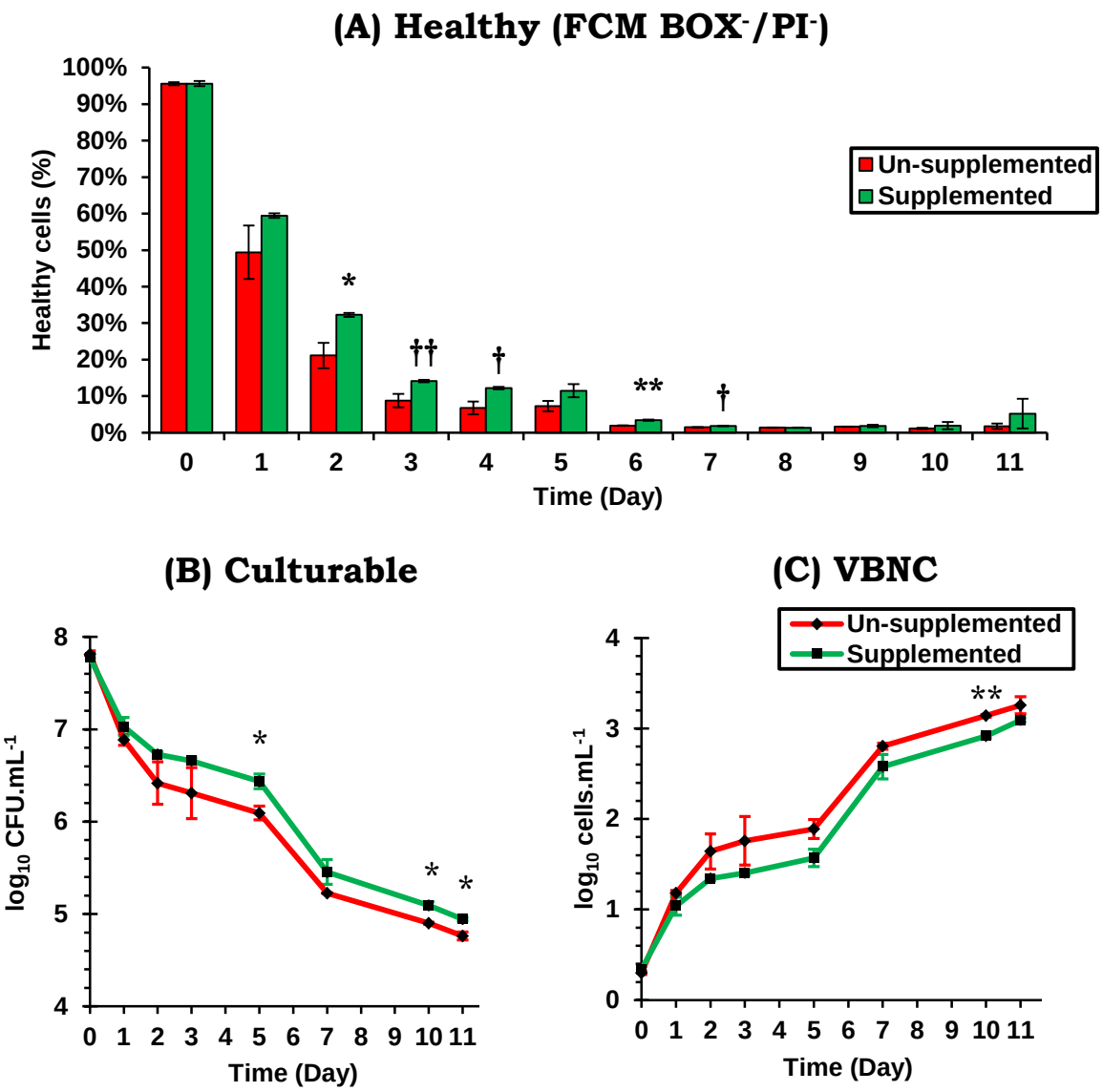


Figure 3.13: The effects of amino acid supplementation of OJ on the viability and culturability of exponential phase *E. coli* K-12 MG1655 (OD_{650nm} 0.5) in OJ during 11 days incubation at 4 °C

(A) The method of this experiment was similar to what was described in Figure 3.2; however, for this experiment, exponential phase cells were added to OJ (with or without amino acids) instead of MOJ. Cells that were not stained with either of Bis-oxonol (BOX) or propidium iodide (PI), were considered healthy. BOX^+/PI^- and BOX^+/PI^+ cells were considered injured (depolarized membrane) and dead respectively. Error bars are the ± standard deviation of the mean values. (*: $p < 0.05$; **: $p < 0.01$; †: $p = 0.05$; ††: $p = 0.06$, all compared to un-supplemented OJ samples at the marked time point. (n = 2)
(B) Culturable cells were considered those that could form a colony on a nutrient agar plate after 48 h incubation at 37 °C. Viable cells are the total number of PI^- cells and include not only the healthy cells but also those with depolarized membrane (i.e., injured cells of BOX^+/PI^-). Total cell population includes both the viable and non-viable (dead BOX^+/PI^+) cells.
(C) The number of viable but non-culturable (VBNC) cells was calculated by subtracting the Log_{10} number of culturable cells from viable cells. Error bars are the ± standard deviation of the mean values (*: $p < 0.05$, compared to un-supplemented OJ) (*: $p < 0.05$; **: $p < 0.01$; all compared to un-supplemented OJ samples at the marked time point).

result described above, the rate of decrease in healthy population was noticeably lower in OJ compared to MOJ samples. There was a significant reduction in the healthy population in OJ samples regardless of the amino acid supplementation. At all time-points, compared to control un-supplemented OJ, the mean percentage of healthy cells was greater in amino acid-supplemented samples. The observed differences were found to be significant at days 2 and 6 post-inoculation ($p < 0.05$ and $p < 0.01$ respectively). The lack of significance at days 3, 4 or 7 was believed to be due to small number of samples used (n = 2; p-values of 0.05, 0.06 and 0.05 respectively). In general, the overall decrease in viability of the cells in un-supplemented and supplemented samples during the course of the study was not found to be statistically significant.

Culturability (Plate Count)

Similar to what was observed in case of MOJ, the supplementation of OJ with amino acid resulted in an increase in the mean $\log_{10}$ number of culturable cells (Figure 3.13(B)). The $\log_{10}$ number of culturable cells in un-supplemented and amino acid-supplemented OJ samples was found to be statistically significant at days 5, 10 and 11. However, the overall decrease in culturability of the cells in un-supplemented and supplemented samples during the course of the study was not statistically significant ($p = 0.09$).

VBNC Cells

Despite the observed increase in the healthy and/or culturable cell populations of *E. coli* in amino acid-supplemented samples, the total $\log_{10}$ number of viable

cells in all OJ samples remained relatively constant throughout the experiment (7.95–8.13 $\log_{10}$ cells.mL^{-1}). Consequently, the pattern of change in $\log_{10}$ of VBNC population (i.e. $\log_{10}$ viable minus $\log_{10}$ culturable) closely resembled that of culturable cells [Figure 3.13(C)]. Similar to what was observed in amino acid-supplemented MOJ, at each time points, the mean $\log_{10}$ of VBNC cells was lower in supplemented cells, however the difference was only found to be statistically significant at day 10 ($p < 0.01$). On the whole, no significant difference was observed between the overall results obtained for supplemented and control samples.

3.3.7 Morphological Studies of the Cells in MOJ and OJ

Using FCM made it possible to study not only the viability of the *E. coli* cells in MOJ and OJ, but also the MOJ- and/or OJ-induced change in light scatter properties of the cells. As mentioned in the previous chapter, in FCM the scattered light from the cells is detected in two way: (a) FSC-A which is used as a rough indicator of the cell size and (b) SSC-A which depends on the surface roughness and/or granularity of the cells (Shapiro, 2000, 2003). [Figure 3.14] shows the median FSC-A and SSC-A of the healthy, injured or dead mid-log-phase *E. coli* cells in MOJ and OJ samples at 4 °C. In general, the median FSC-A and SSC-A of the injured (BOX^+/PI^-) and dead (BOX^+/PI^+) cells were significantly greater than the values observed for healthy cells (BOX^-/PI^-). This was assumed to be due to DNA-damage induced stress response of the cells leading to elongation and filamentation of the cells. This issue will be discussed in more detail later.

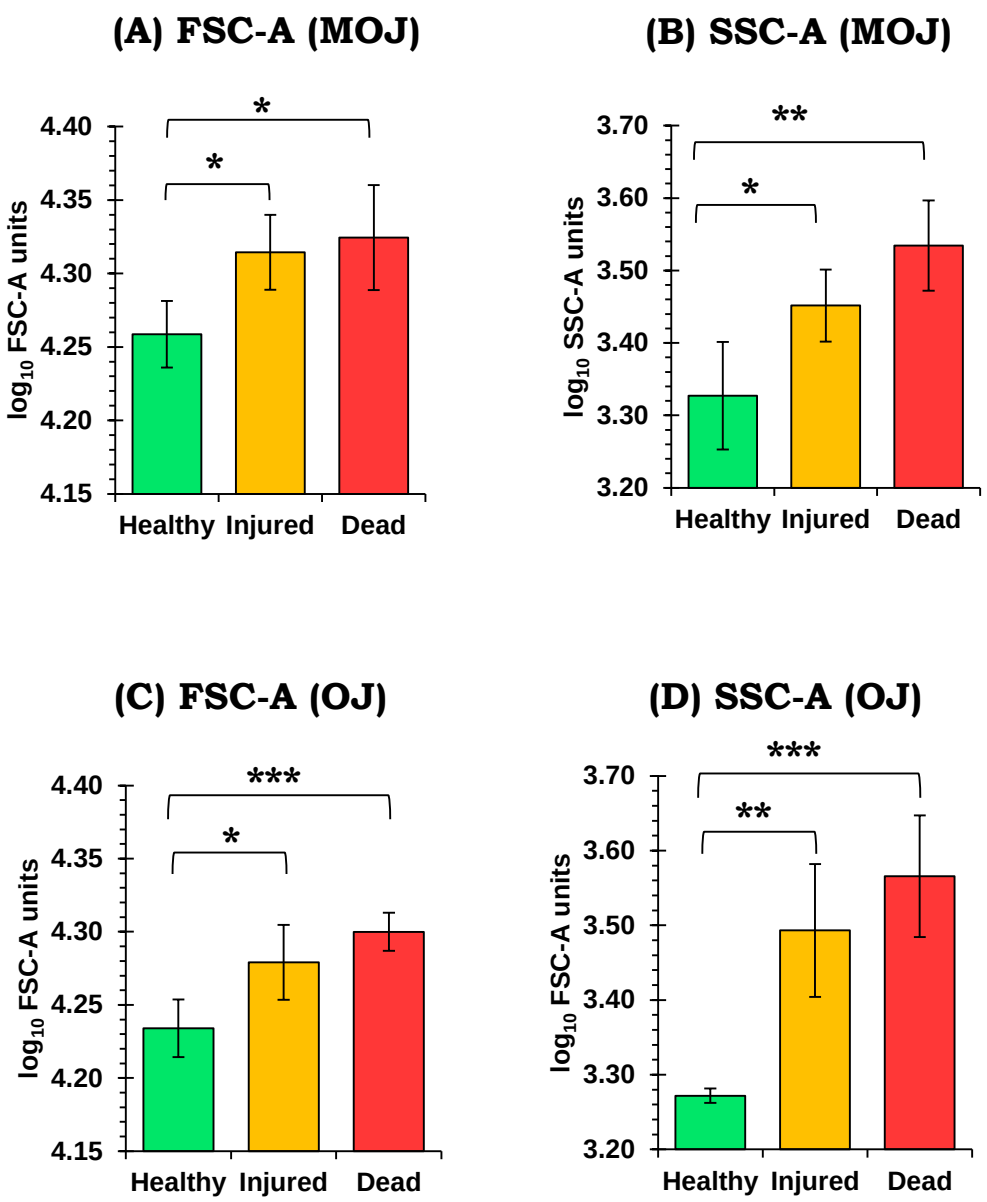


Figure 3.14: Median FSC-A and SSC-A of *E. coli* K-12 MG1655 in OJ and MOJ

The Figure shows the median log10 FSC-A and SSC-A of healthy, injured and dead cells in OJ and MOJ The values are the median $\log_{10}$ FSC-A or SCC-A units (i.e., channel number) reported by the FCM. Error bars are the ± standard deviation of the median values. (*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$) (n = 4)

Considering the significant difference between the light scatter properties of BOX^- (healthy cells) and BOX^+ (injured or dead) cells, a close correlation between the pattern of change in median FSC-A or SSC-A of the cells and the viability state of the cells could be observed. [Figure 3.15(A)] and (B)] respectively, show the relationship between the changes in median FSC-A or SSC-A of the cell population and the percentage of "non-healthy" BOX^+ cells (i.e. injured and dead) immediately after inoculation in MOJ containing various concentration of ascorbic acid (compared to time 0 h pre-inoculation). Compared to control un-supplemented MOJ samples, a significantly greater percentage of non-healthy cells was observed in MOJ samples supplemented with 5 or 10 $g.L^{-1}$ of ascorbic acid ($p < 0.01$ and $p < 0.001$ respectively). This increase was matched with a significant increase in the median FSC-A and SSC-A of the cells in 5 or 10 $g.L^{-1}$ ascorbic acid supplemented MOJ samples (both $p < 0.05$ compared to un-supplemented samples).

[Figures 3.16(A) & (B)] show the change in median FSC-A and SSC-A of the cells in MOJ and OJ samples (with or without amino acid supplementation) during the course of the experiment respectively. Compared to the MOJ samples, the rate of increase in either of the median FSC-A or SSC-A was significantly lower in OJ samples. With regard to the median SSC-A of the cells inoculated in MOJ, there was a dramatic increase during the first 24 h of the inoculation followed by relatively similar values for all samples regardless of the amino acid supplementation. Moreover, there was a significant difference between the

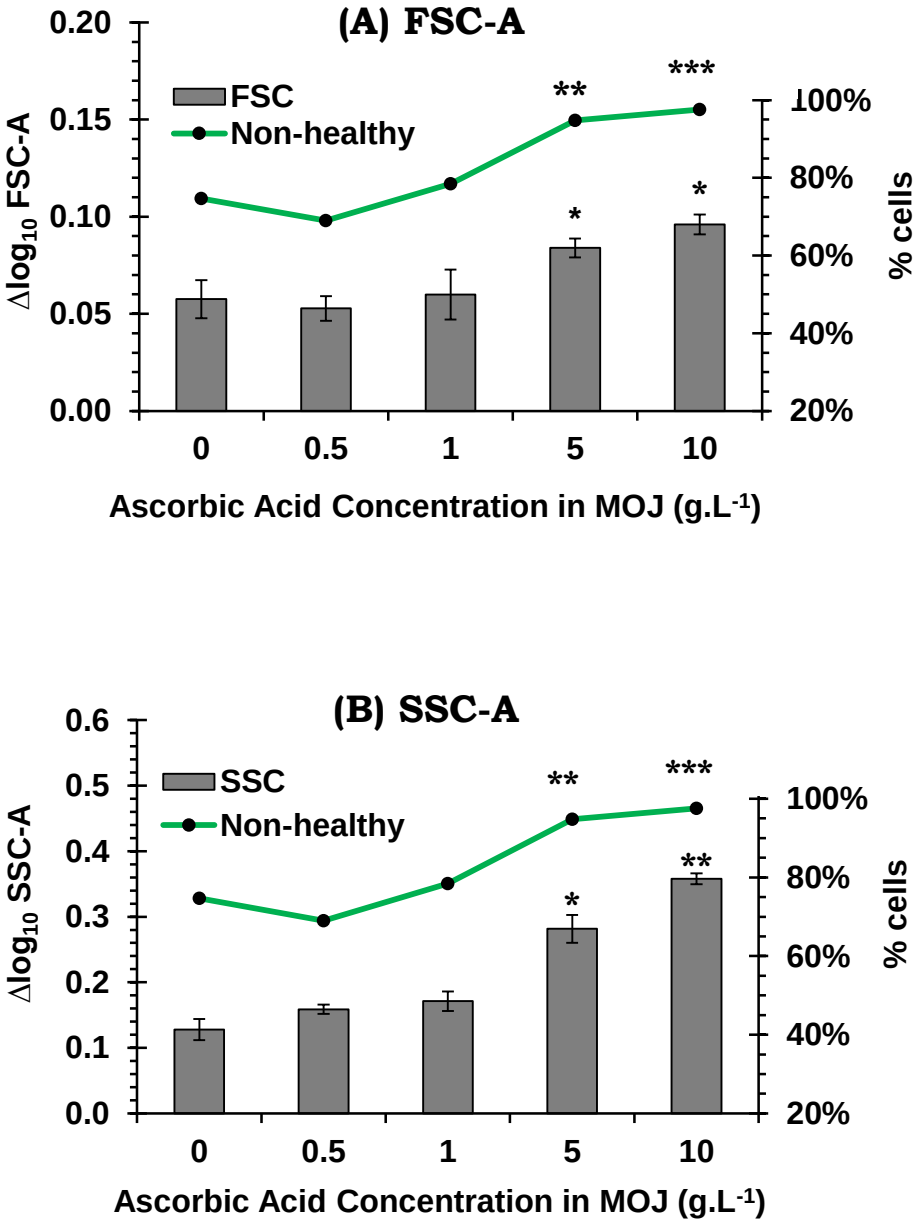


Figure 3.15: Relationship between the change in light scatter properties of the cells and the percentage of non-healthy cells

This figures shows the relationship between the ascorbic acid-induced change in **(A)** FSC-A and **(B)** SSC-A of the cells and the percentage increase in the percentage of non-healthy (BOX^+) cells. Error bars are the ± standard deviation of the median values. (*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$ compared to un-supplemented MOJ samples).

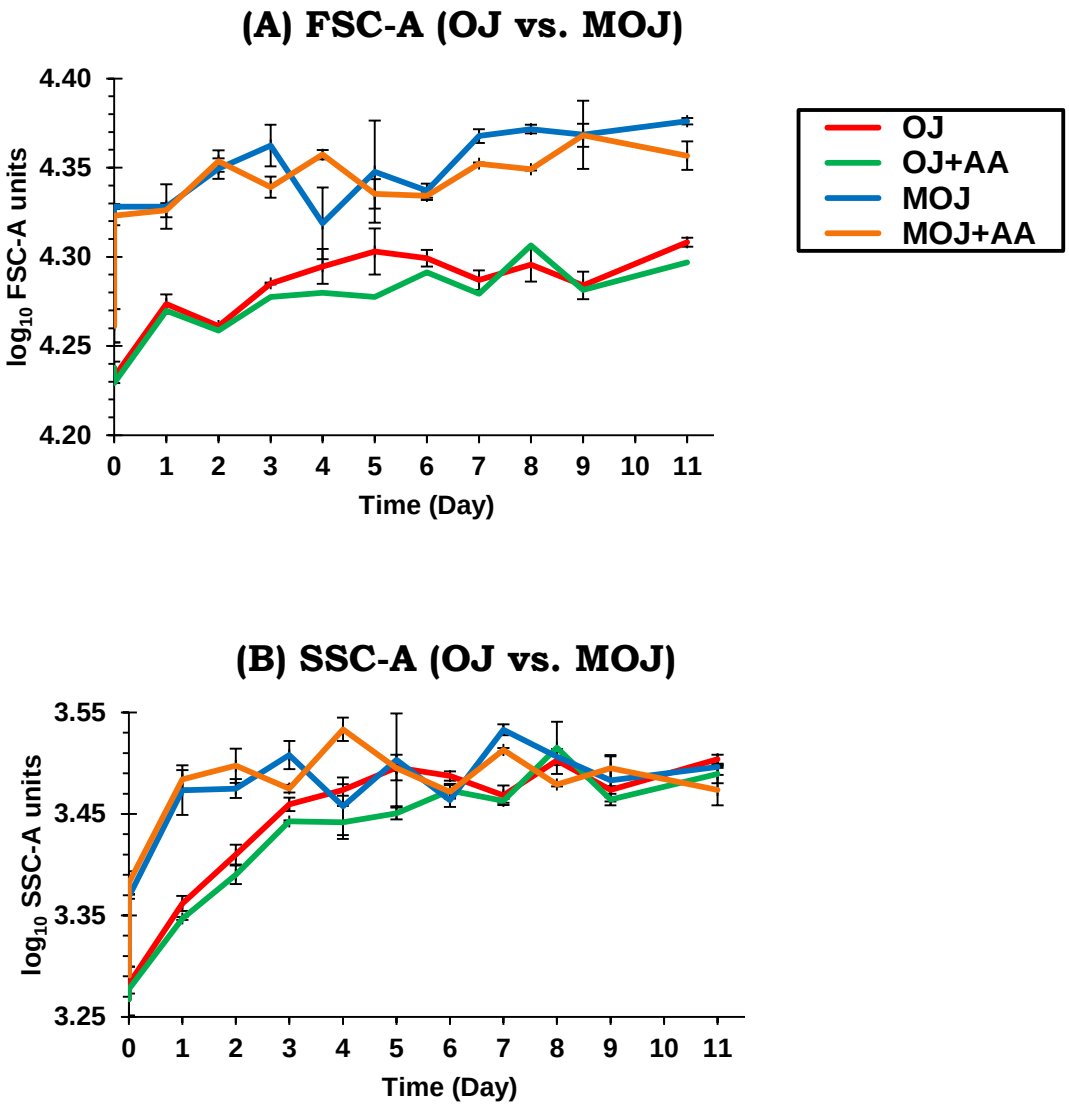


Figure 3.16: The effects of amino acid supplementation of OJ on the optical properties of exponential phase *E. coli* K-12 MG1655 (OD_{650nm} 0.5) in MOJ during 11 days incubation at 4 °C

Figures **(A)** and **(B)** respectively show the overall change in the FCM forward scatter (FSC-A) and side-scatter (SSC-A) of the *E. coli* (all cells regardless of their viability state). The values are the median Log_{10} FSC-A or SCC-A units (i.e., channel number) reported by the FCM at each time-point. Error bars are the ± standard deviation of the median values. (*: $p < 0.05$; compared to un-supplemented OJ samples at the marked time point). Figures **(C)** and **(D)** compare the results shown in Figures 3.14 (A) and (B) with that of 3.16(A) and (B)

results observed in OJ and MOJ up to day 6 post-inoculation (p = 0.01). However from this time onwards, relatively similar median SSC-A values for *E. coli* cells was observed in both the OJ and MOJ samples.

This was interesting, considering that the percentage of healthy cells population remained relatively constant in MOJ and OJ after day 1 and day 6 respectively ([Figure 3.11(A)] and [Figure 3.13(A)] respectively). [Figure 3.17] show the regression analysis of the relation between the median SSC-A or FSC-A of *E. coli* cell population inoculated in OJ (data previously shown in [Figure 3.16(A)] against the percentage of healthy cells (data previously shown in [Figure 3.13(A)]). Each plot consists of 48 data points, the values obtained for four OJ samples at 12 time-point during the course of the study. As expected and similar to what was observed in MOJ samples [Figure 3.15], there was a strong linear negative correlation (R^2 = 0.93) between the percentage of healthy cells and the median SSC-A of the cells in OJ [Figure 3.17(A)]. In other words, reduction in the percentage of healthy cells corresponded with an increase in $\log_{10}$ median SSC-A of the cell population. In contrast to the SSC-A parameter, the correlation between the median FSC-A of the cell population and their viability state was weaker ([Figure 3.17(B)]; R^2 = 0.73). The weaker correlation between the median FSC-A of the cell population and the percentage of healthy cells could be due to greater contribution of the injured cells to the overall number of non-healthy BOX^+ cells in OJ. As was shown in [Figure 3.14], compared to FSC-A, there was a significantly greater difference between the median SSC-A of the healthy and injured cells ($p < 0.05$ and $p < 0.01$

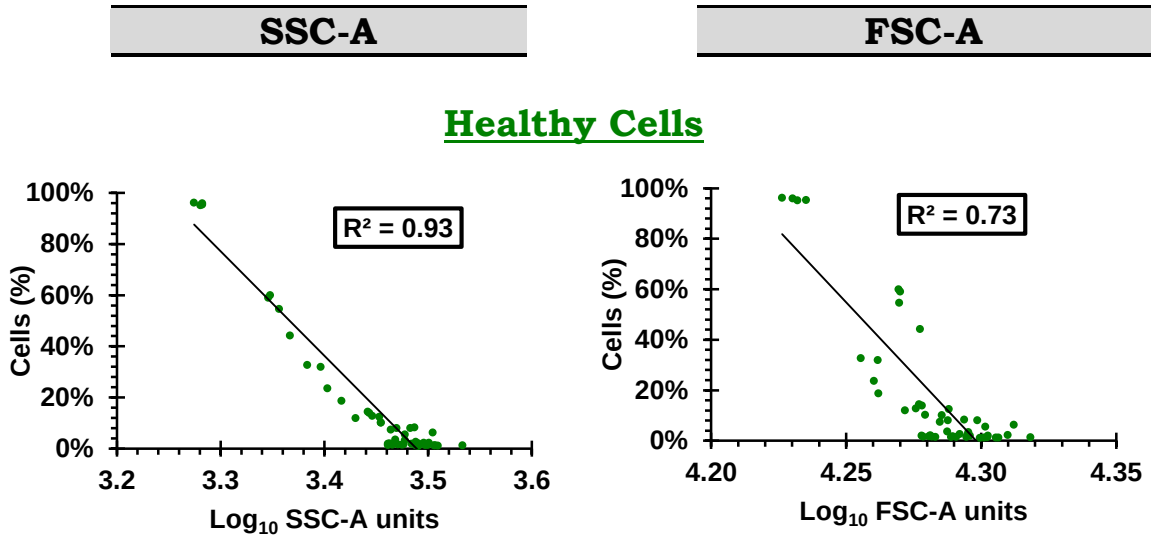


Figure 3.17: The correlation between the percentage of healthy cells and the light scatter properties of the cells in OJ.

Dot plots shown here were generated by plotting the percentage of healthy, injured or dead *E. coli* cells in OJ samples against the FSC-A or SSC-A the cell population. Each dot consists of 48 dots representing the values obtained for four OJ samples (with or without amino acids) up to day 11 post-inoculation (12 data points including time 0 h).

respectively). This could have led to greater correlation between the median SSC-A of the cell population and the percentage of healthy cells, compared to median FSC-A.

In summary, the results showed a close agreement between the median SSC-A of the *E. coli* cells population and their viability state in OJ and MOJ.

3.4 Discussion

3.4.1 Growth Phase and Incubation Temperature

Growth Phase

In general, compared to log-phase and early-stationary-phase cells (OD 0.5–2), stationary and late-stationary-phase cells (OD 4 and 6) were found to be more resistant in MOJ and OJ both at 4 °C and 22.5 °C [Figures 3.2 and 3.3]. These results were in agreement with those reported in the literature showing a greater acid resistance of the stationary-phase cells compared to log-phase cells (Small *et al.*, 1994; Arnold & Kasper, 1995; Chang & Cronan Jr., 1999; Price *et al.*, 2004). Arnold & Kasper (1995) studied the survival (culturability) of mid-log, late-log and stationary-phase of several *E. coli* strains inoculated in acidified tryptone soy broth (pH 2). The results of their study showed that compared to log-phase cells, a significantly greater survival was observed for stationary-phase cells. On the other hand, the survival of late-log-phase *E. coli* in an acidic environment was strain-dependant in that study. For instance, the late-log-phase of *E. coli* O157:H7 strains of 84-01 and TB285 were more resistant to acidified TSB than mid-log-phase cells while the opposite was observed in case

of late-log O157:H7 strain of ATCC 43895. The strain-dependant resistance of late-log-phase cells to acidic condition could explain the significant reduction in the mean number of culturable late-log-phase cells (OD 0.75 and 1) in OJ and MOJ samples [Figure 3.3].

The acid resistance (AR) mechanisms of stationary-phase *E. coli* are AR1, AR2 and AR3 (see Section 1.3.2). The activation of AR1 requires the absence of glucose from the growth medium and the growth of the cells in mildly acidic condition (pH 5.5) prior to acid stress, while the activation of AR2 and AR3 systems need the growth of the cells in glucose-containing complex media as well as the presence of exogenous glutamate and arginine during the acid stress (Richard & Foster, 2003). The 2×LB used in this study for growing the cells contained glucose (in the form of yeast extract) which could have suppressed the AR1 system. In addition, the presence of 20 $g.L^{-1}$ of tryptone led to alkalinisation (pH 8-9, data not shown) of the growth medium most likely due to the catabolism of amino acids and excretion of excess ammonium (Chang & Cronan, 1999; Sezonov *et al.*, 2007). Moreover, the MOJ used at this stage of the study did not contain any amino acids necessary for the activation of AR2 and AR3. Therefore, the observed increase in acid resistance of stationary-phase cells was presumed to have been regulated by RpoS, a sigma subunit of RNA polymerase (also known as σ^{38} or σ^{s}), which is responsible for the general stress response of *E. coli* (Hengge-Aronis, 1993; Cheville *et al.*, 1996). Low pH condition (pH < 5) could lead to an increase in translation of *rpoS* mRNA and inhibition of RpoS proteolysis (Hengge-Aronis, 2002).

Moreover, the RpoS-regulated acid stress response does not require the prior exposure of the cells to acidic condition (Cheng & Kaspar, 1998).

Incubation Temperature

With regard to the incubation temperature of the samples, significantly greater adverse effects were observed on the culturability as well as viability of the cells at 22.5 °C compared to 4 °C [Figures 3.2 and 3.3]. It has been suggested that once acid stress response in *E. coli* is induced, storage at cold temperature can improve the survival of *E. coli* in acidic environment (Lin *et al.*, 1996). Several studies have demonstrated enhanced resistance of *E. coli* to acidic conditions at low temperatures (Clavero & Beuchat, 1996; Zhao *et al.*, 1993; Uljas & Ingham, 1998). For instance, Uljas & Ingham (1998) reported a greater survival of *E. coli* in apple juice and acidified TSB at 4 °C compared to 21 °C. A similar phenomenon has also been reported in case of *S.* Typhimurium inoculated in OJ and yoghurt as well as *Listeria monocytogenes* in fermented acidic salami (Álvarez-Ordóñez *et al.*, 2013; Gounadaki *et al.*, 2007)

It has been suggested that the reduction in survival of the cells in acidic foods at high temperatures could be due to greater dissociation of organic acids inside the cells leading to greater microbial inactivation (Álvarez-Ordóñez *et al.*, 2013). According to the weak acid theory, the undissociated form of organic acid diffuses through the cellular membrane where it is converted into dissociated form due to trans-membrane pH gradient (ΔpH). Consequently, this leads to greater intracellular concentration of proton and weak acid anions

(Salmond *et al.*, 1984). In order to maintain the pH_i and to prevent the deleterious effects of low pH_i on cell structure, enzymatic activity and nucleic acids, cells actively extrude protons by employing ATP. This results in depletion of cellular energy leading eventually to cell death (Davidson & Harrison, 2003; Lu *et al.*, 2011). This phenomenon which is also referred to as metabolic exhaustion has been extensively discussed by Leistner (2000) who reported greater reduction of *salmonella* in fermented sausages and *Listeria innocua* in water-in-oil emulsions at ambient temperatures than under refrigeration.

According to Stratford & Eklund (2003), in order to diffuse through the plasma membrane and lower the cytoplasmic membrane, organic acids need to be have small molecular size (less than 3 carbons such as acetic acid) or to be lipophilic. However, citric acid and malic acid not only have more than three carbons (six and four respectively), but are also hydrophilic (octanol-water partition coefficient or log P_{oct} of -0.172 and -1.26 respectively) (Stratford & Eklund, 2003; Brittain, 2001). As a result, this mechanism was not assumed to be the reason behind the lower percentage of healthy cells at 22.5 °C.

A possible reason behind the observed decrease in the percentage of healthy cells at 22.5 °C of the cells could be the greater metabolic activity of the cells at this temperature compared to 4 °C. According to Bloomfield *et al.*, (1998), the stress-induced growth arrest of the cells, leads to de-coupling of the cell division from metabolism. In cells with high metabolic activity, this results in rapid generation of lethal concentration of reactive oxygen species (ROS) inside

the cells. However, due to growth-arrest, cells are unable to detoxify themselves leading to loss of culturability, sub-lethal injury or cell death.

Despite this, at time 0 h post-inoculation and contrary to what was observed throughout the experiment, the population of healthy cells in MOJ samples containing stationary-phase cells was significantly lower at 4 °C compared to 22.5 °C [Figure 3.2]. This was assumed to be due to stationary-phase-induced changes in the composition of the membrane during their growth in 2×LB (pre-inoculation in MOJ). It is known that moving to the stationary-phase results in an increase in the saturated fatty acid composition of the membrane and the conversion of unsaturated fatty acids to cyclopropane derivatives (DiRusso & Nyström, 1998). Cyclopropane fatty acid formation in stationary-phase cells of *E. coli* has been suggested to play a major role in increased resistance of the cells to acidic condition (Chang & Cronan Jr., 1999). However, the increase in the saturated fatty acid content of the membrane could adversely affect the cold shock response of the cells by reducing the flexibility of the membrane and the physical state of the lipid bi-layer in *E. coli* (De Mendoza & Cronan Jr., 1983). Therefore, it could be hypothesized that the stationary-phase-induced increase in saturated fatty acids composition of the membrane adversely affected the capability of *E. coli* to resist the cold shock in MOJ at time 0 h post-inoculation. However, it is also important to remember that in the wider context of survival, the incubation of the cells at 4 °C showed a significantly positive effect on the overall physiology of the cells in MOJ and OJ compared to 22.5 °C as previously shown in section 3.3.2.

3.4.2 OJ vs. MOJ

Compared to the results obtained for OJ samples, a significantly lower number of healthy or culturable cells were observed in MOJ samples. This was despite the fact that both MOJ and OJ had relatively similar pH and osmolality. Moreover, the concentration of sugars, organic acid and potassium in MOJ was within the ranges naturally found in OJ. Therefore, it is reasonable to suggest that other components of OJ could have played a role in viability and/or culturability of *E. coli*. A similar difference between the microbial survival in a model juice system and real fruit juice has been reported by Nualkaekul & Charalampopoulos, (2011). These researchers developed a mathematical model for the predicting the survival of *Lactobacillus plantarum* based on the results obtained in model solutions containing various concentrations of citric acid, ascorbic acid and different pH values. This model however, was not successful for predicting the survival of this microorganism in all fruit juices. They attributed this discrepancy to the presence of other compounds such as dietary fibre, protein and polyphenols in fruit juices. In another study performed by Uljas & Ingham, 1998, the survival of *E. coli* in apple juice was significantly greater than the level observed in acidified model solution. They also proposed that compounds other than organic acid could have played a role in viability of *E. coli* in apple juice. The possible role of OJ particles and hesperidin the primary polyphenols of OJ on physiology of *E. coli* in OJ has been investigated in Chapter 4.

3.4.3 Viability vs. Culturability

As was shown in [Figure 3.4], inoculation of *E. coli* in OJ and MOJ had little effect on total $\log_{10}$ number of viable cells as determined by FCM. On the other hand, a significant increase was observed in the number of VBNC cells particularly in MOJ samples and at 22.5 °C. In case of both the MOJ and OJ, VBNC cells consisted of not only the injured cells but also healthy cells with intact polarized membrane.

Very few studies have investigated the existence of VBNC state in foods particularly acidic fruit juices (Rowan, 2004; Nicolò *et al.*, 2011). For instance, to the best knowledge of the author, so far only one study has investigated the induction of VBNC state in *E. coli* as a direct result of the physicochemical characteristics of an acidic fruit juice (i.e., grapefruit juice;). It is known that subjection of *E. coli* to various stress conditions as well as transition to stationary-phase can induce the *rpoS*-regulated general stress response. Consequently, this results in a reduction or cessation of growth in *E. coli* through down-regulation of genes responsible for translation and ribosome biogenesis (Hengge-Aronis, 2000 & 2002; Jozefczuk *et al.*, 2010). In turn, the induction of VBNC state in bacteria has been suggested to be due to stress-induced de-coupling of growth and metabolism, leading to elevated intracellular generation of ROS in stressed cells upon their transfer to a rich growth medium (Bloomfield *et al.*, 1998; Oliver, 2005). The important role of RpoS in the stress response of *E. coli* to stationary-phase starvation, oxidative stress and cold shock has previously been reported (Hengge-Aronis, 1993 & 2002; Vidovic *et*

al., 2011). This could explain why generally greater number of culturable cells was observed in case of stationary-phase cells and those inoculated at 4 °C [Figure 3.3]. The induction of RpoS in these cells, could have led to an improved response of *E. coli* to generation of intracellular ROS and therefore, a greater number of culturable cells. A similar mechanism was reported by Boaretti *et al.*, (2003) who showed a significantly shorter persistence of *E. coli* as VBNC cells for the rpoS-mutant cells compared to the parental strains.

In the current study, while there was a significant reduction in the culturability of the cells in MOJ or OJ, the total number of viable cells (PI^-) remained fairly constant. A similar relation between the number of viable and culturable cells has been reported for various microorganisms including *Salmonella spp.*, *E. coli*, *L. monocytogenes* and *Vibrio vulnificus* in foods, water and environmental samples under various stress conditions (Cho & Kim, 1999; Lesne *et al.*, 2000; Dinu & Bach, 2011; Liu *et al.*, 2008; Cunningham *et al.*, 2009; Oliver, 2005; Oliver *et al.*, 2005).

3.4.4. Sugars and Organic Acids

The change in sugar content of MOJ within the ranges naturally found in OJ, did not have any significant effect on either of the viability or culturability of *E. coli* [Figure 3.5].

Increase in sugar contents of MOJ from 60 $g.L^{-1}$ to 120 $g.L^{-1}$ resulted in an increase in mean osmolality of the MOJ from 423 $mOsmol.kg^{-1}$ to 640

mOsmol.kg^{-1} respectively. It has been stated that the optimal osmolality for growth of *E. coli* is at 300 mOsmol.kg^{-1}; however, while it can tolerate osmolalities of between 30 and 3,000 mOsmol.kg^{-1}, the growth rate decreases upon deviation from the optimal osmolality (Cayley & Record, 2004). Consequently, it could be suggested that the increase in sugar concentration of MOJ, most likely led to a greater osmotic stress in *E. coli*. Nevertheless, the resultant osmotic stress was not strong enough to affect either the viability or culturability of the cells in MOJ considering the wide range of osmolalities it can tolerate. It is also reasonable to presume that the greater availability of sugars such as glucose and fructose and therefore the nutrients which can be utilized by *E. coli* negated the possible adverse effects of osmotic shock on *E. coli* in case of medium- and high-sugar MOJ solutions.

Unlike what was observed for MOJ solutions with different sugar content, the increase in organic acid content of MOJ from 6 g.L^{-1} to 18 g.L^{-1}, led to a significant decrease in culturability and viability of *E. coli* [Figure 3.6]. The range of osmolalities and pH for these solutions was similar to the ones containing different amount of sugars. Considering the lack of statistically significant difference between the rate of survival of *E. coli* in MOJ samples with different sugar content (hence different osmolalities) it could be suggested that the observed adverse effects on viability in MOJ solution with high organic acid concentrations was due to the antimicrobial properties of the organic acid and not the osmolality.

Although statistically significant, however the observed adverse effects of 3-fold increase in organic acids on $\log_{10}$ number of healthy or culturable cells during the course of the study were relatively small (0.23 and 0.58 $\log_{10}$ reduction respectively). This could be due to different antimicrobial mechanisms of citric and malic acids. Bjornsdottir *et al.*, (2006) investigated the effects of citric acid and malic acid on survival of *E. coli* at pH 3.2 solutions at 25 °C. They showed that while the gradual increase in concentration of citric acid from 1 mM to 60 mM (fully-protonated) corresponded with a decrease in culturability of *E. coli*, the supplementation of the solution with 5–10 mM fully-protonated malic acid led to a significant increase in culturability of the cells. Considering the concentration of fully-protonated organic acids in MOJ solutions used in the current study, and by assuming the same effect at 4 °C, it could be hypothesized that while increase in citric acid concentration of MOJ decreased the culturability, the addition of 4.6 mM and 9.1 mM fully-protonated malic acid in MOJ led to an improvement in culturability of *E. coli*. In other words, the counteraction of these organic acids could explain the reason behind the relatively small differences that were observed between the results obtained for MOJ with different organic acid concentrations.

3.4.5. Ascorbic Acid

Ascorbic acid has been shown to exhibit both antioxidant and antimicrobial effects against *E. coli* in acidic environment (Van Opstal *et al.*, 2006; Tajkarimi & Ibrahim, 2011). In the current study, supplementation of MOJ with 0.5–10 $g.L^{-1}$ ascorbic acid led to a significant decrease in the percentage of dead cells in

MOJ (BOX^+/PI^+) during the course of the study [Figure 3.9(A)]. Nevertheless, simultaneously, the population of healthy cells (BOX^-/PI^-) also decreased, most notably at time 0 h post-inoculation [Figure 3.9(B)]. Increase in ascorbic acid concentration of MOJ reduced the pH and increased the osmolality of the solution [Table 3.4]. Although the possibility could not be excluded that these parameters played a role in ascorbic acid-dependant physiology of *E. coli* in MOJ, however, they were not assumed to be the primary cause behind the observed results. For instance, increase in ascorbic acid concentration of MOJ from 1 $g.L^{-1}$ to 5 $g.L^{-1}$ led to a significant reduction in the number of healthy cells at time 0 h post-inoculation [Figure 3.9(B)] despite the lack of statistical significance between their pH or osmolality.

The overall increase in viability of *E. coli* in ascorbic acid-supplemented MOJ could be attributed to the increased oxidative stress response of *E. coli*. Richter & Loewen (1981) investigated the role of ascorbic acid in induction of catalase activity in *E. coli*. The result of their study showed that the supplementation of the growth medium (LB) with 0.57–5.7 mM ascorbic acid led to a significant induction of catalase synthesis in *E. coli*. Within 30 min of the supplementation of growth medium with 5.7 mM (≈ 1 $g.L^{-1}$) ascorbic acid, the catalase levels in *E. coli* increased by eight-fold. This was believed to be due to oxidation of ascorbic acid leading to generation of H_2O_2 which was shown to be capable of inducing catalase synthesis within 15 s of its addition to the growth medium. The elevated level of catalase was attributed to the increased generation of H_2O_2, the by-product of the ascorbic acid oxidation. The stress-induced growth arrest in

metabolically active cells could lead to generation of ROS in bacteria (Bloomfield *et al.*, 1998). Therefore, it is reasonable to assume that the increase in concentration of ascorbic acid led to greater increase in intracellular concentration of H_2O_2 hence a greater oxidative stress response. Based on this assumption, it could be hypothesized that the increase in ascorbic acid content of MOJ and the resultant increase in H_2O_2 concentration was responsible for the initial reduction in the number of healthy cells in MOJ samples supplemented with 5 $g.L^{-1}$ or 10 $g.L^{-1}$ ascorbic acid. However, the greater induction of catalase could have improved the survival of injured cells, leading to lower number of dead cells.

The increase in catalase activity of the cells could also explain the concurrent increase in viability and culturability of the cells (Figure 3.10(A)) and the reduction in VBNC population (Figure 3.10(B)), particularly when MOJ was supplemented with 5 $g.L^{-1}$ or 10 $g.L^{-1}$ ascorbic acid. The supplementation of solid growth medium with catalase as low as 200 catalase units per plate has been shown to restore the culturability of VBNC *E. coli* cells by degrading stress-induced intracellular H_2O_2 (Mizunoe *et al.*, 1999). This raises the question of whether or not the possible ascorbic acid-induced increase in catalase activity of *E. coli* was sufficient to reduce the extracellular level of H_2O_2. Ma & Eaton (1992) showed that the catalase activity in *E. coli* against exogenous H_2O_2 depends on the cell density of the medium. While the H_2O_2-sensitivity of a wild-type *E. coli* K-12 and its catalase mutant was similar in low-density cell suspensions (5×10^2 $cells.mL^{-1}$), the wild-type strain was more

resistant to exogenous H_2O_2 when the cell density was increased to 5×10^7 cells.mL^{-1}. They proposed that the collective catalase activity of wild-type strain could reduce the level of exogenous H_2O_2 and even cross-protect the catalase-mutant strains in the medium. The population of viable cells in MOJ samples in this study was greater than 5×10^7 cells.mL^{-1} throughout the experiment. Therefore, it is plausible to suggest that the aggregate catalase activity of the cells in ascorbic acid-supplemented MOJ solutions combined with the increase in catalase activity of the cells could have played a role in their increased viability and/or culturability.

3.4.6. Amino Acids

In general, supplementation of MOJ with amino acids led to a significant increase in the number of viable (healthy and injured) cells up to time 6 h post-inoculation [Figure 3.11]. The statistically significant increase in culturability of *E. coli* in amino acid-supplemented MOJ [Figures 3.12(A) and (B)] between time 0 h and 6 h post-inoculation was also in agreement with the FCM results. In case of OJ samples, although at all time-points, compared to control un-supplemented OJ, the mean percentage of healthy cells was greater in amino acid-supplemented samples, however amino acid supplementation of OJ did not improve the overall viability or culturability of the cells during the course of the study [Figure 3.13].

With regard to the acid stress response of the log-phase *E. coli*, there is very little information in the literature on the long-term survival of these cells

following the acid shock. It has been suggested that unlike the acid resistance (AR) mechanisms of AR2 and AR3 in stationary-phase cells, which require the presence of exogenous glutamate and arginine respectively, the acid tolerance response (ATR) of log-phase cells is amino acid-independent (Richard & Foster, 2003). Rowbury *et al.*, (1999) on the other hand, showed that the supplementation of log-phase cells with amino acids at pH 7.0 could induce the acid habituation (AH) resistant system, leading to an improvement in survival of the cells upon acid challenge at pH 3.0 for 7 min. However, due to significant difference between the methods used in their study and this one, it was difficult to compare the two results. Nevertheless, it is possible that amino acid supplementation of MOJ also played a similar role in the acid stress response of *E. coli*.

In a study reported by Reinders *et al.*, (2001), they showed that supplementation of a model apple juice at pHs of 3.2 and 3.8 with 15 $mg.L^{-1}$ proline was capable of increasing the culturability of *E. coli* by 0.7 and 1.0 $\log_{10}$ $CFU.mL^{-1}$ respectively. They hypothesized that proline could have acted as an osmoprotectant, protecting *E. coli* against high osmotic stress in model apple juice. The concentration of proline in MOJ used in the current study was more than 52 times the level of proline used by Reinders *et al.*, (2001). Considering the similar pH and comparable sugar content of the two solution (MOJ and model apple juice), it is possible that proline exerted a similar protective effect on *E. coli* in MOJ. Amezaga & Booth (1999) have also reported the important role of extracellular free proline in mediating the protection of *E. coli* against

hyperosmotic stress. However, based on the results of the current study, it is not possible to attribute the observed improvement in physiological parameters of the cells to a particular amino acid.

3.4.7. Light Scatter Properties of the Cells

It has been stated that in bacteria, the FSC-A could be used as rough indicator of the cells size while SSC-A could be used not only as an indicator of the cell size but also the changes in granularity of the cells as well as cell wall properties (Roostalu *et al.*, 2008; Shapiro, 2003). Therefore, it is reasonable to presume that the observed increase in the median FSC-A [Figure 3.14(A) and (C)] and median SSC-A [Figure 3.14(B) and (D)] of injured and dead cells is indicative of an increase in the size of the cells. A possible reason for the apparent increase in cell size could be the DNA damage-induced stress response in *E. coli* leading to elongation and filamentation of the cells. Jeong *et al.*, (2008) demonstrated that subjection to acidic condition could result in breakage of DNA strands in *E. coli*. It is also known that the exposure to DNA damaging agents such as H_2O_2 and the resultant DNA damage could induce the so-called "SOS" stress response in *E. coli* with the aim of repairing the damaged DNA (Walker *et al.*, 2000; Konola *et al.*, 2000). During the SOS response's DNA repair process, the cell division is inhibited in order to prevent the transfer of mutated DNA to the daughter cells (Justice *et al.*, 2006). The induction of cell division inhibitors in turn lead to elongation and filamentation of the cells (Bi & Lutkenhaus, 1993; Yitzhaki *et al.*, 2012). Therefore, it could be proposed that subjection of *E. coli* to acidic condition of MOJ induced the SOS stress response

in *E. coli*, leading to the filamentation of the cells.

The results of this study showed the potential for the use of median SSC-A of the cell population as an indicator of cellular viability in MOJ and OJ [Figure 3.17]. There was a strong negative correlation between the population of healthy cells and the median SSC-A but not FSC-A of the cells in both MOJ and OJ samples. This was in agreement with the suggestion made by Shapiro (2003) that care must be taken when inferring relationship between the FSC-A and the cell viability and dead cells. The use of FSC-A and SSC-A as a way of determining the viability of the eukaryotic cells (human lymphocytes and hamster fibroblasts) has previously been reported by McGann *et al.*, (1988). However, to the best of the author's knowledge, no study has so far reported the use of SSC-A as a potential indicator of population of healthy bacteria with intact membrane.

3.5. Conclusions and Further Work

In summary, the results showed that compared to log-phase cells, stationary-phase cells were more resistant to acidic condition of MOJ and OJ. Moreover, the increase in incubation temperature of the samples from 4 °C to 22.5 °C led to a greater decrease in population of healthy and/or culturable cells. The differences observed between the results obtained for MOJ and OJ samples were most likely due to the presence of minor components of the OJ not present in MOJ. Further work is needed to study the exact nature of these compounds and to identify those responsible for the observed discrepancy. Moreover, in this

study the pH of MOJ was kept relatively constant. The effects of change in pH of OJ or MOJ within the ranges naturally found in OJ on the physiological response of *E. coli* also deserve further study.

The results also showed that the change in sugar or organic acid concentration of MOJ had no or little effect on the viability and/or culturability of the cells. On the other hand, ascorbic acid and amino acids supplementation of MOJ resulted in a statistically significant improvement in both viability and culturability of *E. coli* in MOJ up to time 6 h post-inoculation. It would be interesting to utilize these information for designing a predictive model in order to predict the physiological response of *E. coli* in OJ.

Although, the results of the current study showed that inoculation of *E. coli* in MOJ or OJ could induce the VBNC state, it should be noted that for the purpose of this study, culturability was defined as the capability of the cells to form colonies on a single type of solid growth medium (i.e., nutrient agar) and the possibility of resuscitating these cells was not investigated. It has been shown that while some VBNC *E. coli* are capable of resuscitation and regaining their culturability, some are not and the resuscitation depends on the various environmental and chemical stimuli (Wesche *et al.*, 2009; Arana *et al.*, 2007; Pinto *et al.*, 2011). It is also known that supplementation of the various growth media with ROS scavengers such as catalase and sodium pyruvate could improve the culturability of the stressed non-culturable cells (Mizunoe *et al.,* 1999; Pinto *et al.,* 2011). Therefore, further work is needed to investigate the

possible recovery of these cells using different recovery methods. This could involve using various minimum and/or rich solid growth media as well as inclusion of various ROS scavengers such as catalase and sodium pyruvate in these media. FCM data could be used as a suitable guide for developing appropriate recovery and resuscitation media for OJ-induced VBNC cells. For instance by using a cell sorter FCM, it should be possible to collect each FCM-gated population in a separate container, hence facilitating the investigation of the culturability of each population by plating them on separate plates.

Based on the work of Ma and Eaton (1992), it was hypothesized that the observed increase in viability of *E. coli* in ascorbic acid-supplemented MOJ solutions could have been due to high cell density of the samples. Therefore, it would be interesting to investigate whether or not the ascorbic acid supplementation could result in similar effects in MOJ samples with low *E. coli* cell density. This is critically important considering the very low dose of infection of entero-haemorrhagic *E. coli* (Kaper *et al.*, 2004). As mentioned above, the supplementation of MOJ with 5 or 10 $g.L^{-1}$ ascorbic acid or amino acids caused a significant improvement in viability of the cells up to time 6 h post inoculation. Moreover, the time required for achieving 5 $\log_{10}$ reduction in culturability of the cells in MOJ was increased from 3 days to 5 and 10 days for MOJ samples supplemented with 5 or 10 $g.L^{-1}$ ascorbic acid respectively. Therefore, the possible role of ascorbic acid in improved culturability of *E. coli* should be taken into consideration by OJ manufacturers when developing a HACCP plan for ascorbic acid-supplemented OJ in order to ensure 5-$\log_{10}$

reduction of *E. coli* in these products.

As discussed above, the SOS and oxidative stress responses of the cells could have played a role in their survival in OJ. Therefore, it would be interesting to investigate the expression of the genes such as *recA*, *oxyR* and *soxS* which are responsible for regulating these responses (Cox, 2007; Storz *et al.*, 1990; Amábile-Cuevas & Demple, 1991). Furthermore, it was hypothesized that a greater generation of ROS at 22.5 °C compared to 4 °C due to metabolic exhaustion of the cells could be the cause of the greater decrease in the percentage of healthy cells at 22.5 °C. This could be investigated further by changing the redox conditions of the OJ samples stored at 4 °C and 22.5 °C for instance by inclusion or exclusion of oxygen (e.g., vigorous aeration of the OJ or deaeration respectively) or antioxidants (e.g., ascorbic acid).

In this study it was suggested that the stationary-phase-induced increase in saturated fatty acids composition of the membrane could be behind the reduced resistance of *E. coli* to cold shock in MOJ at time 0 h post-inoculation. This hypothesis deserved to be investigated further by studying the fatty acid composition of *E. coli* immediately after their inoculation in OJ according to the protocol described by Shaw & Ingram (1965).

It is important to emphasise that, in this study, only one *E. coli* strain (i.e. K-12 MG1655) was used. This was mainly because the metabolism, complete genome sequence and gene regulation of this strain is known and well

characterized (Feist *et al.,* 2007; Blattner *et al.,* 1997). Moreover this strain has previously been used as a surrogate strain for *E. coli* O157:H7 in food microbiological studies for the purpose of developing predictive microbiology models (Valdramidis *et al.,* 2007). However it is important to note that the behaviour of pathogenic *E. coli* such as *E. coli* O157:H7 in OJ or under various stress conditions could be significantly different from those observed in this study. For instance, Vidovic *et al.*, (2011) compared the cold stress response of strain K-12 with that of *E. coli* EHEC O157:H7 B-1 following the transfer of the cells from 37 °C to 15 °C and subsequent incubation at 15 °C. They showed that compared to the former, significantly greater growth was observed for EHEC strain 6 h post-stress ($p < 0.05$). It has also been demonstrated that EHEC O157:H7 are more resistant to acidic conditions. This is believed to be due to the presence of a large number of O-island genes in *E. coli* O157:H7 strain compared to K-12 strain some of which have been shown to be regulated in response to acid stress (Carter *et al.,* 2012; King *et al.,* 2010). On the other hand, Lin *et al.*, (1996) demonstrated that some commensal (non-pathogenic) strains of *E. coli* such as EF312, EF313 and EF314 exhibited relatively similar acid mechanisms (AR1, AR2 and AR3) and acid resistance to various patient isolated EHEC strains. Considering the findings of the aforementioned studies, it is of utmost importance to repeat some of the results of this study (for instance the effects of ascorbic acid or amino acids supplementation of MOJ on the physiology of *E. coli*) using EHEC strains (where facilities permit) or commensal strains with similar acid resistance to EHEC strains.

In this study, the effects of the composition of MOJ on the physiology of *E. coli* were investigated using exponentially growing cells. Considering that the stationary-phase cells were shown to be more resistant to the acidic condition of MOJ, this could lead to underestimation of the rate of survival of stationary-phase cells. Therefore, it is important to repeat the results using stationary-phase cells.

Finally, as previously mentioned (see Section 1.2.2) numerous studies have been conducted in order to study the antimicrobial activity of orange essential oil compounds against *E. coli* and *Salmonella* (Bisignano & Saija, 2002; Fisher & Phillips, 2006; Di Pasqua *et al.*, 2007; Espina *et al.*, 2013). From these studies, it is not clear if the presence of essential oil compounds in OJ could have played a role in lower reported incidents of OJ-associated outbreak of pathogenic *E. coli*. In the current study, the effects of essential oil on the physiological state of *E. coli* in MOJ were not investigated. Considering the studies mentioned above, further work is needed to investigate the effect of change in essential oil and/or volatile compounds of OJ on the viability of *E. coli*. This could include addition of these compounds to the MOJ (within the ranges naturally found in OJ, Table 1.5) and conducting experiments similar to the ones reported here for sugars and organic acids.

Chapter 4

The Effects of Orange Juice Clarification on the Physiology of *E. coli* in Orange Juice

4.1 Introduction

One of the major production stages of orange juice (OJ) is clarification. The main purpose of this step is to remove the excess seeds, orange fruit pieces, pieces of membrane as well as bitter essential oil compounds such as limonene present in the freshly squeezed OJ (Rutledge, 1996). Depending on the type of OJ product (e.g., with or without bits), up to 12% (w/v) pulp is added back to the juice before packaging (Berlinet *et al.*, 2007). OJ can also be supplemented with homogenised pulp in order to meet the demand of the consumers for smoother OJ products (Sorenson & Bogue, 2005). OJ contains many different types of antimicrobial compounds such as volatile and essential oil compounds (mainly limonene), as well as flavanones (primarily hesperidin) (Garg *et al.*, 2001; Di-Pasqua *et al.*, 2007; Bisignano & Saija, 2002). It has also been shown that the majority of these compounds, especially the latter two, are mainly present in the pulp and cloud of OJ (Ben-Shalom & Pinto, 1999; Brat *et al.*, 2003). As a result, it was hypothesized that OJ clarification and the resultant removal of these compounds could lead to improved survival of *E. coli* in OJ. The main aim of the current study was to test this hypothesis by monitoring the effects of OJ clarification on both the culturability and viability of *E. coli* using plate counting and FCM techniques respectively.

4.2 Results

4.2.1 Pulp Content of the Orange Juice

In order to study the role of orange juice (OJ) pulp on the culturability of *E. coli*, first it was necessary to measure the pulp content (w/w) of OJ. This was done by first centrifuging the OJ and then dividing the weight of the pellet by the total weight of pre-centrifuged OJ. The moisture/dry mass content of the pulp was determined by drying the pulp in a 90 °C incubator, until no change in its weight was observed. The pulp content of the freshly squeezed OJ used in this study (henceforth referred to as 5F-OJ) was 5.03 ± 0.60 % (w/v). The dry mass content of the pulp was around was 18.08 ± 0.36% (w/w). This information were subsequently used for supplementing pulp-free OJ generated by centrifugation (un-supplemented 0% pulp OJ or 0S-OJ) with 5% (5S-OJ) or 10% (10S-OJ) pulp. This was done in order to study the effects of centrifugation step and addition of extra pulp on the culturability of *E. coli* respectively. Total removal of pulp (0S-OJ) and/or subsequent supplementation of 0S-OJ with 5% or 10% pulp (5S-OJ and 10-OJ respectively) did not affect the pH of OJ (pH range 3.25 ± 0.01, $p = 0.60$).

4.2.2 Characterization of OJ Cloud Particles

Studying the effect of cloud particles on the culturability and viability of *E. coli* required their characterization on the basis of size distribution in OJ and also their physical and/or chemical characteristics. Within the following text, “FOJ” stands for filtered OJ and the numbers preceding it refer to the pore size of the

filter used for clarifying the pulp-free OJ in µm. The centrifuged pulp-free OJ (0S-OJ) was filtered through sterile filter papers with pore sizes of 11 µm, 8 µm, 1.6 µm or 1.2 µm resulting in the generation of 11-FOJ, 8-FOJ, 1.6-FOJ and 1.2-FOJ respectively. Filtration of 0S-OJ with 0.7 µm or 0.22 µm was not practical due to blockage of filters with large OJ cloud particles. As a result, 1.2-FOJ which lacked these particles was used instead of 0S-OJ for preparation of 0.7-FOJ and 0.22-FOJ. The cloud particle size distribution of OJ filtered with filter papers of various pore sizes was measured by laser diffraction using a Mastersizer [Figure 4.1].

Depending on the filtration regime, three distinct cloud populations could be observed: (a) small particles with diameters of less than 0.7 µm which were assumed to be mainly needle-like crystals of hesperidin; (b) small to medium size particles of between 0.7 µm and 3 µm presumably the carotenoid-containing chromoplastids and small rag fragments; and (c) large particles of greater than 3 µm most likely the large rag fragments (Mizrahi & Berk, 1970). In general, the results indicated that the smaller the pore size of the filters, the greater the removal of particles. The volume median diameter (D_{50}) or the mid-point size of cloud particles decreased when the pore size of filter papers was decreased from 11 µm to 1.2 µm (6.408 and 0.358 respectively) [Table 4.1]. However, compared to 1.2-FOJ, there was a 0.245 µm increase in the D_{50} of 0.7-FOJ samples from 0.358 µm to 0.603 µm. The higher D_{50} values obtained for 0.7-FOJ was considered to be due to the tendency of the laser diffraction technique for overestimating the size of non- spherical needle-like particles.

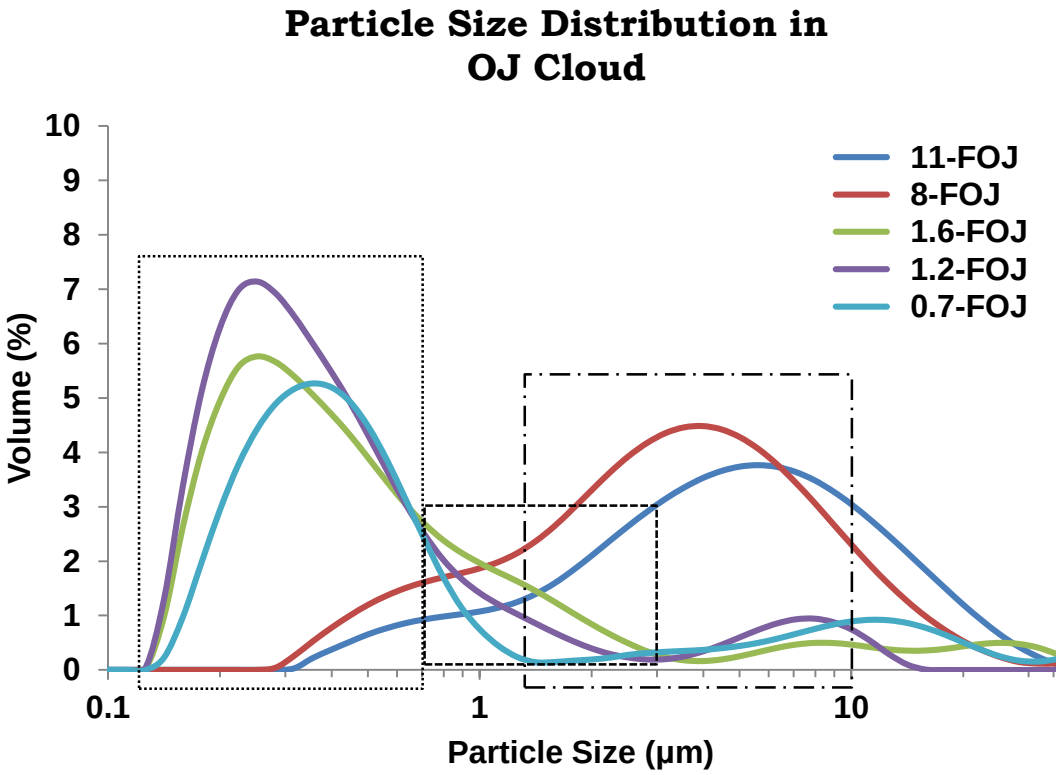


Figure 4.1: Particle size distribution in orange juice (OJ) cloud

The graph shows the cloud particle size distribution in OJ samples filtered with filter papers of different pore sizes. FOJ stands for filtered OJ and the number preceding it refer to the pore size (µm) of the filter used for filtering the sample. Based on their size, OJ particles could be divided into three overlapping population of small, medium and large, represented with a dot, dashed and long dashed-dot boxes respectively. The Y-axis or the volume percentage is a Mastersizer generated value for the ratio volume sample to volume dispersant.

Table 4.1: Volume median diameter or D_{50} of OJ cloud particles

FOJ Sample	D_{50} (µm)
11-FOJ	6.408 ± 1.273
8-FOJ	3.593 ± 0.275
1.6-FOJ	0.460 ± 0.143
1.2-FOJ	0.358 ± 0.083
0.7-FOJ	0.603 ± 0.286

This Table shows the median diameter of the particles reported by the Mastersizer for the experiment described above in [Figure 4.1]

This observation further supported the assumption that 0.7-FOJ contained mainly the needle-like hesperidin crystals. The assumptions about the nature of these particles were made based on the cloud particle characterization experiments presented in this study as well as those of Mizrahi & Berk, (1970) who reported the characteristics of different fractions of cloud particles [Figure 4.2].

The visual examination of the filter papers after the filtration of 50 mL of 0S-OJ (in case of filter papers of 1.2 µm to 11 µm) or 1.2-FOJ (in case of 0.7 µm) was also performed [Figure 4.3]. The aim here was to confirm the assumptions made about the nature of cloud particles by correlating their physical appearance on the surface of the filter papers to their size [Figure 4.2]. The observations demonstrated that 8 µm and 11 µm filter papers were largely covered with visible OJ particles with little colour change. As a result, 8-FOJ and 11-FOJ were considered to contain the majority of cloud particles, including large and small rag fragments, chromoplastids and hesperidin crystals. On the other hand, 1.6 µm and particularly 1.2 µm filter papers were covered with not only the rag fragments but also orange pigments presumably the chromoplastids. Filtration of the 1.2-FOJ with 0.7 µm filter paper resulted in the retention of much lower amount of chromoplastids as well as a white layer on the surface of the filter, most likely the white crystals of hesperidin. Therefore, it was presumed that 1.6-FOJ consisted of both small rag fragments and chromoplastids, whereas 1.2-FOJ contained very small amount of chromoplastids. Therefore it could be suggested that large cloud population

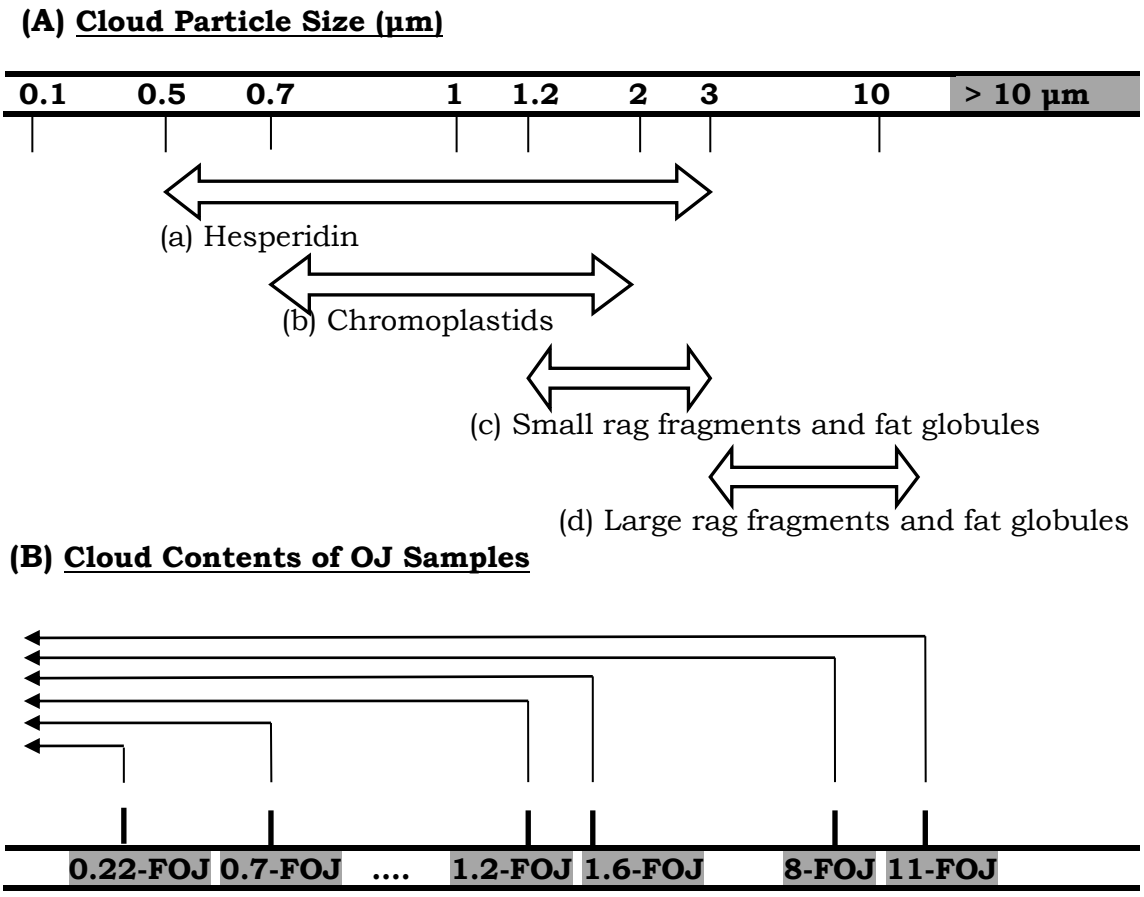


Figure 4.2: Characterization of cloud particles in filtered OJ (FOJ)

(A) The figure shows the size range of different parts of OJ cloud. These assumptions were made based on the cloud characterization experiments (Section 4.2.2) as well as the results reported in the literature. The cloud particles can be divided into: (a) hesperidin needle-like crystals of 0.5 µm to 3 µm long and 0.05 µm to 0.2 µm thick); (b) chromoplast plastids containing carotenoids of between 0.7 µm to 2 µm; (c) small amorphous rag fragments of between 1.2 µm and 3 µm; and (d) large amorphous rag fragments of larger than 3 µm and 10 µm. Particles of larger than 10 µm are not considered to be a cloud particle.

(B) The figure shows the cloud composition of FOJ samples filtered with filter papers of different pore sizes. The number preceding "FOJ" refers to the pore size (µm) of the filter used for filtering the sample. The arrow covers the range of particles present in the sample. Filtration with 0.22 µm filter paper removes the majority of hesperidin crystals, although some could pass through the filter due to their needle-like structure. Filtration with 0.7 µm and 1.2 µm filter papers on the other hand, results in near complete removal of chromoplastids and small rag fragments. 1.6-FOJ contains not only the hesperidin and chromoplastids, but also small rag fragments. In addition to the aforementioned particles, 8-FOJ and 11-FOJ also contain large rag fragments.

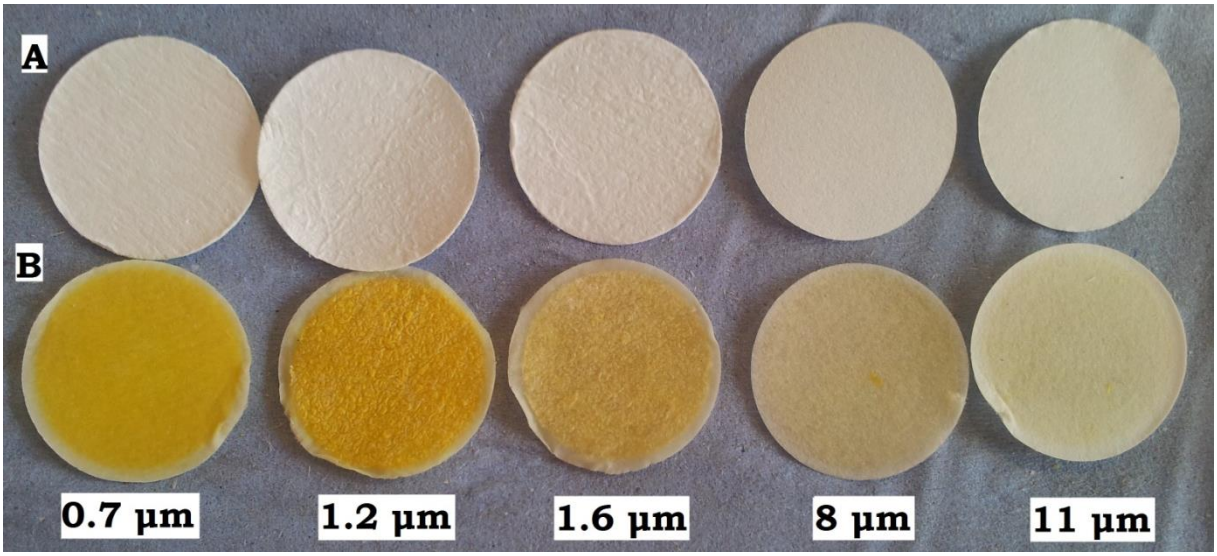


Figure 4.3: Pre- and post-filtration state of filter papers used for preparation of filtered orange juice (FOJ)

The figure shows the pre-filtration **(A)** and post-filtration **(B)** state of filter papers used for preparation of filtered orange juice. The labels below the filter papers show their pore size. Filter papers with the pore size of 1.2 µm to 11 µm were used for filtration of pulp-free orange juice, whereas 0.7 µm filter was used for filtration of the 1.2-FOJ (OJ previously filtered with 1.2 µm filter paper). 11 µm and 8 µm were mainly covered with rag fragment with little colour change, while 1.6 µm and particularly the 1.2 µm filter papers were covered with not only the rag fragments but also orange chromoplastids (containing carotenoids). 0.7 µm filter paper on the other hand was covered mainly with chromoplastids.

consisted of mainly rag fragments, whereas the medium and small population contained mainly the chromoplastids and hesperidin respectively.

Based on the results described above, it was hypothesized that autoclaving filtered OJ samples containing particles of larger than 0.7 µm (i.e., proteinous rag fragments, fat globules and chromoplastids) would result in the degradation of these compounds, hence production of dark browning products. [Figure 4.4] compares the colour of filtered OJ in test tubes before and after being autoclaved at 121 °C for 15 minutes. All pre-autoclaved filtered OJ samples were transparent orange and their absorbance at 600 nm (measured by spectrophotometer) progressively decreased when filter papers with smaller pore size were used (0.353 ± 0.003 and 0.068 ± 0.002 absorbance unit for 11-FOJ and 0.22-FOJ respectively: [Table 4.2]). Autoclaving of OJ filtered with 11 µm, 8 µm, 1.6 µm and 1.2 µm filters yielded the production of very dark brown products, whereas in case of OJ filtered with 0.7 µm filter, the sample was bright orange indicating the lack of rag or chromoplastids. In case of the latter, there was also a large amount of white precipitate at the bottom of the test tube typical of hesperidin crystals (Ben-Shalom & Pinto, 1999). Compared to 0.7-FOJ samples, however, filtration of pre-autoclaved 1.2-FOJ with a 0.22 µm filter (0.22-FOJ) led to a considerable reduction in the amount of white precipitate in post-autoclaved 0.22-FOJ test tubes. Autoclaved 0.7-FOJ and 0.22-FOJ were both transparent and looked similar to the samples of autoclaved model orange juice (MOJ) described in Chapter 3 [Table 3.1]. In summary, it was shown that it could be possible to remove different types of OJ

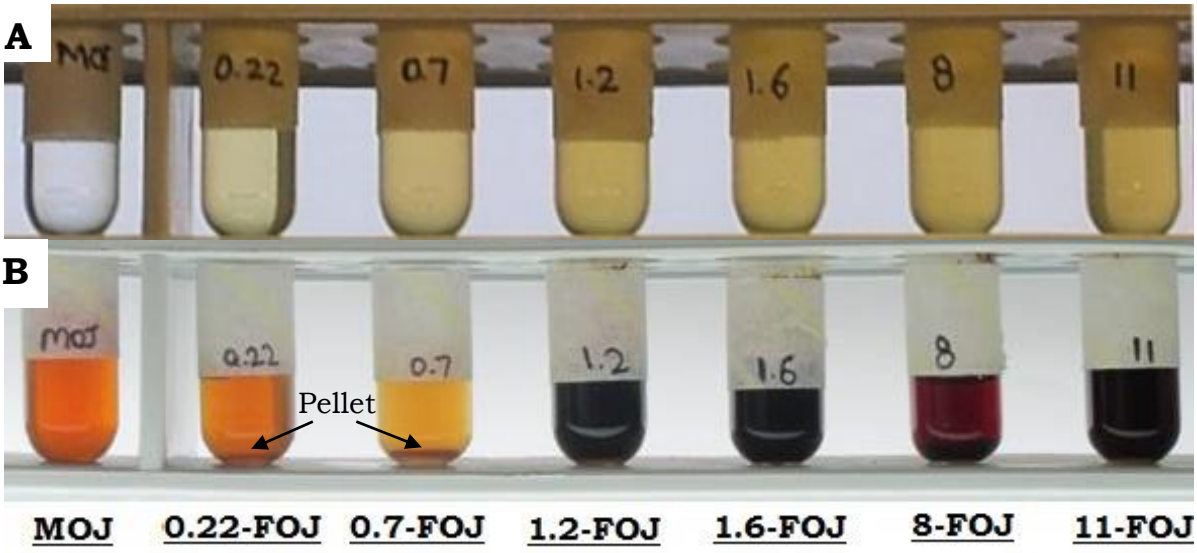


Figure 4.4: Autoclaved filtered orange juice (FOJ) and model orange juice (MOJ)

The figure shows the change in colour and pellet content of FOJ or MOJ samples in test tubes before **(A)** and after **(B)** being autoclaved at 121 °C for 15 minutes. The values shown on the test tube are indicative of the pore size of the filter used for filtering the OJ. The absorbance of the filtered OJ samples (measured with a spectrophotometer at 600 nm) was progressively decreased when filter papers with smaller pore sizes was used for OJ filtration indicating effectiveness of the filtration regime. Autoclaving OJ samples with particle sizes of larger than 1.2 µm resulted in the production of dark browning products. For samples with particle sizes of less than 1.2 µm, the production of browning products was lower and the solution remained transparent. There was a considerable amount of white pellet in 0.7-FOJ and to a lower extent in 0.22-FOJ tubes, typical of hesperidin crystals.

Table 4.2: The absorbance of OJ cloud particles (pre-autoclaved)

Sample	Absorbance (600 nm) (AU)
MOJ	0.000 ± 0.001
0.22-FOJ	0.068 ± 0.002
0.7-FOJ	0.174 ± 0.001
1.2-FOJ	0.264 ± 0.000
1.6-FOJ	0.303 ± 0.001
8-FOJ	0.357 ± 0.002
11-FOJ	0.353 ± 0.003

particles (e.g., large and small rag fragments, chromoplastids and hesperidin) using filter papers of different pore size.

4.2.3 The Effects of OJ Pulp on Culturability of *E. coli* at 4 °C and 37 °C

This experiment was performed in order to investigate the role of OJ pulp on the culturability of *E. coli* K-12 MG1655 inoculated in OJ samples at 4 °C or 37 °C. In order to achieve this aim, 3×10^9 cells of mid-logarithmic phase ($OD_{650\ nm}$ = 0.5) *E. coli* K-12 MG1655 were added to 15 mL of OJ samples containing 0, 5% or 10% pulp. Samples were stored at 4 °C or 37 °C for a maximum of 12 days. The number of culturable cells was determined at various time points by decimal serial dilution of the samples in maximum recovery diluent (MRD) and plating 100 µL of the appropriate dilution (or neat samples) on nutrient agar plates. A statistically significant ($p < 0.05$) inverse relationship was observed between the concentration of pulp and culturability of *E. coli* at 4 °C [Figure 4.5]. Total removal of pulp in case of 0S-OJ increased the culturability of *E. coli*; however, the difference only became significant after day one ($p < 0.05$). This difference was particularly noticeable after day five when the gap between 0S-OJ and pulp-containing OJ samples (5F-OJ, 5S-OJ and 10S-OJ) widened, reaching at least 2.2 $\log_{10}$ CFU.mL^{-1} difference by day twelve ($p < 0.01$). On this day, in case of samples of OJ with 5% or 10% pulp, no colony grew on plates when neat samples were plated (CFU.mL^{-1} < 10). Comparing the results obtained for pre-centrifuged or post-centrifuged OJ samples containing 5% pulp (5F-OJ and 5S-OJ respectively) showed that centrifugation itself did not affect

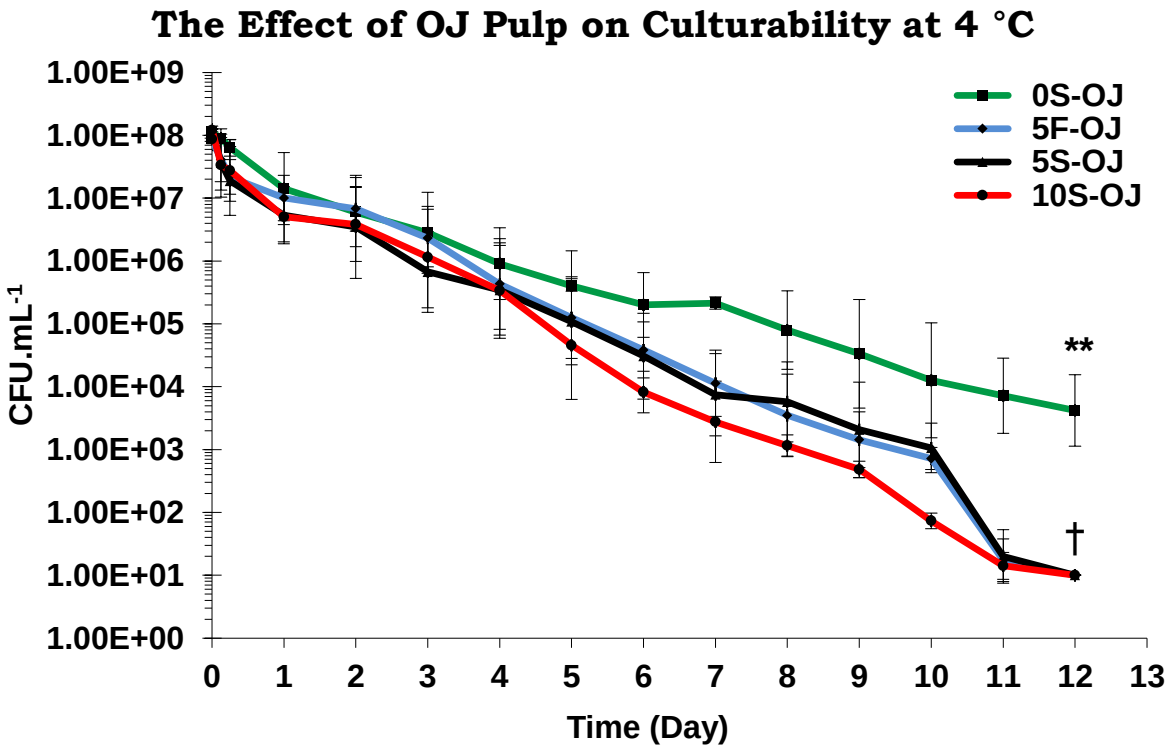


Figure 4.5: The culturability of *E. coli* K-12 MG1655 subjected to OJ with various pulp contents at 4 °C

The figure shows the role of OJ pulp on culturability of *E. coli* K-12 MG1655 inoculated in OJ samples at 4 °C. 3×10^9 cells of mid-logarithmic phase (OD_{650} = 0.5) *E. coli* were added to 15 mL of OJ samples containing 0, 5% or 10% pulp. Samples were stored at 4 °C for 12 days. The number of culturable cells was determined at various time points, by decimal serial dilution of the samples in maximum recovery diluent (MRD) and plating 100 µL of the appropriate dilution (or neat samples) on nutrient agar plates. Error bars are the ± standard deviation of the mean value obtained at each time point. The experiment was repeated at least twice each with a duplicate

†: At the marked time point (time 24 h of 5F-OJ, 5S-OJ and 10S-OJ), no colony grew on nutrient agar plates when 100 µL of neat samples were plated (< 10 $CFU.mL^{-1}$); **: $p < 0.01$ compared to 5% or 10% pulp-containing samples

OJ Sample	Condition
0S-OJ	Pulp-free centrifuged **OJ** (**0**% Un-**S**upplemented)
5F-OJ	**F**reshly squeezed **OJ** (Naturally containing **5**% pulp)
5S-OJ	Centrifuged **OJ** (**S**upplemented with **5**% pulp)
10S-OJ	Centrifuged **OJ** (**S**upplemented with **10**% pulp)

the culturability of *E. coli* (p = 0.98). In addition, increasing the pulp content of OJ from 5% to 10% resulted in a noticeable reduction in the number of culturable *E. coli* especially between day 5 and 11, however, this difference was not statistically significant (p-values of 0.14 and 0.05 for 5F-OJ and 5S-OJ respectively).

At 37 °C [Figure 4.6], the clarification of OJ generated similar results to those observed at 4 °C. Compared to 5% or 10% pulp-containing OJ samples, centrifugation and complete removal of the pulp (0S-OJ) caused a significant increase in the culturability from time 3 h post-inoculation (p < 0.05 for all comparisons). Except for 0S-OJ samples, no colony was observed on nutrient agar plates when neat samples were plated after 6 h of incubation regardless of the treatment condition (< 10 CFU.mL^{-1}). As at 4 °C there was no significant difference between the culturability of *E. coli* subjected to OJ with 5% (either natural or supplemented) or 10% pulp. However, the time required for the loss of culturability (< 10 CFU.mL^{-1}) was decreased dramatically from 12 days at 4 °C to only 6 h at 37 °C (p < 0.0001).

4.2.4 The Effects of OJ Cloud on Culturability of *E. coli* at 4 °C and 37 °C

Similar to what was described above for pulp, in order to investigate the role of OJ cloud on culturability of *E. coli*, 3×10^9 cells of *E. coli* K-12 MG1655 were added to 15 mL of 0.7-FOJ (containing hesperidin but free from small and large rag fragment and chromoplastids), 1.6-FOJ (containing all cloud particles

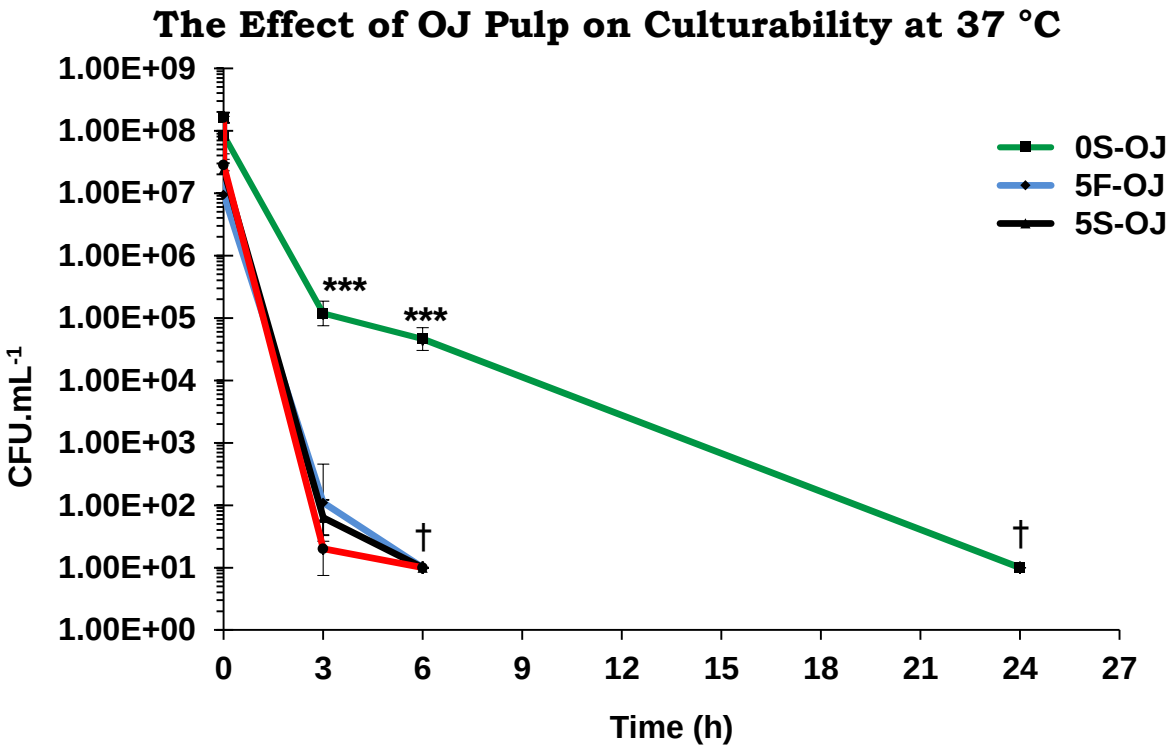


Figure 4.6: The Culturability of *E. coli* K-12 MG1655 subjected to OJ with various pulp contents at 37 °C

The figure shows the role of OJ pulp on culturability of *E. coli* K-12 MG1655 inoculated in OJ samples at 37 °C. The experimental method was similar to what was described for 4 C samples (Figure 4.5), with the difference of incubating the samples at 37 °C. Error bars are the ± standard deviation of the mean value obtained at each time point. (***: $p < 0.001$ compared to 5F-OJ, 5S-OJ and 10S-OJ at the marked data-point).

†: At the marked time point (time 6 h of 5F-OJ, 5S-OJ and 10S-OJ; time 24 h of 0S-OJ), no colony grew on nutrient agar plates when 100 µL of neat samples were plated (< 10 $CFU.mL^{-1}$).

OJ Sample	Condition
0S-OJ	Pulp-free centrifuged **OJ** (**0**% Un-**S**upplemented)
5F-OJ	**F**reshly squeezed **OJ** (Naturally containing **5**% pulp)
5S-OJ	Centrifuged **OJ** (**S**upplemented with **5**% pulp)
10S-OJ	Centrifuged **OJ** (**S**upplemented with **10**% pulp)

except large rag fragment) and 11-FOJ (containing all cloud particles). The results were compared to those obtained for pulp-free OJ i.e., OJ-0S containing hesperidin, chromoplastids as well as small and large fragments. Samples were stored at 4 °C or 37 °C and their culturability was studied for a maximum of 12 days by plating the samples on nutrient agar plates.

Similar to the results described above for pulp, at 4 °C the presence of OJ cloud particles had a significant adverse effect on the culturability of *E. coli* MG1655 [Figure 4.7]. However, the effect was only statistically significant when particles of larger than 0.7 µm but smaller than 1.6 µm were removed. While twelve days incubation at 4 °C led to 5.91 ± 0.40, 5.67 ± 0.39 and 5.79 ± 0.41 $\log_{10}$ CFU.mL^{-1} reduction in the culturability of *E. coli* in 0S-OJ, 11-FOJ and 1.6-FOJ samples respectively, this was nearly one $\log_{10}$ lower for 0.7-FOJ samples (4.86 ± 0.04; $p < 0.001$). No significant difference was observed between the culturability results obtained for 0S-OJ (unfiltered pulp-free), 11-FOJ (containing all cloud particles) and 1.6-FOJ (free from large rag fragments) samples. Nonetheless, the reduction in the cloud particle content of these samples decreased the rate of reduction in culturability of *E. coli*. For instance, the difference between the culturability of *E. coli* in 0S-OJ, 11-FOJ, 1.6-FOJ and that of 0.7-FOJ was significant during the last ten, nine and seven days of incubation respectively ($p < 0.05$). In other words, although removal of particles of larger than 0.7 µm had a significantly positive effect on the culturability of *E. coli*, however the removal of other particles such as rag fragments and chromoplastids did improve the culturability of *E. coli*. At 37 °C [Figure 4.8], removal of the cloud particles of OJ

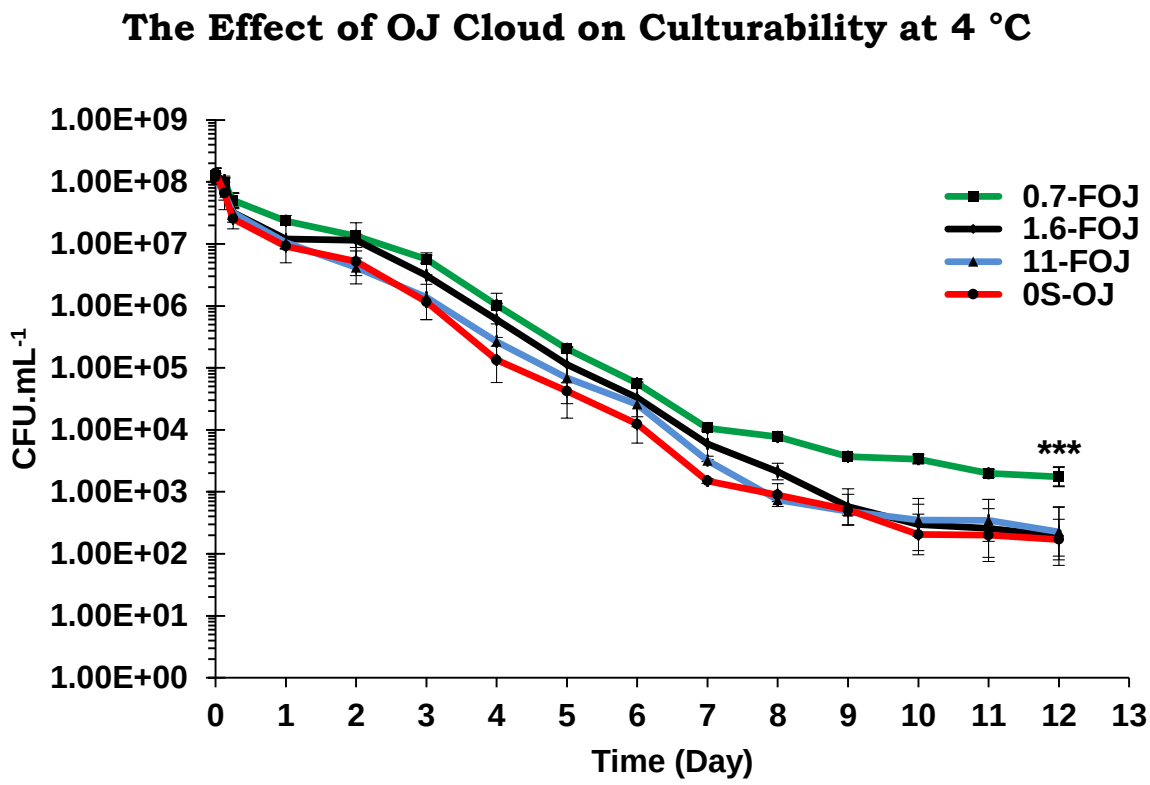


Figure 4.7: The Culturability of *E. coli* K-12 MG1655 subjected to filtered orange juice (FOJ) with various cloud contents at 4 °C

The figure shows the effect of cloud particles on culturability on culturability of *E. coli* K-12 MG1655 inoculated in OJ samples containing various cloud contents and incubated at 4 °C. The values preceding "-FOJ" it is indicative of the pore size of the filter used for filtering the orange juice (OJ). 0S-OJ stands for the centrifuged unfiltered pulp free OJ. The experimental method was similar to what was described for 4 C samples (Figure 4.5) with the difference of using pulp-free filtered OJ samples instead of pulp containing OJ. Error bars are the ± standard deviation of the mean value obtained at each time point. There was a significant difference between the CFU.mL^{-1} of culturable cells when particles of larger than 0.7 was removed (***: $p < 0.001$ compared to 0S-OJ).

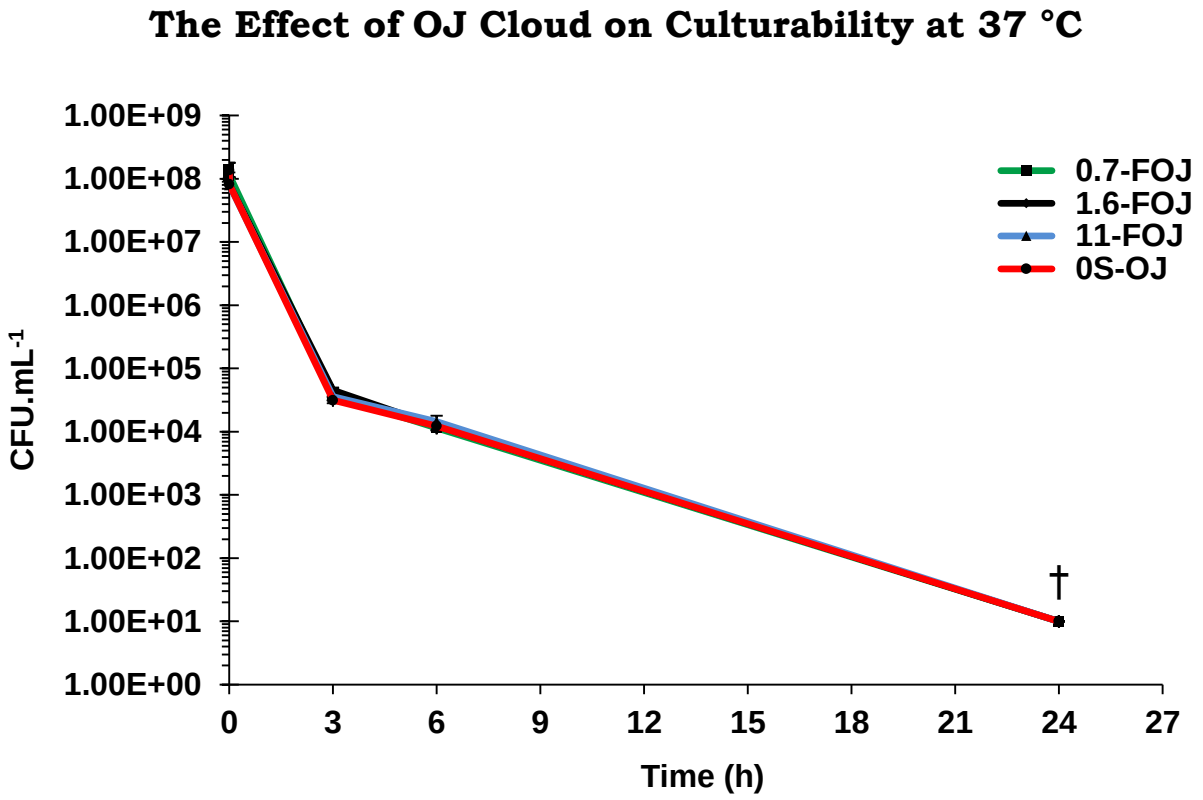


Figure 4.8: The Culturability of *E. coli* K-12 MG1655 subjected to filtered OJ (FOJ) with various cloud contents at 37 °C

The figure shows the effect of cloud particles on culturability on culturability of *E. coli* K-12 MG1655 inoculated in OJ samples containing various cloud contents and incubated at 4 °C. The values preceding "-FOJ" is indicative of the pore size of the filter used for filtering the orange juice (OJ). 0S-OJ stands for the centrifuged unfiltered pulp free OJ. The experimental method was similar to what was described for 4 °C samples (Figure 4.7) with the difference of incubating the samples at 37 °C. Error bars are the ± standard deviation of the mean value obtained at each time point. No noticeable difference was observed between results of different FOJ samples.

†: At the marked data point, in case of all samples, no colony grew on nutrient agar plates when 100 µL of neat samples were plated (< 10 $CFU.mL^{-1}$)

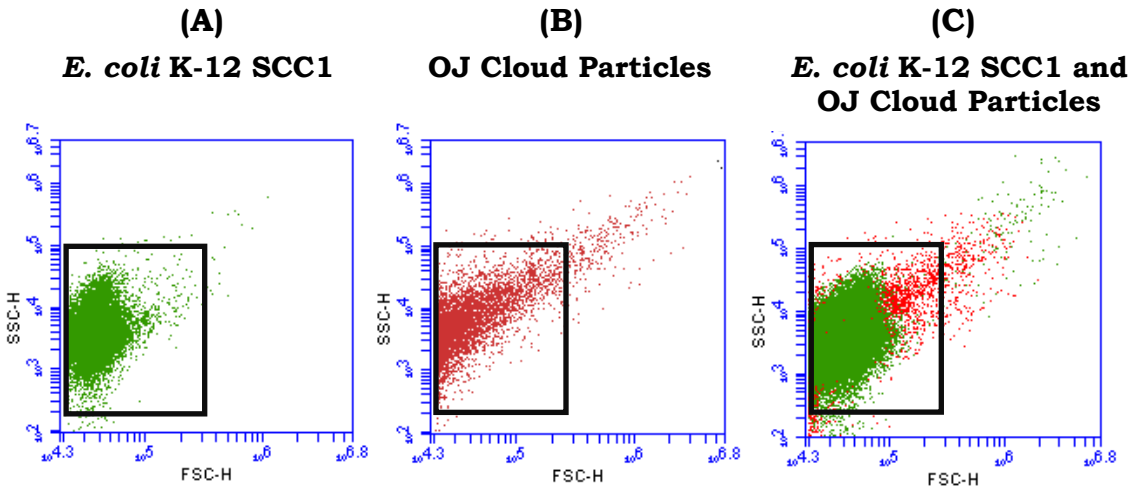
did not enhance the culturability of the *E. coli* any more than what could be achieved with removal of pulp as reported above. Furthermore, after 24 hours, regardless of the cloud particle content of OJ samples, no colony grew on plates when neat samples were plated (< 10 CFU.mL^{-1}), indicating that the effect of temperature on culturability was significantly greater than that of cloud particles.

4.2.5 Identification of *E. coli* and Cloud Particles with FCM

The purpose of these studies was to evaluate the viability of *E. coli* in filtered OJ containing various concentrations and/or types of cloud particles using FCM. The application of FCM technique made it possible to identify not only the healthy cells, but also the injured and dead cells as well as enable rapid cell counting. The effects of pulp on viability of *E. coli* in OJ could not be studied with this technique due to the large size of pulp particles and the risk of blocking the FCM system with particles of larger than 40 µm. The viability experiments were performed by analysing filtered OJ samples containing 2×10^8 cells.mL^{-1} of mid-exponential phase (OD_{650} = 0.5) *E. coli* K-12 MG1665 or SCC1 (2×10^8 cells.mL^{-1} in 15 mL of OJ) and incubating the sample for a maximum of 48 hours and 24 hours at 4 °C or 22.5 °C respectively. At every time point samples were diluted in PBS and analyzed on the flow cytometer. Samples were stained with one or both viability dyes of propidium iodide (PI) and bis-oxonol (BOX) in order to detect the dead or membrane-depolarized (injured and dead) cells respectively.

Filtered OJ samples used for viability experiments contained not only *E. coli* but also various levels of OJ cloud particles depending on the filtration regime used. As a result, it was important to determine not only the number of *E. coli* present in the sample but also the number of OJ cloud particles. The key problem with this approach, however, was that cloud particles were within the same size range as *E. coli*, making it difficult to discriminate the two based on their optical properties such as forward and/or side scatters [Figure 4.9]. Auto-fluorescence of *E. coli* cells was also similar to those of cloud particles. For instance, the measured median green (FL1-A) and red (FL3-A) fluorescence unit for cloud particles was 176 ± 20 and 310 ± 16 respectively (n = 42). The FL1-A and FL3-A values for *E. coli* K-12 MG1655 was very similar and found to be 200 ± 11 and 301 ± 4 respectively. Consequently, it was not possible to determine the true number of *E. coli* cells present in the inoculated filtered OJ sample based on either the fluorescence or light scatter properties.

In order to overcome this obstacle, it was decided to first measure the concentration of cloud particles present in filtered OJ. This was done by acquiring diluted (5%, 10%, 20%, 40%, 60%, 80% in PBS) and undiluted filtered OJ on the flow cytometer for 1 minute (data rate of between 1000-2500 events per second). The results showed a significant decrease in the number of cloud particles present in the samples when the pore size of filters was progressively decreased from 11 µm to 0.22 µm ($p < 0.001$) [Figure 4.10]. No difference was observed between the number of cloud particles in 8-FOJ and 11-FOJ ($p = 0.98$) indicating that the filtration with 8 µm filter had little effect



Parameter		Median log_{10} values	
FSC-H	4.56	4.51	4.58
SSC-H	3.41	3.76	3.52

Figure 4.9: Identification of *E. coli* cells and OJ particles based on their light scatter properties

The figure shows the FCM forward scatter (FSC-H) versus side scatter (SSC-H) dot plots of 20,000 events of **(A)** *E. coli* K-12 SCC1 cells diluted in PBS, **(B)** OJ cloud particles in OJ filtered with 11 µm filter paper (11-FOJ) and **(C)** *E. coli* cells and cloud particles in 11-FOJ. *E. coli* (green dots) and OJ cloud particles (red dots) had similar FSC-H and SSC-H values, making it difficult to discriminate cells from cloud particles.

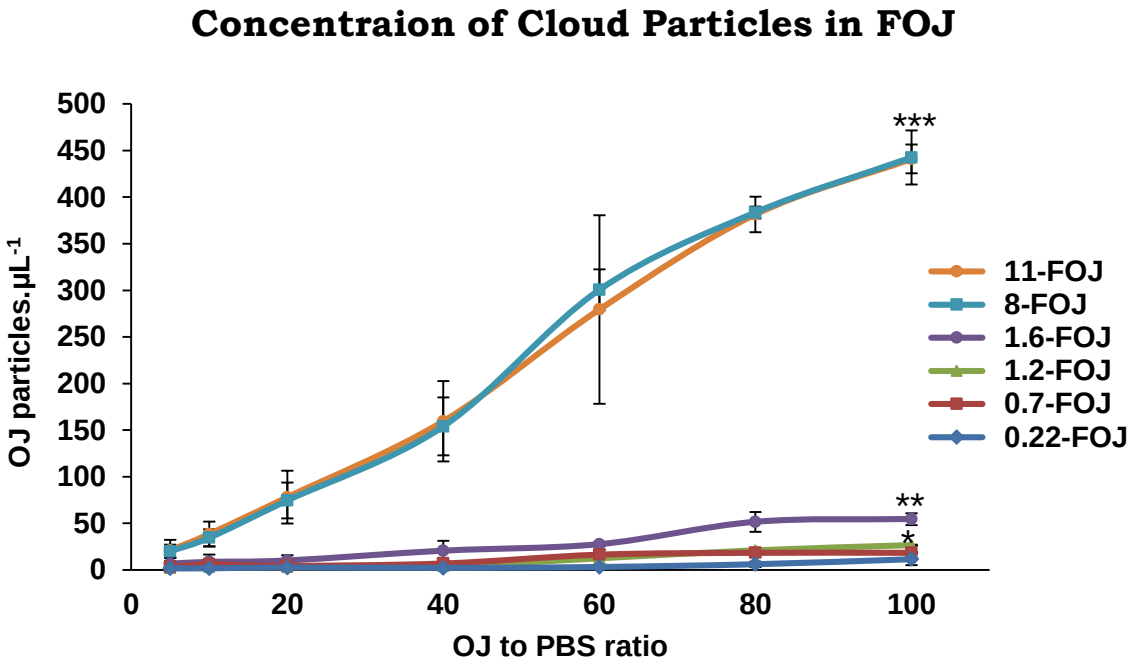


Figure 4.10: Measuring the concentration of OJ cloud particles in different dilutions of various FOJ samples

The values preceding "-FOJ" is indicative of the pore size of the filter used for filtering the OJ. 0.22-FOJ and 0.7-FOJ were prepared by filtering 1.2-FOJ, others with filtering the pulp-free OJ. The X-axis and Y-axis show the dilution of OJ in PBS and the number of OJ particles detected by FCM, based on their forward and side scatters (FSC-H/SSC-H) respectively. Error bars are the ± standard deviation of the mean value obtained at each time point.

Table 4.3: FCM Settings

Desired Number of Cells	**Flow speed**		**Data Rate**	**Acquisition Time**	**Sample Volume Collected**	**Events Concentration**
Events	µL.min^{-1}	µL.s^{-1}	Events.s^{-1}	s	µL	Events.µL^{-1}
20,000	13	4.62	Min: 1,000	20	4.33	4,618
			Max: 2,500	8	1.73	11,545

In FCM studies, 20,000 cells were collected at flow speed of 13 µL per minute. The minimum and maximum data rate of 1,000 and 2,500 events per second corresponded to 4,618 and 11,545 events per µL of sample.

on reducing the cloud particle content of centrifuged OJ compared to 11-FOJ. The regression analysis (coefficient of determination of R^2) of the data showed that FCM was highly sensitive for detecting the variation in the concentration of particles present in the sample (R^2 = 0.99).

Accordingly, these results also made it possible to calculate the number of cloud particles that were present in filtered OJ samples inoculated with *E. coli*. Based on the preliminary experiments (data not shown), the maximum acceptable data acquisition rate and the flow speed for FCM was set at 2500 events per second and 13 µL per minute respectively [Table 4.3]. This corresponded to analysis of not more than 11,545 events per µL (approximately 10^4 events per µL). To achieve a similar cell concentration, filtered OJ samples containing approximately 2×10^8 *E. coli* cells per mL (2×10^5 *E. coli* cells.μL^{-1}) were required to be diluted 1:20 in PBS (5%) before analysis. Consequently, by knowing the number of particles present in an OJ sample, it was possible to predict both the number of cloud particles as well as *E. coli* cells. For instance, acquiring 20,000 events from a 5% diluted sample of 11-FOJ inoculated with *E. coli* cells meant acquiring 19,979 *E. coli* cells and 21 cloud particles (± 11 cloud particles or *E. coli*), corresponding to approximately 99.9% *E. coli* cells in the sample.

4.2.6 Effects of OJ Cloud on Viability of *E. coli* K-12 MG1655 at 4 °C

The effect of cloud particles on the viability of *E. coli* K-12 MG1655 in filtered OJ at 4 °C was investigated [Figure 4.11]. The green fluorescent viability dye of

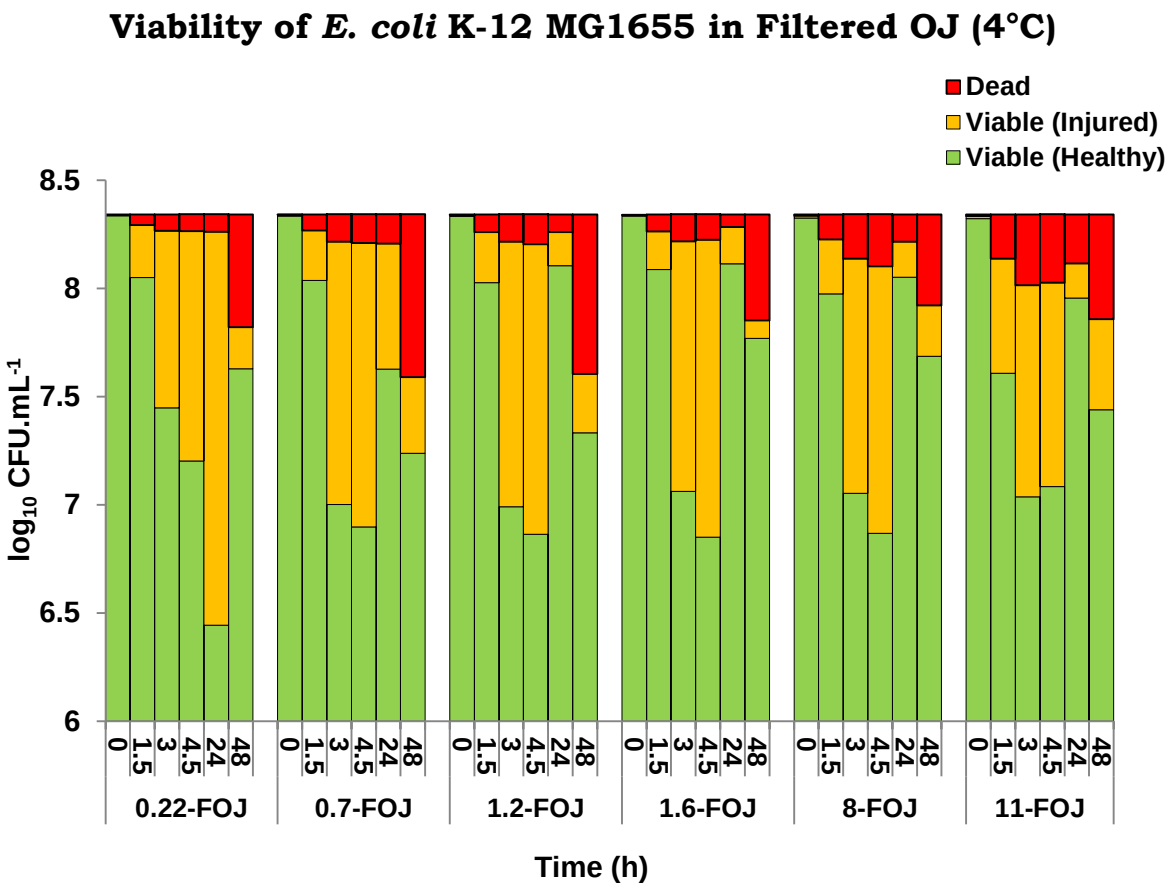


Figure 4.11: The viability of *E. coli* K-12 MG1655 in filtered OJ (FOJ) at 4 °C

The figure shows the number (log_{10} cells.mL^{-1}) of healthy GFP$^+$ *E. coli* K-12 MG1655 cells in filtered OJ samples throughout incubation at 4 °C for 48 hours. The values preceding "-FOJ" is indicative of the pore size of the filter used for filtering the OJ. Samples were incubated at 4 °C instead of 22.5 °C. Cells were stained with bis-oxonol (BOX) and PI. The former stains cells with depolarized membrane (injured or dead) wheras the latter stain only the DNA of dead cells. In this graph, log_{10} cells.mL^{-1} of healthy (BOX$^-$/PI$^-$), injured (BOX$^+$/PI$^-$) and dead cells (BOX$^+$/PI$^+$) have been shown as green, orange and red respectively.

BOX was utilized along with PI in order to detect not only the healthy (BOX^-/PI^-) and dead cells (BOX^+/PI^+) but also cells with depolarized membrane (i.e., injured BOX^+/PI^-). Healthy and injured cells which exclude the PI dye (PI^-) were considered to be viable.

In general, compared to cloud-free OJ (0.22-FOJ), the presence of OJ cloud particles in filtered OJ especially those with particle size of less than 8 µm, resulted in a significant decrease in the number of healthy *E. coli* by time 4.5 h post-inoculation ($p < 0.01$ for all samples excluding 11-FOJ with p-value = 0.06). Nevertheless, the rate of decrease in the number of healthy cells appeared to be dependent on the type of particles present in the OJ. For instance, at time 1.5 h post-inoculation, the number of healthy cells in 11-FOJ samples was significantly lower compared to other OJ samples, suggesting a greater antimicrobial effectiveness of large rag fragments ($p < 0.01$). At time 3 h, however, no significant difference was observed between the healthy population among all OJ samples containing cloud particles. This was mainly due to greater rate of decrease in the number of healthy cells in 0.7-FOJ, 1.2-FOJ, 1.6-FOJ and 8-FOJ between time 1.5 h and 4.5 h. Consequently, by time 4.5 h, the latter samples contained a significantly lower number of healthy cells compared to 11-FOJ as well as cloud-free 0.22-FOJ.

An increase in the $\log_{10}$ number of healthy cells at time 24 h at 4 °C was observed. During the same time period the $\log_{10}$ number of viable cells remained relatively constant in all OJ samples. Therefore, it could be suggested

that this increase was most likely due to resuscitation of sub-lethally injured cells. The reason for this increase was not clear, however it was postulated that the cold shock-induced cell aggregation as well as the physical protection of the cell from acidic condition of OJ by cloud particles (excluding 0.22-FOJ) could have led to a greater recovery of injured cells. Their combined effects could also explain why compared to cloud-containing OJ samples, the apparent recovery of injured cells was delayed for 24 hours in virtually cloud-free OJ samples (0.22-FOJ).

Nevertheless, the observed increase at time 24 h (excluding 0.22-FOJ samples) was temporary and by time 48 h, there was a significant decrease in the number of viable cells in all samples regardless of the cloud content of OJ. In addition, at time 48 h, 0.7-FOJ had significantly lower number of viable cells compared to cloud-free 0.22-FOJ ($p < 0.05$) as well as OJ samples containing small and large rag fragments (1.6-FOJ, 8-FOJ and 11-FOJ, $p < 0.01$ for all). Therefore it was hypothesized that that hesperidin crystals exhibit a significant adverse effect on the long-term viability of *E. coli* in OJ at 4 °C in the absence of large cloud particles.

4.2.7 Effects of OJ Cloud on Viability of *E. coli* K-12 SCC1 at 22.5 °C

The effect of cloud particles on the viability of *E. coli* in OJ samples incubated at room temperature (22.5 °C) was investigated. As mentioned before this temperature was chosen in order to study the effect of improper storage of OJ on the survival of *E. coli*. Using the GFP-generating *E. coli* K-12 SCC1 also made

it possible to take advantage of the pH sensitivity of the GFP (Kneen *et al.*, 1998) for investigating the effects of cloud particles on the internal pH (pH_i) of the *E. coli* inoculated in OJ.

Figure 4.12 shows the gradual change in the viability of GFP-generating strain SCC1, stained with the DNA-staining viability dye propidium iodide (PI) using a dot plot of green fluorescence (FL1-A) versus red fluorescence (FL3-A) in 11-FOJ at room temperature. A similar pattern of change in viability was found in case of all OJ sample and therefore 11-FOJ is shown as a representative sample. Cells which were GFP positive (GFP^+) were considered healthy (shown with green dots). Cells that were not stained with PI were considered viable which included not only the healthy cells but also cells that had low or no GFP content. The latter two formed two distinct populations (shows with blue and orange dots respectively) and were assumed to be consisted of acid stressed cells with internal pH of less than 5, as will be discussed later. Cells that had been stained with PI were considered as dead (shown with red dots). At time 0 h, most cells were GFP^+, however upon addition of cells to OJ a dramatic increase in the number of either dead or low GFP/GFP^- cells was observed. The results obtained for all samples are described below.

Figure 4.13 compares the effects of each filtration regime on the number of healthy cells in OJ during 24 hours of incubation at room temperature. At time 0 h (immediately post-inoculation in OJ) there was no noticeable difference between the populations of healthy cells regardless of the samples'

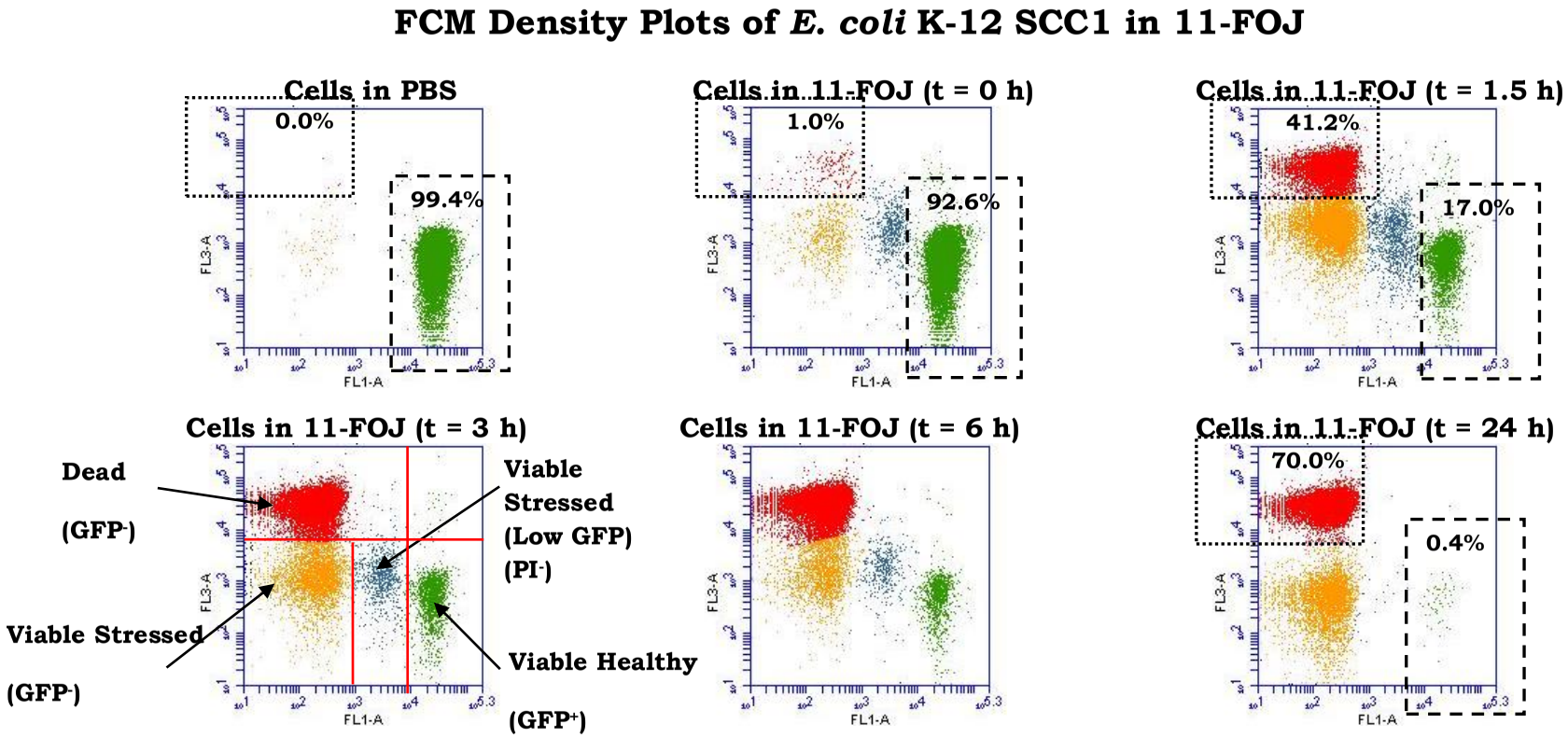


Figure 4.12: Fluorescence density plots showing the change in viability of *E. coli* SCC1 K-12 SCC1 in orange juice (OJ) filtered with 11 µm filter paper (11-FOJ)

The figure shows the dot plots of FL1-A (green fluorescnece of GFP) versus FL3-A (red fluorescence of PI) for *E. coli* K-12 SCC1 subjected to an OJ filtered with 11 µm (11-FOJ) during 24 hours incubation at room temperature (22.5 °C). Plots show the data for 20,000 cells each represented with a single dot. Green, blue, orange and red dots represent GFP^+ (healthy), low GFP, GFP^-/PI^- (presumably injured or stressed) and GFP^-/PI^+ (dead) cells respectively. The values inside the dashed or dot boxes shows the percentage of healthy and dead cells respectively. The trend in change in viability of cells observed for other FOJ samples was similar though with a different rate. Therefore, for this figure 11-FOJ was chosen as the representive sample.

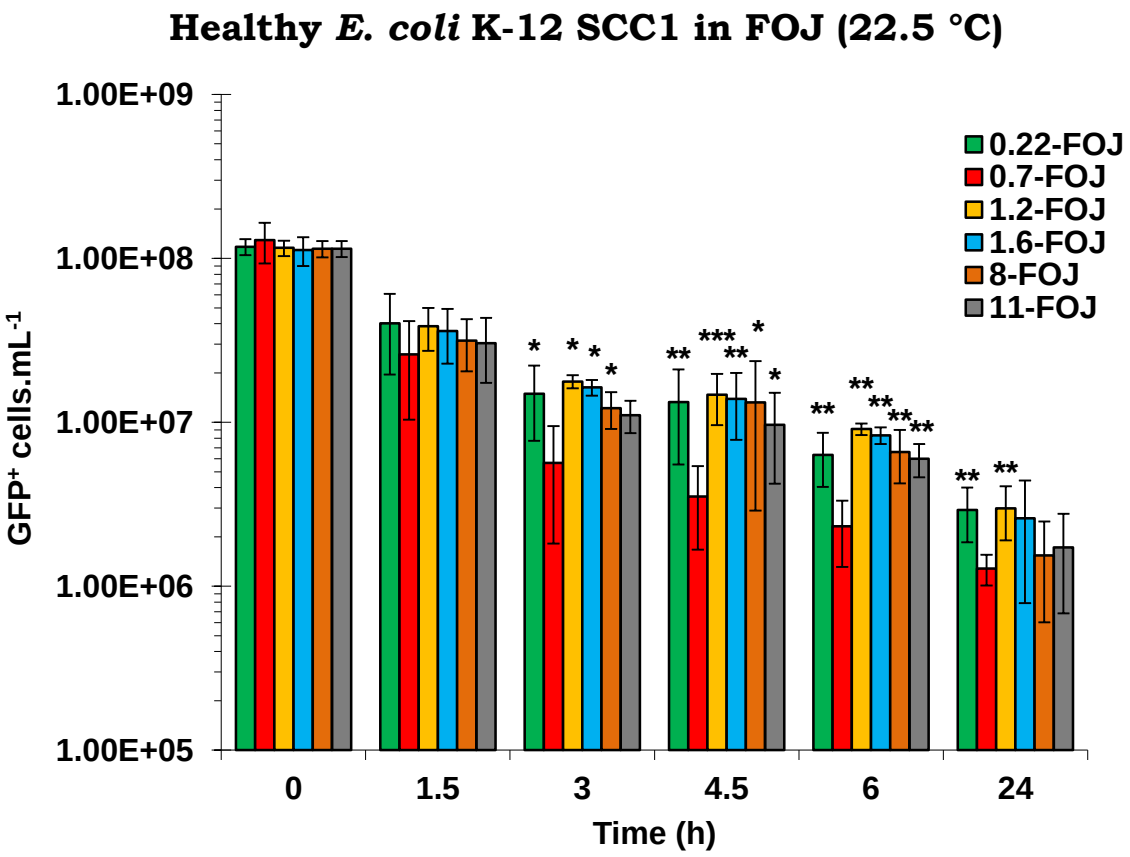


Figure 4.13: The number of GFP⁺ (healthy) *E. coli* K-12 SCC1 in filtered OJ (FOJ)

The figure shows the number (cells.mL^{-1}) of healthy GFP⁺ *E. coli* K-12 SCC1 cells in FOJ samples throughout incubation at 22.5 °C for 24 hours. The values preceding "-FOJ" is indicative of the pore size of the filter used for filtering the OJ. 15 mL of filtered OJ were inoculated with 3×10^9 cells of *E. coli* K-12 SCC1. Samples were incubated at 22.5 °C and the number of healthy GFP+ cells (green population in Figure 4.12) was measured using FCM. Error bars are the ± standard deviation of the mean value obtained at each time point [*: $p < 0.05$; **: $p \leq 0.01$; ***: $p \leq 0.001$ (all compared to 0.7-FOJ at the marked data-point)].

cloud content. However, within the next 24 hours of incubation, two filtration regime dependent trends in cell viability were observed. At 1.5 h post-inoculation, an interesting nevertheless non-significant trend in the number of healthy cells in filtered OJ was noted. Reducing the cloud content and maximum particle size of OJ samples from 11 µm to 1.2 µm (11-FOJ and 1.2-FOJ) resulted in a gradual 0.10 $\log_{10}$ increase in the mean concentration of healthy (GFP^+) cells. This was in agreement with the initial assumption that the overall reduction in the number of rag fragments and fat globules containing antimicrobial compounds such as essential oils leads in improved viability of the *E. coli* in OJ. In contradiction of this assumption, however, in case of 0.7-FOJ samples (containing mainly hesperidin), the filtration of particles of between 0.7 µm and 1.2 µm (primarily chromoplastids) caused a noticeable reduction (0.17 $\log_{10}$) in the mean number of healthy GFP^+ cells. This reduction in viability was presumed to be either due to (a) abolishment of the physical protection of *E. coli* cells from acidic condition of OJ by large rag fragments and chromoplastids and/or (b) greater antimicrobial efficacy of hesperidin in the absence of particles of larger than 0.7 µm. The latter could also explain why removal of hesperidin particles in case of 0.22-FOJ samples restored the population of healthy cells to the levels observed in 1.2-FOJ samples.

The results obtained at time 3 h reaffirmed the assumption made about the non-significant trend that was observed at time 1.5 h. At this time point, significantly greater number of healthy cells was observed for 1.2-FOJ and

1.6-FOJ compared to 11-FOJ sample confirming the hypothesis that the removal of fat globules and rag fragments could improve the viability of the *E. coli* in OJ (both $p < 0.05$). Conversely, compared to 1.2-FOJ, 1.6-FOJ and 8-FOJ samples, the number of healthy cells in 0.7-FOJ sample were significantly lower (0.50, 0.46 and 0.33 mean $\log_{10}$ reduction respectively, all $p < 0.05$). Moreover, removal of hesperidin particles resulted in a significant 0.42 $\log_{10}$ recovery in the number of healthy cells. These results reiterated two assumptions made at time 1.5 h about the greater antimicrobial activity of hesperidin in the absence of chromoplastids and rag fragment. At time 4.5 h and 6 h post-inoculation, the population of healthy cells in 0.7-FOJ remained significantly lower compared to other OJ samples regardless of the latters' cloud content ($p < 0.01$ at 6 h post-inoculation). However, in the case of samples with particle size of larger than 1.6 µm (i.e. 8-FOJ and 11-FOJ), the statistical significance was lost at time 24 h post-inoculation. This suggested a possible increase in the effectiveness of large rag fragments in reducing the pH_i of the cells, hence the number of healthy cells.

No significant difference between the number of healthy cells in 8-FOJ and 11-FOJ samples and similarly between 1.2-FOJ and 1.6-FOJ samples was observed at each time point throughout the experiment. This was not surprising considering the similar nature of cloud particles in these samples confirmed earlier by the cloud characterization experiments [Figure 4.3 & 4.4).

Post-hoc analysis of these data revealed further information about the different kinetic rate of change in the number of healthy cells caused by different fractions of cloud such as hesperidin, chromoplastids and rag fragments. [Figure 4.14] shows the rate of reduction in the number of healthy cells in filtered OJ samples containing various cloud content. The rate was determined by calculating the $\log_{10}$ cells.mL^{-1} reduction in the population of healthy GFP^{+} between each time point and dividing this value by the duration of time between two sampling points (i.e., 1.5 h or 18 h). The rate (-dN$_{(GFP+)}$.dt^{-1}) was expressed as $\log_{10}$ reduction in the number of healthy *E. coli* cells per millilitre per hour (-$\log_{10}$ cells$_{(GFP^{+})}$.mL^{-1}.h^{-1}).

Regardless of the cloud content of the samples, the greatest mean rate of decrease in the number of healthy cells was observed during the first one and a half hour of the experiments. This was assumed to be mainly due to acid shock and lower resistance of *E. coli* to acidic condition of OJ during this period. Concurrent to acid stress, the presence of cloud particles also appeared to play a role in reducing the number of healthy cells. For instance, the mean rate observed for 0.7-FOJ samples was between 0.11 and 0.19 $\log_{10}$ Cells$_{(GFP^{+})}$.mL^{-1}.h^{-1} higher than the values observed for other samples. This showed that the removal of particles of larger than 0.7 µm could potentially increase the effectiveness of hesperidin in reducing the number of healthy cells; however the differences observed were not significant (*p*-values of between 0.22 and 0.45 compared to 0.7-FOJ).

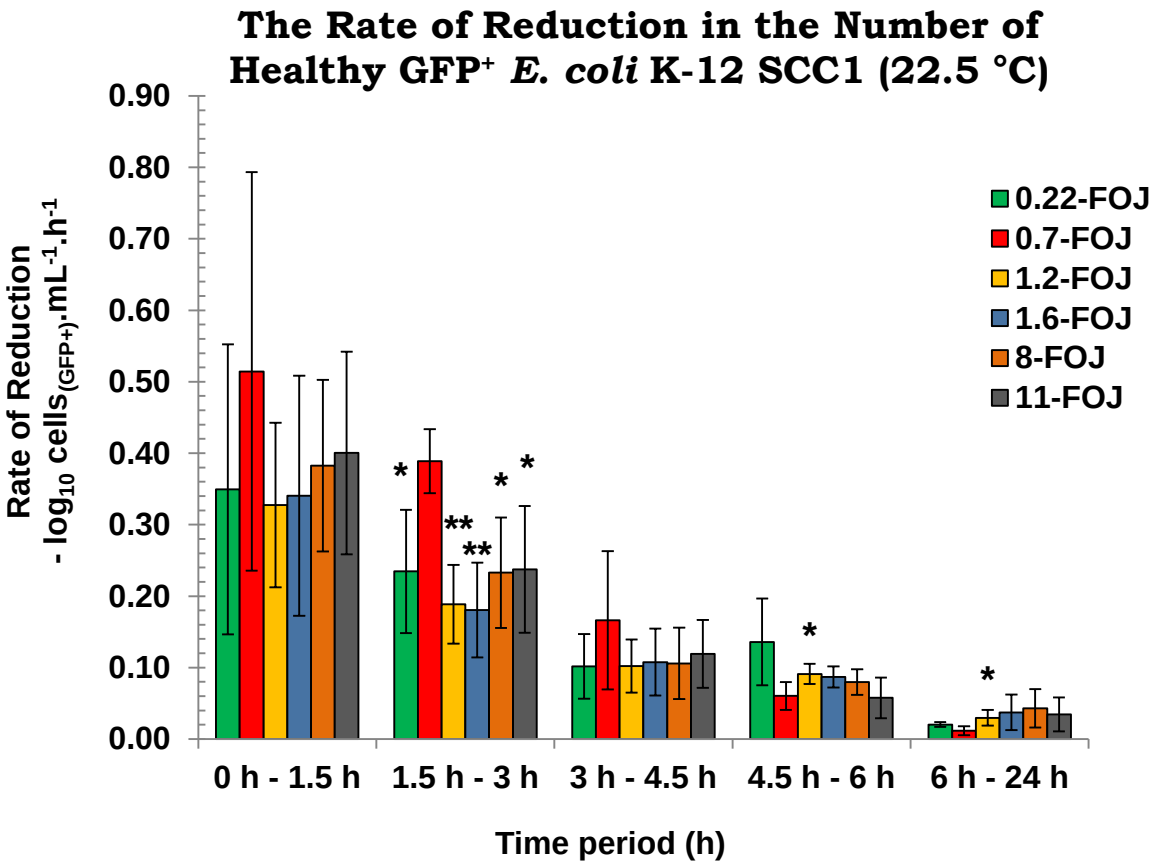


Figure 4.14: The rate of reduction in the number of GFP⁺ (healthy) *E. coli* K-12 SCC1 in filtered OJ (FOJ) at 22.5 °C

The figure shows the rate of reduction ($-\log_{10}$ cells$_{(GFP^+)}$.mL^{-1}.h^{-1}) in the number of healthy GFP⁺ *E. coli* K-12 SCC1 cells in filtered OJ samples throughout incubation at 22.5 °C for 24 hours. The values preceding "-FOJ" is indicative of the pore size of the filter used for filtering the OJ. The rate was determined by calculating the $\log_{10}$ cells.mL^{-1} reduction in the population of healthy GFP⁺ between each time point and dividing this value by the duration of time between two sampling points (i.e., 1.5 h or 18 h). Error bars are the ± standard deviation of the mean value obtained at each time point. [*: $p < 0.05$; **: $p \leq 0.01$ (all compared to 0.7-FOJ at the marked data-point)].

Compared to 0 h to 1.5 h, between time 1.5 h and 3 h post-inoculation, there was a noticeable reduction of between 0.11 and 0.16 $\log_{10}$ $Cells_{(GFP^+)}.mL^{-1}.h^{-1}$ in the rate of reduction of healthy cells. Nevertheless this reduction was only significant in case of 8-FOJ and 11-FOJ samples (p < 0.05). During this time period, significantly greater rate was observed for 0.7-FOJ samples (0.39 $\log_{10}$ $cells_{(GFP^+)}.mL^{-1}.h^{-1}$, p < 0.05 compared to 0.22-FOJ, 8-FOJ and 11-FOJ; p < 0.01 compared to 1.2-FOJ and 1.6-FOJ). This confirmed the greater effectiveness of hesperidin crystals in reducing the number of healthy cells in the absence of large particles. In case of 8-FOJ and 11-FOJ (both containing large rag fragments), the lower level of statistical significance as well as greater mean rate of reduction compared to 1.2-FOJ and 1.6-FOJ samples (both lacking large rag fragments) showed a possible adverse effect of large rag fragments on the viability of *E. coli* between time 1.5 h and 3 h. Nevertheless, the rate of reduction obtained for OJ samples containing cloud particles of larger than 0.7 µm (during this time period was very similar to the one observed for the cloud-free 0.22-FOJ indicating that these particles had no effect on the number of healthy cells.

This trend continued between time 3 h and 4.5 h post-inoculation, during which a sharp decrease in the rate was observed in case of all samples except 11-FOJ (p < 0.05 for 0.22-FOJ; p < 0.01 for other samples except 11-FOJ with p = 0.09). This could consequently point out to overall increase in the resistance of *E. coli* cells to acidic condition, giving rise to their improved capability in maintaining their pH_i. Despite the lack of statistical significance

between the rates observed for each sample, the greatest mean rate was achieved in 0.7-FOJ. Therefore it could be said that hesperidin crystals were the most effective cloud particles in reducing the number of healthy cells during the first 4.5 hours of the study.

Interestingly, from time 4.5 h to 6 h post-inoculation, cloud particles appeared to exhibit a modest protective effect against the acid-induced decrease in internal pH of *E. coli* in OJ. The mean rate observed in cloud-free 0.22-FOJ was noticeably higher than those observed in cloud-containing samples regardless of the cloud content (p = 0.08 compared to both the 0.7-FOJ and 11-FOJ). It is possible, therefore, to hypothesize that cloud particles could physically protect the cells from the acidic environment of the OJ. This could explain why the mean rate of reduction in the number of healthy cells was steadily decreased when maximum particle size of OJ was increased from 1.2 µm to 11 µm. Paradoxically, the greater interaction of the cells with cloud particles of larger than 1.2 µm could also be the main rationale behind the greater rate of reduction in the number of healthy cells in 1.2-FOJ, 1.6-FOJ, 8-FOJ and 11-FOJ between time 6 h and 24 h. The reduction rates observed in 1.2-FOJ samples was significantly greater than the values obtained for 0.7-FOJ sample between time 4.5 h to 24 h ($p < 0.05$). Greater physical contact with chromoplastids, rag fragments and essential oil-containing fat globules could have led to a greater damage to cell membrane and therefore greater rate of reduction in the number of healthy cells.

In summary, based on the results described above, it could be suggested that, the acidic condition of the OJ was the main contributing factor in reducing the internal pH of the cells throughout the study. The rate of reduction in the number of healthy cells was significantly decreased by the end the experiment, indicating greater resistance of the cells to acidic condition of the OJ. Nevertheless during the first 4.5 hours of experiment particles of smaller than 0.7 μm (most likely the hesperidin crystals) were more effective than larger cloud particles in reducing the internal pH of the cells. On the other hand, cloud particles appeared to have a protective effect against the acid induced reduction in pH_i of the cells between time 4.5 h to 6 h post-inoculation. Finally, during the next 18 hours the study (time 6 h to 24 h), particles of larger than 1.2 μm appeared to be more effective than hesperidin crystals in reducing the number of healthy cells.

So far, only the effects of OJ cloud particles on the number of healthy *E. coli* was discussed. However, as mentioned before, viable cells include not only the GFP^+ healthy cells, but also the stressed (with $pH_i < 5$) and potentially injured cells with low or no GFP^-. [Figure 4.15] shows the overall viability of SCC1 strain in filtered OJ with various cloud content. Green, orange and red columns show the $\log_{10}$ cells.mL^{-1} healthy (GFP^+), stressed (low GFP/GFP^- and PI^-) and dead cells (PI^+) respectively. Up to time 6 h post-inoculation, no significant difference in the overall number of viable cells was observed at each time point between cloud-free 0.22-FOJ and other OJ samples regardless of their cloud content. The only exception to this was the lower number of viable cells in 0.7-

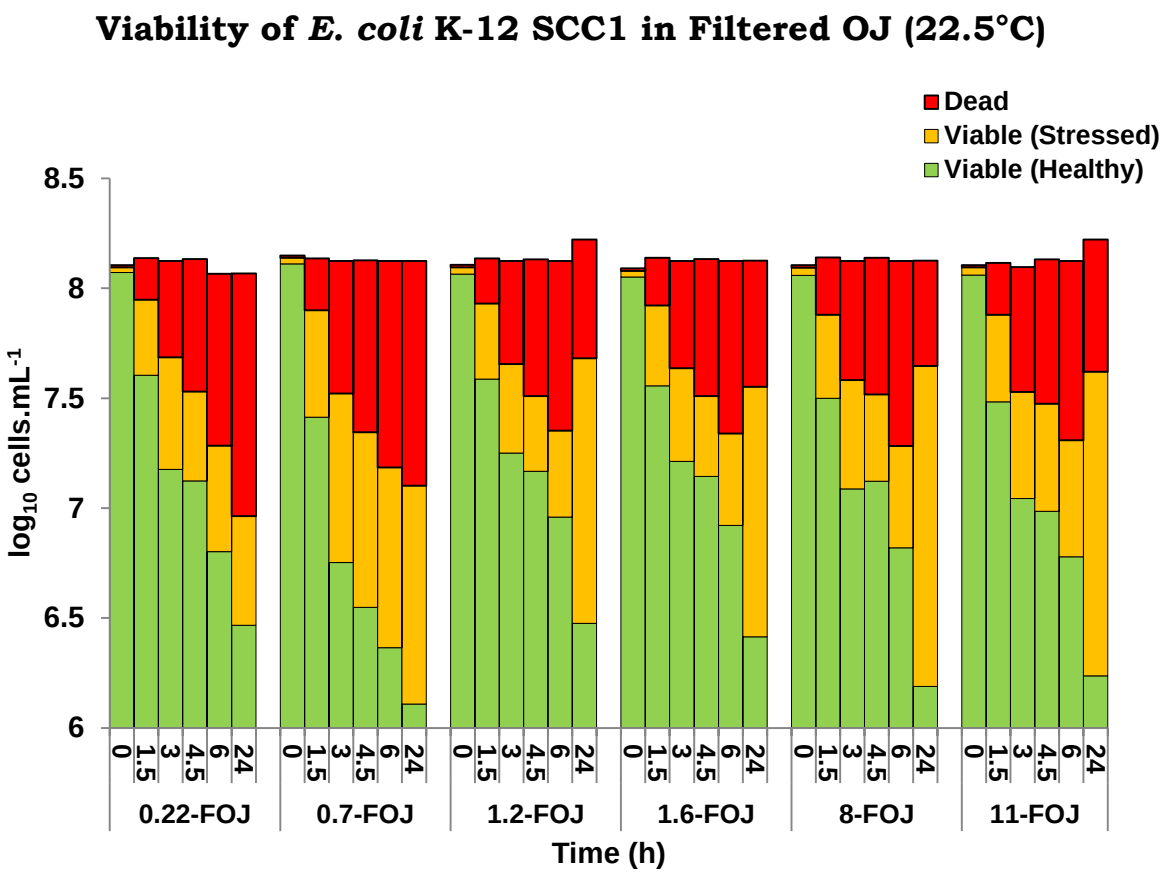


Figure 4.15: The viability of *E. coli* K-12 SCC1 in filtered OJ (FOJ) at 22.5 °C

The figure shows the number (log_{10} cells.mL^{-1}) of healthy GFP$^+$ *E. coli* K-12 SCC1 cells in filtered OJ samples throughout incubation at 22.5 °C for 24 hours. The values preceding "-FOJ" is indicative of the pore size of the filter used for filtering the OJ. Cells were stained with propidium ioide (PI) in order to stain dead cells. In this graph, log_{10} cells.mL^{-1} of healthy (GFP$^+$), GFP$^-$/PI$^-$ [presumably stressed with internal pH or pH_i < 5, Richard & Foster (2004)] and GFP$^-$/PI$^+$ (dead) have been shows as green, orange and red respectively. The number of healthy cells have already been discussed in Figure 4.13.

FOJ compared to 0.22-FOJ samples at 3 h post-inoculation (7.53 ± 0.09 $\log_{10}$ cells.mL^{-1} and 7.69 ± 0.02 $\log_{10}$ cells.mL^{-1} respectively, $p < 0.05$). These result showed that although cloud particles and particularly hesperidin crystals were effective in reducing the number of healthy cells, this did not necessarily translate into greater number of dead cells. In other words, up to time 6 h, the cloud particles had no significant role on killing the *E. coli* and therefore, the only major contributing factor to cell death was assumed to be the acidic condition of the OJ. In contrast, compared to time 6 h, at 24 h post-inoculation, a cloud content dependent difference in cell viability was observed. In cloud-free 0.22-FOJ sample, the number of viable cells was significantly decreased from 7.28 ± 0.13 to 6.96 ± 0.09 ($p < 0.01$). On the other hand, in 0.7-FOJ samples consisting of primarily the hesperidin crystals, no noticeable change in total number of viable cells was observed. The presence of particles of larger than 0.7 µm in filtered OJ caused a noticeable, nonetheless non-significant increase in the number of viable cells. Mean difference between the number of viable cells at time 6 h and 24 h for samples with particles of larger than 0.7 µm, ranged from 0.21 to 0.36 $\log_{10}$ cells.mL^{-1} ($p < 0.05$ for 1.2-FOJ, 8-FOJ and 11-FOJ). In summary, based on the results mentioned above, it could be argued that, throughout 24 hours incubation of the OJ samples at 22.5 °C, although different cloud particles are capable of reducing the viability of *E. coli* at various time points, however they could also play a role in protecting the *E. coli* from the acidic condition of OJ.

4.3 Discussion

4.3.1 The Pulp Content of Orange Juice

The pulp content of the OJ used in this study was slightly lower than the 6% to 12% range reported by Berlinet *et al.*, (2007) for industrially manufactured OJ. However factors such as the type of cultivar, seasonal variation and method of preparation could affect the pulp and/or cloud content of OJ. For instance, Brat *et al.*, (2003) and Rega *et al.*, (2004) reported the pulp content of the Spanish Naveline variety to be around 4.2% and 12% respectively. This was despite the fact that both groups had used the same processing conditions for removal of the OJ pulp. Therefore, both the 5% and 10% pulp supplementation conditions used in this study were within the ranges reported in the literature.

4.3.2 Measurement of Particle Size

With respect to the cloud particles, three distinct populations were observed [Figure 4.1]. According to Mizrahi & Berk, (1970), OJ cloud particles can be divided into four main groups: large amorphous rag fragments of 2 µm to 10 µm; fat globules attached to the rag particles; chromoplastids containing carotenoids (approximately 1 µm) and sharp needle-like hesperidin crystals of 0.5 to 3 µm long and 0.05 to 0.2 µm thick. The ranges reported for cloud particles by these researchers were very similar to what was observed in the current study. In addition, the pattern of particles size distribution in filtered OJ samples was in agreement with the tri-modal distribution reported by Ackerley & Wicker, (2003) and Fernández-Vázquez *et al.*, (2013).

Further support for the assumptions made about the nature of cloud particle

could be derived from the differences that were observed between the colour and precipitate content of the autoclaved OJ samples [Figure 4.4]. Expectedly, autoclaving OJ samples containing proteinous rag fragments and fat globules (8-FOJ and 11-FOJ) as well as colour pigments (1.2-FOJ and 1.6-FOJ) resulted in the production of burnt or very dark browning products. Hence it could be said that the lack of browning products in 0.7-FOJ and 0.22-FOJ samples is indicative of the absence of rag fragments and chromoplastids. The significant amount of white precipitate at the bottom of the test tube of 0.7-FOJ is typical of hesperidin crystals and has been previously reported by Mizrahi & Berk, (1970) and Ben-Shalom & Pinto, (1999). Therefore, it seems that filtration of OJ with 0.7 µm filter paper removes the majority of large and medium size cloud particles, leaving only the free fine crystals of hesperidin. The filtration with 0.22 µm syringe filters on the other hand removes the majority of these crystals resulting in the formation of a virtually cloud-free OJ.

The apparent higher D_{50} value obtained for 0.7-FOJ compared to 1.2-FOJ [Table 4.1] could be due to reduced sensitivity of the laser diffraction technique for measuring the size of irregular non-spherical particles (Malvern, 2013). This technique is based on the assumption that the particles are spherical, whereas in OJ, particles of less than 1.2 µm mainly consist of hesperidin crystals up to 10 µm long (Mizrahi & Berk, 1970). The orientation and the flow alignment of the long needle-like crystals during passage through the laser could have contributed to the larger D_{50} values reported by the Mastersizer for 0.7-FOJ.

4.3.3 Culturability Studies

In general, there was an inverse relationship between the pulp content of OJ and the culturability of *E. coli* isolated from OJ samples incubated at 4 °C [Figure 4.5]. It was initially hypothesized that the entrapment of *E. coli* by OJ pulp particles could have prevented their presence, hence their growth on the surface of the solid growth media. However, increase in the pulp concentration of OJ up to 20% (data not shown) did not affect the initial number of culturable cells at time 0 h. Therefore, the observed reduction in the culturability throughout the experiment was attributed to the antimicrobial activity of the pulp particulates and not necessarily the physical entrapment of the cells by the pulp.

The most abundant volatile compound in OJ pulp which has been shown to exhibit antimicrobial activity — within the ranges naturally present in OJ — is limonene (Brat *et al.*, 2003; Espina *et al.*, (2013). However based on the work of Fernández-Vázquez *et al.*, (2013), this compound was not considered to be the primary cause of the antimicrobial action of OJ pulp. In their study, the latter demonstrated that the removal of OJ pulp and its subsequent add back to the pulp-free OJ led to a significant increase in the concentration of limonene. They attributed this increase to the release of lipophilic limonene from the lipid fraction of the pulp and cloud. In the current study, the concentrations of essential oils and volatile compounds were not measured. Nevertheless, it is plausible to assume that compared to pre-centrifuged 5% OJ (5F-OJ), post centrifuged 5% OJ (5S-OJ) should have contained a greater concentration of

limonene. In turn, this should theoretically have resulted in lower culturability of *E. coli* inoculated in 5S-OJ. The lack of difference between the culturability of *E. coli* isolated from 5F-OJ and 5S-OJ samples, therefore suggests that the presence of limonene was not the main reason for the observed pulp-induced reduction in culturability of *E. coli*.

This result may however, be explained by the synergic action of antimicrobial volatile compounds in OJ (Caccioni *et al.*, 1998). For instance, Jordan *et al.*, (2001) set up a study to determine the effects of pulp reduction on volatile compounds of OJ. They showed that removal of pulp results in a significant decrease in not only the non-polar terpenic hydrocarbons such as limonene, but also low-polar aldehydes and alcohols of decanal and linalool respectively. As mentioned before (see Section 1.2.2), compared to limonene the latter two have been shown to exhibit a greater antimicrobial activity against *E. coli* (Inouyi *et al.*, 2001a, 2001b; Liu *et al.*, 2012). Consequently, it could be suggested that overall reduction in the non-polar and low-polar essential oils and volatile compounds content during the pulp reduction stage was the probable reason behind the observed increase in the culturability of *E. coli*.

The results obtained for pulp-free 0S-OJ samples at 37 °C [Figure 4.6], further support the hypothesis that reduction in the pulp content of OJ, plays an important role in increasing the survival and culturability of *E. coli* regardless of the incubation temperature. The significant difference observed for the culturability of *E. coli* at 4 °C and 37 °C, was consistent with the general trend

discussed earlier in the previous chapter [Figure 3.4] for the lower rate of reduction in culturability of the cells at 4 °C. The reduction in pH of OJ at 37 °C (due to greater dissociation of organic acids), degradation of ascorbic acid, production of furfural and browning products, and also the competition with natural microflora were considered to be the main reasons for a significantly greater decrease in the survival of *E. coli* at 37 °C (Kaanane *et al.*, 1988; Álvarez-Ordóñez *et al.*, 2013; Roig *et al.*, 1999; Uljas *et al.*, 2001).

With regard to the cloud particles, increase in cloud content of OJ samples incubated at 4 °C, was associated with a noticeable decrease in the mean $\log_{10}$ CFU.mL^{-1} of culturable cells throughout the experiment [Figure 4.7]. However, the difference between the number of culturable cells isolated from 0.7-FOJ and 0S-OJ was only significant for the results obtained between the day three and day twelve. On day 12 of the incubation, the difference between the number of culturable cells isolated from 0S-OJ (pulp-free) and 0.7-FOJ was smaller than what was observed between the of 0S-OJ and 5F-OJ [Figure 4.5]. This demonstrated that OJ cloud particles are less effective than OJ pulp particles in reducing the culturability of *E. coli* at 4 °C.

In respect of 37 °C samples [Figure 4.8], the results obtained for all different conditions were almost identical. This lack of difference could again be attributed to the combined effects of the lower antimicrobial efficacy of the cloud particles (compared to the pulp), as well as increased inactivation of *E. coli* at 37 °C.

4.3.4 Viability (FCM) Studies

The results of the culturability experiments at optimal growth temperature of *E. coli* (37 °C) showed a rapid reduction in culturability of *E. coli* in OJ. As a result, in order to facilitate the studying of the viability during the first 24 hours of incubation, it was decided to incubate the OJ samples at room temperature (22.5 °C) instead of 37 °C. Both the 4 °C and 22.5 °C were also considered to be more relevant to food safety by simulating respectively the proper (refrigerated) and improper (ambient temperature) home storage of OJ by consumers.

In general, the results of the FCM experiment provided a better picture of the effect of OJ clarification on the viability of *E. coli* in OJ. As it was shown in Figure 4.12, the inoculation of filtered OJ with GFP^+ *E. coli* K-12 SCC1 to OJ resulted in a significant decrease in the number of GFP^+ cells and generation of three distinct GFP^- populations. The observed change in GFP fluorescence could be attributed to the change in internal pH (pH_i) of the *E. coli*. Richard & Foster (2004) demonstrated that when *E. coli* cells were subjected to acidic conditions, they behaved similar to other acidophilic bacteria. These microorganisms respond to acid stress by reversing their trans-membrane potential ($\Delta\psi$) (becoming inside positive) and increasing their pH_i in order to reduce the resultant excess proton motive force (PMF). However this phenomenon was only observed in *E. coli* when cells were supplemented with extracellular glutamate and/or arginine. Despite this, the pH_i reported for acid-adapted *E. coli* still remained highly acidic (pH_i of 4.2 and 4.7 for glutamate- and arginine-

supplemented cells respectively). Furthermore, Kneen *et al.*, (1998) reported that GFP could be used as an effective indicator of the change in pH_i of the bacterial cells owing to its high pH sensitivity and specificity. The GFP fluorescence is reduced at pH values of less than 5 due to change in both its protonation and conformational states.

The results of the current study corroborate the work of the aforementioned studies. OJ is a highly acidic solution which contains both the glutamate and arginine amino acids. Therefore it could be hypothesized that *E. coli* inoculated in OJ employs a similar acid resistance strategy in order to import proton and become inside positive, resulting in the generation of positively charged bacteria with acidic pH_i of less than 5. Consequently, it could also be suggested that the generation of GFP^- population is due to the reduction in pH_i to values of below 5. The presence of two distinct viable GFP^- populations (low GFP and GFP^-) was attributed to different degrees of conformational changes in GFP in pH values of below 5. Low GFP cells (with lower degree of change in conformational structure of GFP) progressively became GFP^- most likely due to complete denaturation and inactivation of GFP (GFP^-) and ultimately non-viable (GFP^-/PI^+). Therefore, based on these assumptions, GFP^+ cells were considered healthy, whereas viable (PI^-) cells which were either low GFP or GFP^- were assumed to be acid stressed, acid-adapted and/or sub-lethally injured.

The results for samples incubated at 22.5 °C (Figures 4.13, 4.14 and 4.15) showed that although the overall reduction in cloud content of OJ led to an

improvement in viability, surprisingly a significantly lower number of healthy GFP^+ cells were observed for samples of 0.7-FOJ during the first 3 hours of incubation. This raised an intriguing question of why filtration with a 0.7 µm filter led to a significantly greater decrease in viability of *E. coli* in OJ. As - mentioned earlier, the cloud of 0.7-FOJ samples consists of mainly the hesperidin crystals. Therefore, it could be hypothesized that these crystals exert a different antimicrobial mechanism from larger particles such as rag fragments, essential oils of fat globules and chromoplastids. Significant increase in the viability of *E. coli* in 0.22-FOJ compared to 0.7-FOJ and 1.2-FOJ also suggests that hesperidin crystals have a significantly stronger interaction with *E. coli*.

In order to explain this phenomenon, it is important to understand the effects of pectin on the electric charge of the OJ cloud particles. Mizrahi & Berk (1970) and Croak & Corredig (2006) measured the electric charge (ζ-potential) of the colloidal cloud particles of the OJ. All particles were shown to be negatively charged at pH values above 2.5 and the ζ-potential increased with increasing the pH. The ζ-potential of the particles within the pH range of OJ was between -5 mV to -25 mV which they attributed to the presence of carboxyl groups of pectin. Similar interaction of negatively charged pectin with cloud particles of apple juice have been shown by Yamasaki *et al.*, (1964). It has been postulated that the coating of cloud particles with pectin is the possible reason for their negative charge and stability in OJ (McLellan & Padilla-Zakour, 2004; Ellerbee, 2009).

According to Brat *et al.*, (2003), nearly 60% of the pectin is associated with the cloud particles. In OJ, the sugar moiety of the crystalline hesperidin (rhamnose or glucose) interacts through hydrogen bonding with the neutral sugar moieties of the galacturonic acid backbone of the pectin to form stable colloidal particles (Ben-Shalom & Pinto, 1999). Upon release of hesperidin from the rag vacuoles into the serum, pectin rapidly acts as the nucleation site for hesperidin by recognizing its sugar moiety. Quick and strong binding of pectin to hesperidin results in the formation of small pectin-bound hesperidin crystals (Ellerbee, 2009).

Interaction of acid-stressed *E. coli* with negatively charged colloidal particles has been reported. In their study, Farahat *et al.*, (2008) showed that in highly acidic condition (pH < 4.5) the positively charged *E. coli* (i.e., with positive ζ-potential) were rapidly adsorbed to negatively charged quartz particles possibly due to electrostatic attraction forces between the quartz and *E. coli*. It is reasonable to presume that larger cloud particles will have a greater magnitude of negative charge due to possessing greater surface area and consequently will have a greater number of interactions with pectin. Based on the Coulomb's law, compared to hesperidin crystals, a greater attractive force between the positively charged bacteria and the larger particles could be assumed. On the other hand, it is known that the negatively charged cloud particles in OJ repel each other, preventing the aggregation and flocculation of the cloud particles (Ellerbee, 2009). Therefore it is possible to postulate that the presence of large

negatively charged cloud particles in close proximity of the bacteria reduces the chance for smaller pectin-hesperidin crystals to get attracted to the bacteria. However, removal of these large particles increases the interaction of negative particles of hesperidin with *E. coli*.

This still leaves the question open as to why hesperidin is a more effective antimicrobial than the essential oils present in the fat globules that have covered the larger cloud particles. Although many studies have mentioned the antimicrobial effects of hesperidin, very little was found in the literature on the question of the mechanisms (Garg *et al.*, 2011, Cushnie & Lamb, 2005). It has been shown that flavonoids to which hesperidin belongs to are capable of inhibiting nucleic acid synthesis, cytoplasmic membrane function and energy metabolism (Cushnie & Lamb, 2005). On the other hand, essential oil compounds such as limonene exert their antimicrobial effects mainly by increasing the permeability of the membrane (Di-Pasqua *et al.*, 2007; Espina *et al.*, 2013). It could be possible that hesperidin employs a combination of the mechanisms mentioned above and therefore is more effective than essential oils.

Here, two more hypotheses are proposed for greater rate of antimicrobial action of hesperidin, both related to its shape and size. It is plausible that during the interaction of hesperidin with *E. coli*, the sharp needle-like structure of hesperidin causes a physical damage to the cell membrane, for instance piercing the cells. This could in turn result in a significantly greater number of

injured cells, leading to subsequent increase in the number of dead cells. Another plausible explanation for antimicrobial activity of hesperidin is the possible action of these compounds as nanoparticles. Numerous nanoparticles within the same size range of hesperidin have been shown to exhibit strong antimicrobial activity against various microorganisms including *E. coli*, mainly due to their high surface area to volume ratio (Hajipour *et al.*, 2012). The use of glucosyl hesperidin in the production of pharmaceutical nanoparticles has also been reported by Tozuka *et al.*, (2011). As a consequence, it could be proposed that the antimicrobial effect of hesperidin crystals is simply because of their small size. Increase in the total number of viable cells at time 24 h for samples of 1.2-FOJ, 1.6-FOJ, 8-FOJ and 11-FOJ could be due to physical protection of the cells from the acidic serum of OJ by large cloud particles, reducing the direct contact of the cells with acid. Similarly, it could be postulated that complete removal of cloud particles in case of 0.22-FOJ results in greater accessibility of the highly acidic serum to the bacteria resulting in a greater decrease in viability.

Protection and growth of food pathogens such as *E. coli* and *Salmonella* in the acidic condition by solid food particles and inside the orange fruit have been reported (Lawrence, 1998; Eblen *et al.*, 2004). This phenomenon could not however explain the significant increase in the number of healthy *E. coli* in all samples of filtered OJ after one or two days incubation at 4 °C (Figure 4.11). The apparent temporary recovery and resuscitation of apparently sub-lethally injured cells in these cases could have been due to a combination of the better

acid stress response at 4 °C, lower degradation of ascorbic acid and lower production of browning products, allowing the recovery of injured cells. Cold shock-induced agglomeration and formation of biofilms could also have helped the recovery of injured cells. This issue was investigated in the current study the result of which has been discussed in the following chapter.

4.4 Conclusions and Future Work

This study demonstrated successful application of FCM for rapid investigation of the viability of *E. coli* in OJ. Furthermore, the presence of OJ particles did not have a significant effect on detection of *E. coli* within the food matrix. The most important limitation of this study however, was the detection limit of the flow cytometer used. Although the S/N in this study was within the typical ranges of the flow cytometer, however the detection of cells was only possible due to presence of very high concentration of cells in OJ.

In summary, the results presented here, confirm the hypothesis that clarification of OJ could result in significant increase in both the culturability and viability of *E. coli*. The most interesting finding of this study was that the filtration with 0.7 µm filter paper resulted in greater decrease in viability of *E. coli* strain possibly due to increased availability of hesperidin crystals. Further work is needed to investigate the culturability of the five distinct viable population of *E. coli* (i.e., GFP^+, low GFP, GFP^-, healthy BOX^-/PI^- and injured BOX^+/PI^-). This could be achieved with the help of a cell sorting FCM instrument which facilitates separation and collection of each population. The

collected cells can then be plated separately on appropriate selective and non-selective media to investigate their culturability. This also opens the opportunity for investigating the possible resuscitation and survival of each population in a model digestive system similar to the study conducted by Yuk *et al.,* (2008). These researchers showed that the inoculation of *E. coli* O157:H7 in OJ with or without pulp had little effect on their subsequent survival in simulated gastric fluid (pH 1.5). For instance while the survival of cells in SGF was significantly different ($p < 0.05$) for cells stored for 4 days in OJ with or without pulp, the overall survival of the cells after 4 hours incubation in SGF was similar regardless of the presence or absence of pulp in OJ.

It would be interesting to explore the role of each component of OJ cloud on its own, on the viability as well as culturability of *E. coli*. This will require the total separation of each type of particle (e.g., rag fragments, chromoplastids and hesperidin) and adding them back to an ultra-filtered cloud free OJ and MOJ. Further exploration of the role of each component, especially hesperidin on the viability and culturability of *E. coli* is also necessary. In this study, the rate of reduction in the number of healthy cells in cloud-free 0.22-FOJ was noticeably higher than those observed in cloud-containing samples regardless of the cloud content of OJ samples. Based on these results it was hypothesized that cloud particles could physically protect the cells from the acidic environment of the OJ. This hypothesis deserves to be investigated further by microscopic observation of the interaction between the cloud particles and the *E. coli* cells by the application of scanning electron microscopy (SEM) and/or transmission

electron microscopy (TEM). This should also make it possible to observe the possible physical damage of the *E. coli* cells by hesperidin particles. Investigating the extent of interaction between the OJ cloud particles with *E. coli* could be achieved by measuring the ζ-potential of the different types of cloud particles separately in order to confirm the hypothesis that there is a greater electromagnetic attractive force between *E. coli* and hesperidin than those of *E. coli* and larger cloud particles.

This study did not directly evaluate the possible role of hesperidin crystals as potential antimicrobial nanoparticles. However, if this hypothesis is proven to be correct, this could open new opportunities for food manufacturer for using hesperidin as a natural antimicrobial compound. Another implication of this study for manufacturers of OJ is that there is a definite need to take into consideration the effects of clarification on viability of OJ-associated food-borne pathogens such as *Salmonella* and *E. coli*. Clarification of freshly squeezed OJ with techniques such as ultrafiltration which removes the majority of cloud particles could potentially result in increased viability of these pathogens in OJ.

Chapter 5

The Effects of Orange Fruit Sanitation Stage on the Physiology of *E. coli* in Orange Juice

5.1. Introduction

According to FAO/WHO Codex Alimentarius CAC/RCP 1-1969 standard, in food safety a "Critical Control Point" or CCP is a "*step at which control can be applied and is essential to prevent or eliminate a food safety hazard or reduce it to an acceptable level*". Orange juice (OJ) production consists of many stages, each with a particular purpose (see Section 1.2.3). However, from the food safety point of view, the most important CCP during the production of freshly squeezed OJ is the sanitation step (Schmidt *et al.*, 1997). This is mainly because, subsequent to orange fruit sanitation, there is no further step such as pasteurization to eliminate the potential pathogens. Available chlorine is the most frequently used sanitizer in food industry for the purpose of washing the surface of the fruits, processing work surfaces and decontaminating the washing water. Available chlorine concentrations of 50 ppm to 200 ppm with a contact time of 1–2 min are commonly used for washing fresh produce including oranges (Parish *et al.*, 2003; Suslow, 2000). Numerous studies have investigated the effectiveness of available chlorine for eliminating *E. coli* from the surface of the orange fruits (Pao & Davis, 1999; Pao *et al.*, 2000; Bagci & Temiz, 2011; Martínez-González *et al.*, 2011). Nevertheless, these studies have

only focused on the culturability of the cells and not their viability. This is critical, considering that available chlorine concentration as low as 0.4 ppm can cause sub-lethal injury and induce a viable but non-culturable (VBNC) state in *E. coli* (Singh *et al.*, 1986; Kolling & Matthews, 2001). VBNC *E. coli* cells including chlorine-injured cells have also been shown to be capable of revival, growth and pathogenicity *in vivo* when the stress conditions are removed (Palmer *et al.*, 1984; Singh *et al.*, 1986). In addition to available chlorine, H_2O_2 and organic acids such as citric, lactic and acetic acids have also been suggested as suitable alternatives to available chlorine for sanitation of fresh produce (Parish *et al.*, 2003). However, H_2O_2, lactic acid and acetic acid are also known to induce VBNC state in *E. coli* (Oliver *et al.*, 2005; Li *et al.*, 2005).

The primary aim of this chapter is to investigate the effects of washing *E. coli* K-12 SCC1 with the aforementioned sanitizers on the viability and physiological state of the cells as well as their culturability, before and after their inoculation in OJ.

5.2. General Washing Protocol

In order to achieve this aim, GFP-generating *E. coli* K-12 SCC1 was used. Using this strain made it possible to study not only the viability and culturability of the cells, but also the sanitizer- and/or OJ-induced changes in the intracellular pH (pH_i) of the cells as previously described in Chapter 4. A single colony of *E. coli* was inoculated in 20 mL of 2×LB and allowed to grow in a shaker-incubator for 18 h at 37 °C with aeration (150 rpm). The overnight culture was

subsequently diluted 1:1000 in 50 mL of fresh 2×LB medium and grown for another 24 h to obtain late-stationary-phase culture. 2 mL of the culture was then transferred to a 2.5 mL centrifuge tube. Cells were harvested by centrifugation (5 min at 16,873 RCF), the supernatant was disposed and the pellet was dispersed in 50 µL of fresh PBS. Next, 1 mL of 30 °C sanitizer prepared 10 min prior to the experiment was added to the cell suspension and gently mixed for 2 min by rotating the tubes in a test tube rotator (20 rpm). 30 °C was chosen in an attempt to mimic the recommended temperature for a sanitizer during the washing stage of the fruits with core temperature of around 22.5 °C (room temperature). The positive temperature difference has been shown to prevent infiltration of pathogens into the fruit pulp (Zhuang *et al.*, 1995). After washing, cells were harvested by centrifugation, washed with PBS and dispersed in 1 mL of fresh PBS. Subsequently, 3×10^9 cells were added to 15 mL of OJ (filtered with 1.2 µm filter paper) in glass universal bottles. Samples were then incubated at 4 °C for 13 days with no shaking. At each time point, samples were diluted in PBS, stained with propidium iodide and analyzed with a flow cytometer. In addition, samples were plated on nutrient agar plates, which were incubated at 37 °C for 48 h in order to determine the number of culturable cells.

5.3. Results

5.3.1. Available Chlorine

[Figure 5.1] shows the effects of washing cells with double distilled water (ddH_2O with no available chlorine as the control) or solutions containing 50

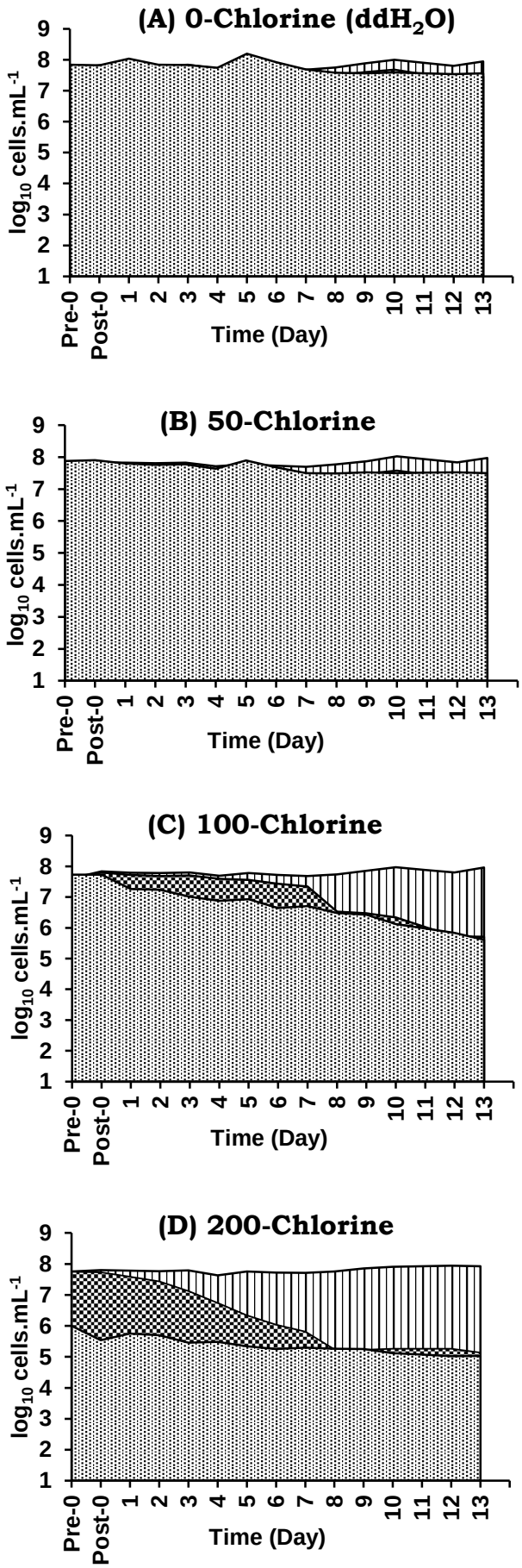


Figure 5.1: The effects of washing stationary phase *E. coli* K-12 SCC1 with different concentrations of chlorine on their viability and culturability in OJ, during 13 days incubation at 4 °C

2 mL of stationary phase *E. coli* were washed with 1 mL of **(A)** double distilled H_2O (0-Chlorine), **(B)** 50 ppm available chlorine (50-Chlorine), **(C)** 100 ppm available chlorine (100-Chlorine) or **(D)** 200 ppm available chlorine (200-Chlorine) for 2 minutes. Cells were harvested by centrifugation, washed with PBS and 3×10^9 cells dispersed in 15 mL of OJ (filtered with 1.2 µm filter paper). Samples were then incubated at 4 °C for 13 days. At each time point, samples were diluted in PBS, stained with propidium iodide and analyzed on the flow cytometer. Samples were also decimally diluted in MRD, plated on nutrient agar plates and incubated at 37 °C for 48 h. The experiment was repeated twice each with a duplicate. The reported values are the mean values of duplicate samples for a representative experiment.

Pre-0: Post-wash, pre-inoculation in OJ
Post-0: Post-wash, post-inoculation in OJ (Both day 0)

ppm, 100 ppm or 200 ppm available chlorine. The chlorine solutions were prepared from a solution of NaOCl containing 12.5% available chlorine. Henceforth, these conditions will be referred to as 0-Chlorine, 50-Chlorine, 100-Chlorine and 200-Chlorine respectively. As previously discussed in Chapter 1, the acid stress in *E. coli* can lead to a significant reduction in pH_i of the cells (Richard & Foster, 2004). In turn, the acidic environment in the cells adversely affects the protonation and conformational structure of the GFP, leading to reduction in FI of GFP^+ cells (Kneen *et al.*, 1998). As a result, throughout the following text, viable GFP^- cells are assumed to be acid stressed cells with pH_i of less than 5. Accordingly, the reduction in GFP fluorescence is presumed to be due to acid-induced conformational changes and denaturation of the GFP. On the other hand, GFP^+ cells are considered healthy cells with near neutral pH_i.

In case of the 0-Chlorine control samples [Figure 5.1(A)], at time 0 h pre-inoculation, more than 99% $\log_{10}$ of the cells were GFP^+ and were presumed to be healthy. However over 13 days of incubation at 4 °C, there was a steady decrease in the GFP^+ population and by day 13, 0.48 ± 0.06 $\log_{10}$ cells.mL^{-1} were found to be GFP^-. With regard to the culturable cells, there was no clear difference between the number of viable and culturable cells during the first week of incubation. However, from day 8, a significantly lower number of culturable cells were observed compared to those of viable cells ($p < 0.01$).

Compared to 0-Chlorine samples, washing cells with 50 ppm available chlorine (50-Chlorine, [Figure 5.1(B)]) had no statistically significant effect on the overall

number of viable, GFP^+ healthy or culturable cells throughout the experiment. On the other hand, washing cells with 100 ppm or 200 ppm of available chlorine [Figure 5.1(C) and (D) respectively] had a highly significant effect on not only the viability, but also the culturability of OJ-inoculated cells. In the case of the 100-Chlorine samples, the number of healthy cells during the first 7 days of the experiment was relatively similar to the levels observed in 0-Chlorine and 50-Chlorine samples. However, between day 7 and day 13, a highly significant decrease in the healthy population was observed compared to 0-Chlorine and 50-Chlorine samples (both $p < 0.0001$). This could suggest that although 100 ppm available chlorine had a greater adverse effect on the overall viability of the cells, up to day 7, cells were capable of tolerating and rectifying the expected available chlorine-induced damages to the cells. With regard to the culturability of the cells in 100-Chlorine samples, at time 0 h, no difference was observed between the number of culturable cells pre- and post-inoculation in OJ. However, during the next thirteen days of the experiment there was a highly significant decrease in culturability of the cells ($p < 0.0001$ compared to both 0-Chlorine and 50-Chlorine).

Unlike the other three conditions, for the case of cells washed with 200 ppm available chlorine (200-Chlorine) there was the highly significant reduction in culturability of the cells before their inoculation in OJ (time 0 h post-washing; $p < 0.0001$). The observed level of decrease in the culturability of *E. coli* (1.75 ± 0.06 $\log_{10}$ cells.mL^{-1}) was within the range of 1–2 $\log_{10}$ reduction reported in the literature for antimicrobial efficacy of 200 ppm available chlorine (Sapers, 2001;

Parish *et al.*, 2003). Inoculation of these cells in OJ and the subsequent incubation at 4 °C for thirteen days caused a further modest decrease of 0.51 ± 0.22 $\log_{10}$ CFU.mL^{-1} similar to the rate of reduction observed in 0-Chlorine and 50-Chlorine samples. Based on the results it could be suggested that 200 ppm available chlorine was more effective than 50 ppm or 100 ppm available chlorine in reducing the initial number of the culturable cells. Nonetheless, unlike the latter two conditions, washing the cells with 200 ppm available chlorine did not increase the rate of reduction in culturability of the cells post-inoculation in OJ. In other words, cells that remained culturable after the initial treatment with 200 ppm available chlorine were more successful in retaining their culturability than those washed with 100 ppm available chlorine, indicating a possible resistance in the OJ.

5.3.2. Hydrogen Peroxide

The method used for studying the effects of H_2O_2 on viability and culturability of *E. coli* K-12 SCC1 was similar to that described above for available chlorine; cells were washed with hydrogen peroxide (1%, 2.5% and 5% prepared from a 30% solution of H_2O_2). These concentrations of H_2O_2 were chosen due to their relevance to sanitation of fresh produce. Currently, 1% H_2O_2 is the only concentration of H_2O_2 approved for use in washing the surface of fresh produce (Sapers & Sites, 2003). Nevertheless, numerous studies have suggested that not only 1% H_2O_2, but also the 2.5% and 5% H_2O_2 could be used as suitable alternatives to available chlorine (Sapers *et al.*, 1999, 2002).

Culturability (Plate Counting)

As it could be seen from [Figure 5.2] there was an inverse dose-dependent reduction in both the culturability and viability of H_2O_2-washed cells inoculated in OJ during 13 days of incubation at 4 °C. With regard to the culturability results, at time 0 h, no significant difference was observed between the culturability of the cells washed with ddH_2O, 1% H_2O_2 or 2.5% H_2O_2, either before or after inoculation in OJ [Figure 5.2(A), 5.2(B) & 5.2(C)]. This indicated that H_2O_2 concentrations of up to 2.5% were no more effective than ddH_2O in reducing the initial number of culturable cells. Nevertheless, 5% H_2O_2 [Figure 5.2(D)] was found to be significantly more effective than 200 ppm available chlorine [Figure 5.1(D)] in reducing the culturability of the cells ($p < 0.01$). Moreover, compared to ddH_2O-washed cells, the post OJ-inoculation $\log_{10}$ reduction in population of culturable cells during the course of the study was significantly higher when cells were washed with 1% H_2O_2 ($p < 0.05$), 2.5% H_2O_2 ($p < 0.01$) and 5% H_2O_2 ($p < 0.05$) respectively. The apparent lower effectiveness of the 5% H_2O_2 compared to 2.5% H_2O_2 in reducing the culturability of OJ-inoculated cells could indicate a possible adaptation of the cells which survived the initial stress of 5% H_2O_2 pre-inoculation in OJ.

Viability (FCM Studies)

With regard to the viability, at time 0 h, no difference was observed between the number of GFP^+ cells pre- or post-inoculation in OJ, regardless of the sanitation regime used. However, compared to ddH_2O-washed cells, the post-inoculation reduction in GFP^+ population was found to be statistically significant in H_2O_2-

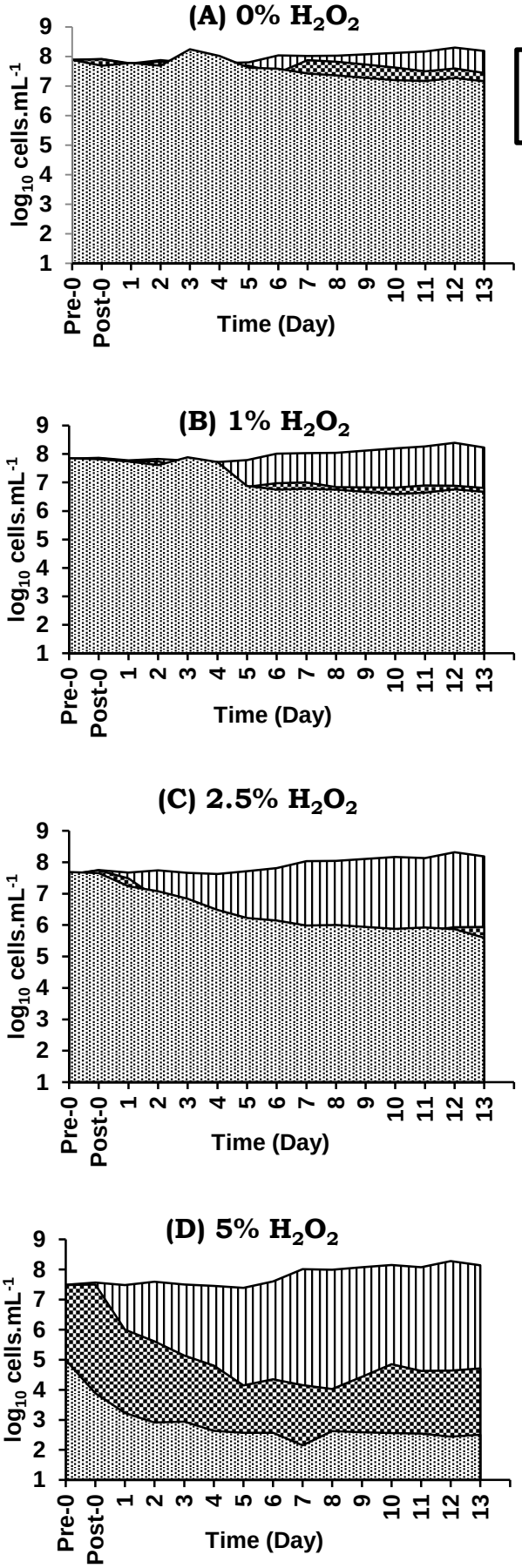


Figure 5.2: The effects of washing stationary phase *E. coli* K-12 SCC1 with different concentrations of H_2O_2 on their viability and culturability in OJ, during 13 days incubation at 4 °C

The method was similar to what was described in Figure 5.1; however, instead of using chlorine, cells were washed with **(A)** double distilled H_2O (0% H_2O_2), **(B)** 1% H_2O_2, **(C)** 2.5% H_2O_2 or **(D)** 5% H_2O_2 before inoculation in filtered OJ. The experiment was repeated twice each with a duplicate. The reported values are the mean values of duplicate samples for a representative experiment.

Pre-0: Post-wash, pre-inoculation in OJ
Post-0: Post-wash, post-inoculation in OJ (Both day 0)

washed cells. Moreover, compared to $\log_{10}$ reduction of ddH_2O-washed cells between day 0 post-inoculation and day 13, a significantly greater reduction was observed in case of cells washed with 1% ($p < 0.05$), 2.5% ($p < 0.01$) and 5% H_2O_2 ($p < 0.05$) respectively. With the exception of 5% H_2O_2 samples, there was a close agreement between the number of culturable and healthy GFP^+ cells as assessed by FCM. Therefore, it could be suggested that while the culturable cells are mainly consisted of GFP^+ cells, VBNC cells could be either GFP^+ (healthy) or GFP^- (stressed with $pH_i < 5$).

Increase in $\log_{10}$ Number of PI^- (Presumed Viable) Cells

An unexpected increase in the number of PI^- was observed in OJ samples regardless of the type of sanitizer used. However in case of H_2O_2-washed cells, the increase in H_2O_2 concentration of the washing solution from 1% to 5% corresponded with a significant increase in the number of PI^- cells after day 5 post-inoculation in OJ. Figure 5.3(A) shows the changes in the number of PI^- cells throughout the experiment, in OJ samples inoculated with available chlorine-washed cells. With the exception of 100-Chlorine samples, the $\log_{10}$ increase in the number of PI^- cells after day 7 was found to be statistically significant in other samples. Nevertheless, the rate of increase in the number of PI^- cells after day 7 in samples containing available chlorine-washed cells, was similar to that of the control samples containing ddH_2O-washed cells ($p > 0.05$). Therefore, it could be suggested that inoculation of stationary-phase *E. coli* in OJ led to a significant increase in the number of PI^-cells. However, this increase was OJ-induced and the pre-OJ inoculation washing of the cells with

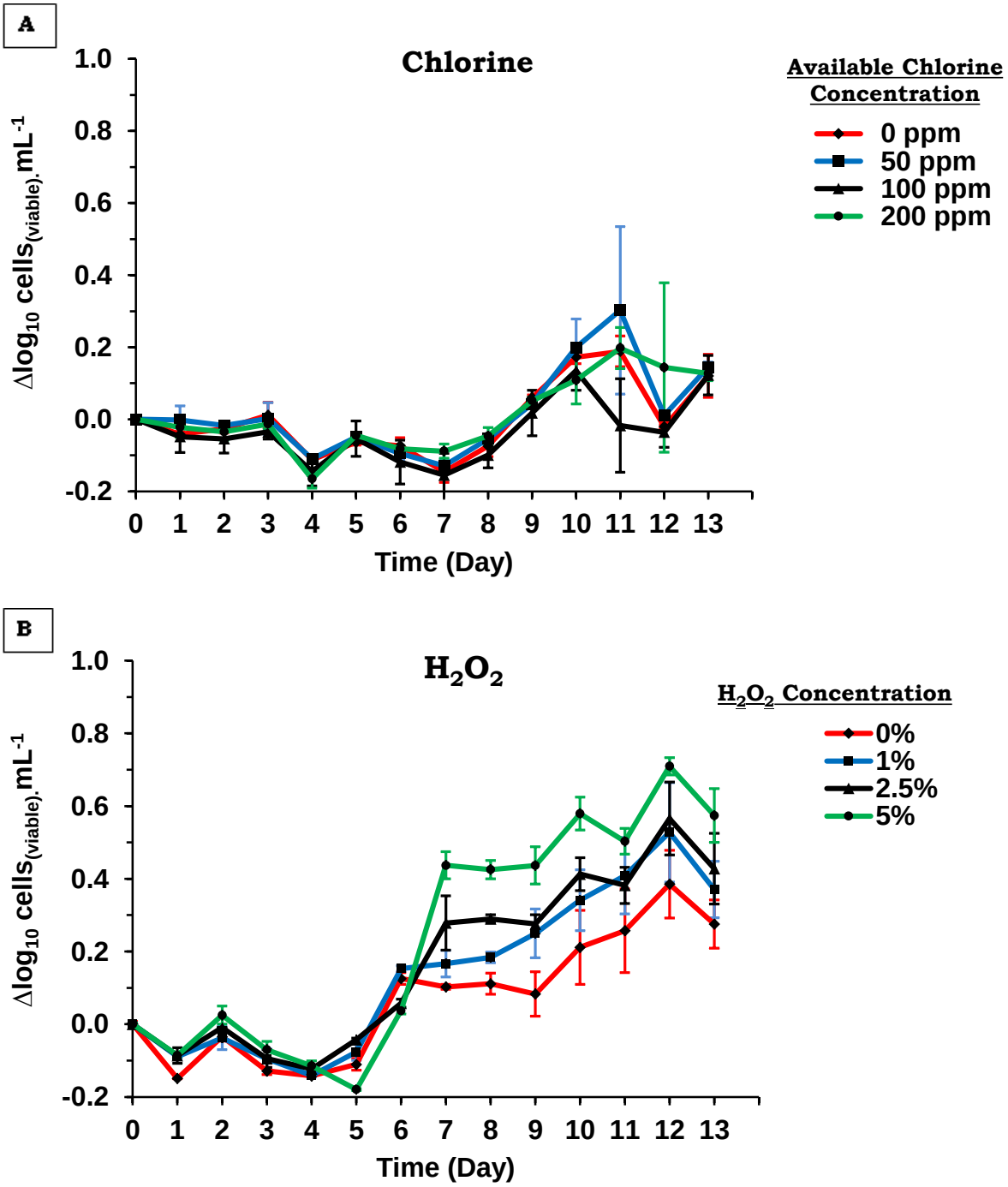


Figure 5.3: The effect of washing *E. coli* K-12 SCC1 cells with different concentrations of (A) NaOCl or (B) H_2O_2 on the total number of viable *E. coli* cells in OJ samples incubated at 4 °C.

The data shown in Figures **(A)** and **(B)** are the total number of GFP^+ and GFP^- viable cells already discussed in Figures 5.1 and 5.2 respectively. PI^- cells were considered to viable cells regardless of their GFP fluorescence (GFP^+ or GFP^-). The number of viable cells at each time was calculated by subtracting the $\log_{10}$ number of viable cells from that of time 0 h post-inoculation and subsequent calculation of the mean value. The experiment was repeated twice each with a duplicate. The reported values are the mean ± standard deviation values of duplicate samples obtained at each time point for a representative experiment.

50–200 ppm available chlorine did not play a role in the observed increase in the population of PI^- cells.

An increase in the total number of PI^- cells was also observed in the case of H_2O_2-washed cells, this time after day 5 post-inoculation (Figure 5.3(B). Nevertheless, from the data it was apparent that this increase was induced by not only the OJ but also the H_2O_2 in a dose-dependent manner. There was a positive correlation between the concentration of H_2O_2 used for washing the cells and the rate of increase in $\log_{10}$ number of PI^- cells between day 5 and day 13. During this time period, compared to ddH_2O-washed cells (control), the observed increase in $\log_{10}$ number of PI^- cells was significantly higher in OJ samples containing cells which had been washed with 1% H_2O_2 ($p < 0.05$), 2.5% H_2O_2 ($p < 0.05$) and 5% H_2O_2 ($p < 0.01$). In summary, inoculation of the cells in OJ and washing the cells with H_2O_2 –but not available chlorine are capable of increasing the number of PI^- cells (presumed viable).

Biofilm Formation

In order to investigate whether or not OJ and MOJ could induce the formation of *E. coli* biofilms, 3×10^9 cells of *E. coli* K-12 SCC1 were inoculated in 15 mL of either of PBS, OJ (filtered with 1.2 µm filter paper) or MOJ in universal bottles. Samples were incubated at 4 °C for 13 days after which the PBS, MOJ or OJ was disposed and the pellet at the bottom of the universal bottle was gently washed with ddH_2O. Subsequently the bottles were stained with a 0.1% solution of crystal violet for 15 min, washed with ddH_2O after which the crystal

violet was dissolved in 2 mL of pure ethanol for 1.5 h. The absorbance of the ethanol was then measured at 570 nm using a spectrophotometer (Favre-Bonté *et al.*, 2003). The absorbance values obtained for MOJ and OJ were significantly greater than those obtained for PBS samples [Table 5.1]. The results showed that OJ and MOJ could induce the biofilm formation in *E. coli*. The reason for the lower level of biofilm formation in OJ compared to MOJ is not clear, however it could be due to the presence of compounds such as fatty acids and sugary fatty acid esters in OJ. These compounds have been shown to be capable of negatively influencing the formation of biofilms in *E. coli* K-12 by reducing the expression of autoinducer-2 or AI-2 (Soni *et al.*, 2008; Furukawa *et al.*, 2010).

Therefore, it is possible that OJ-induced biofilm formation and/or cell aggregation in *E. coli* could have supported the growth of the cells during the first 5–7 days of their inoculation in OJ, resulting in a rapid significant increase in the number of viable cells.

5.3.3. Acidified and surfactant-containing Available Chlorine

When NaOCl is added to water, it dissociates to form the sodium (Na^+) and hypochlorite (OCl^-) ions. The latter then reacts with the hydrogen ions of the water, leading to the formation of hypochlorous acid (HOCl), the active sanitizing form of chlorine (McGlynn, 2004; Equation 5.1).

Equation 5.1:

Table 5.1: Studying the formation of *E. coli* biofilms in PBS, MOJ and OJ by crystal violet staining

Sample*	Absorbance (570 nm) (Absolute units, AU)
Control (No cells)	0.176 ± 0.007
PBS (pH 7.2)	0.274 ± 0.004
MOJ (pH 3.2)	0.631 ± 0.005
OJ (pH 3.2)	0.460 ± 0.022

*: n = 3

15 mL of PBS, MOJ or OJ filtered with 1.2 µm filter paper was transferred into a glass universal bottle and inoculated with 3×10^9 cells of late stationary (24 h culture) *E. coli* K-12 SCC1. Samples were stored at 4 °C for 13 days without aeration. An empty universal bottle was chosen as the control. On day 13, the PBS, MOJ or OJ was disposed, bottles were washed once with 3 mL of sterile ddH_2O, and were stained with 2 mL of 0.1% solution of crystal violet (Basic Violet 3 dissolved in ddH_2O). Samples were incubated at room temperature for 15 minutes after which the solution was disposed and the bottles were washed three times with ddH_2O. The stain was subsequently dissolved in 2 mL of 100% ethanol and its absorbance was measured after 1.5 h at 570 nm using a spectrophotometer. Error bars are the ± standard deviation of the mean value obtained for triplicate samples.

$$NaOCl + H_2O \rightarrow Na^+ + OCl^- + OH^- + H^+$$

$$OCl^- + H^+ + OH^- \leftrightarrow HOCl + OH^-$$

The concentration of HOCl in the available chlorine solution is primarily dependant on the pH. The greatest concentration of HOCl is present in chlorine solutions with pH values of between 4 and 6; however, chlorine at this pH is highly corrosive. At pH values of greater than 6, the equilibrium moves in the direction of conversion to OCl^- reducing the antimicrobial efficacy of the available chlorine. In contrast, in highly acidic solutions (pH < 4), it forms the highly toxic chlorine gas (Cl_2). Therefore, in order to maximize the concentration of HOCl in the chlorine solution without compromising the health and safety, the alkaline solution of available chlorine is generally acidified and maintained within the range of 6.0 and 7.5 (McGlynn, 2004). Moreover, it has been stated that the hydrophobic nature of the surface of orange fruit could reduce the effectiveness of the available chlorine (Martínez-González *et al.*, 2011). As a result, the application of non-ionic surfactants such as Tween-80 in combination with 200 ppm available chlorine has been suggested as a suitable way of increasing the efficacy of the available chlorine (Adams *et al.*, 1989).

The experiment was performed according to the washing protocol described above. However, for this part of the study, 200 ppm available chlorine (henceforth referred to simply as chlorine) was supplemented with 2% citric acid (acidified chlorine) or 100 ppm Tween-80 non-ionic surfactant (surfactant-containing chlorine). Henceforth, OJ samples containing cells washed with PBS,

chlorine, acidified chlorine and surfactant-containing chlorine will be referred to as PBS-OJ, C-OJ, AC-OJ and TC-OJ respectively.

Healthy GFP$^+$ Cells

[Figure 5.4(A)] shows the effects of supplementation of available chlorine solution with acid or surfactant on the $\log_{10}$ number of healthy GFP$^+$ cells pre- and post-inoculation in OJ. Up until day 1, washing *E. coli* cells with acidified chlorine resulted in a significantly lower $\log_{10}$ number of healthy cells in OJ samples (AC-OJ) compared to the other treatment conditions. However from this time onwards, compared to C-OJ and TC-OJ, the population of healthy cells in AC-OJ was significantly higher ($p < 0.05$). With regard to surfactant-containing chlorine, the mean $\log_{10}$ number of healthy cells in TC-OJ samples was lower than those found in C-OJ samples during the course of the study. However the difference between the two conditions was not found to be statistically significant. These results suggested that acidification or addition of surfactant to 200 ppm available chlorine sanitizing solution could increase the susceptibility of *E. coli* to OJ up until time 24 h post-inoculation. However, it appears the initial stress also improved the capability of the survivors to resist the OJ.

Viable Cells

Although compared to the PBS, washing cells with all available chlorine-containing sanitizers resulted in a significantly lower number of healthy cells in OJ, it did not appear to have an adverse effect on the $\log_{10}$ number of PI$^-$ cells

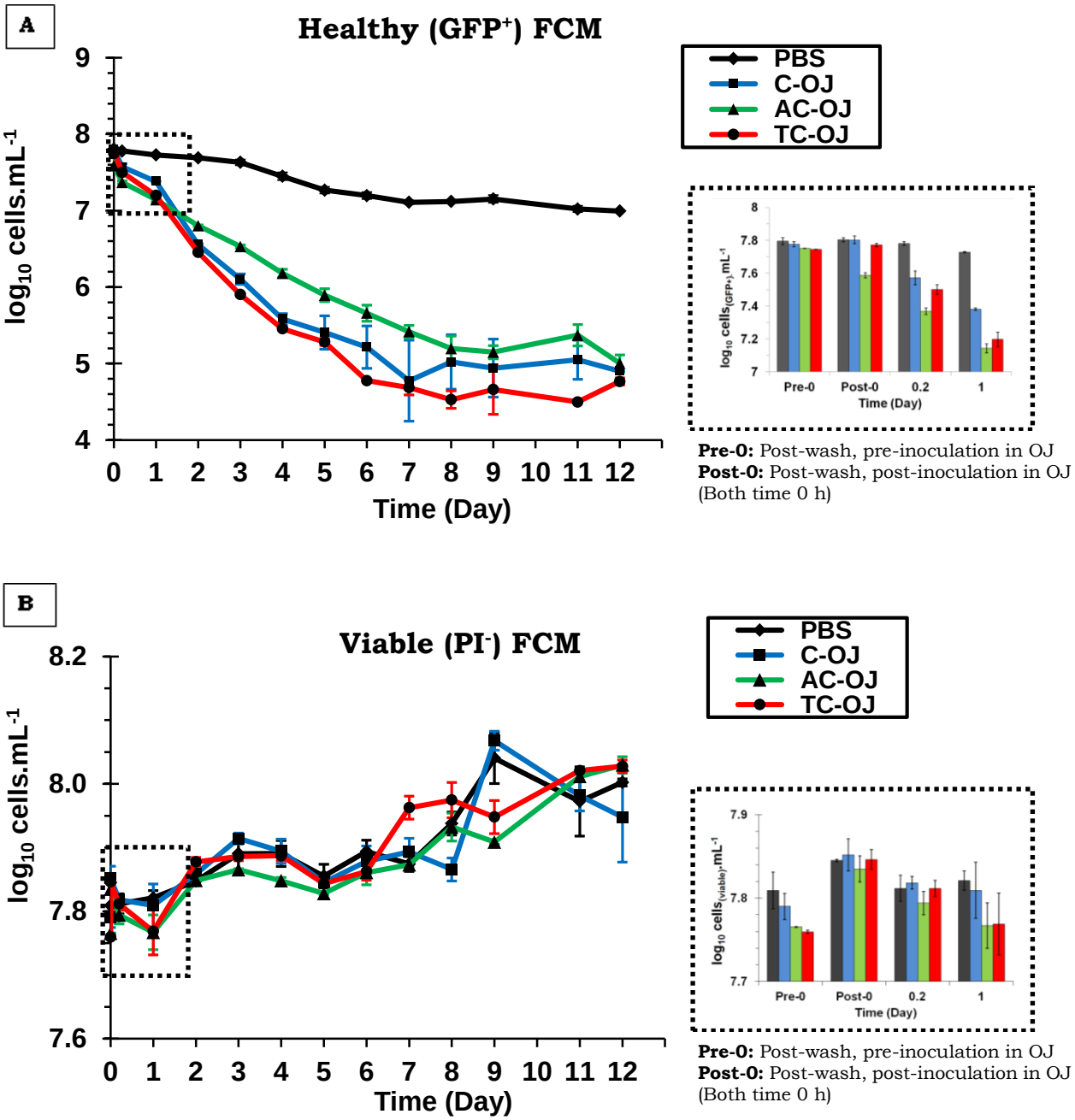


Figure 5.4: The effect of pre-inoculation washing of *E. coli* K-12 SCC1 with PBS, chlorine, acidified chlorine and surfactant-containing chlorine on its viability in OJ during 12 days incubation at 4 °C.

Graph **(A)** shows the total $\log_{10}$ cells.mL^{-1} of GFP$^+$/PI$^-$ (presumed healthy) *E. coli* cells in OJ while graph **(B)** illustrates the $\log_{10}$ cells.mL^{-1} change in the total number of viable cells (GFP$^+$/PI$^-$ and GFP$^-$/PI$^-$). The method used for this experiment was similar to what was described in Figure 5.1; however, in this case, cells were washed with either of PBS (control), 200 ppm available chlorine (C-OJ), 200 ppm available chlorine + 2% citric acid (AC-OJ) or 200 ppm available chlorine + 100 ppm Tween 80 (TC-OJ). The experiment was repeated twice and Reported values are the mean values of duplicate samples for a representative experiment. The experiment was repeated twice each with a duplicate. The reported values are the mean ± standard deviation values of duplicate samples obtained at each time point for a representative experiment.

(presumed to be viable) in the samples [Figure 5.4(B)]. On the contrary, during the course of the experiment, there was a 0.16–0.27 $\log_{10}$ cells.mL^{-1} increase in the number of PI$^-$ cells in OJ samples regardless of the treatment condition. The results are in agreement with the dose-dependent increase in the number of PI$^-$ *E. coli* cells washed with different concentration of H_2O_2. (See Figures 5.3(B)). Moreover, with the exception of the pre-inoculation washing stage of *E. coli* with different sanitizers, post-inoculation treatment was identical for all OJ samples. As a consequence, the results suggest that the observed increase in the number of PI$^-$ cells is due to an increase in the number of viable *E. coli* cells. Nonetheless, there is little supporting evidence in the literature for the growth of *E. coli* at 4 °C particularly in acidic conditions. For instance, Conner & Kotrola (1995) reported the growth of *E. coli* O157:H7 at 4 °C in tryptic soy broth supplemented with 3% and 4% sodium lactate but not in organic acid-supplemented media regardless of the pH (pH 4-7); however, they reported growth for samples which had been incubated at 25 °C. Walderhaug *et al.,* (1999) also reported the growth of *E. coli* subsequent to their infiltration in oranges when they were stored at 21 °C, but not at 4 °C. However, it is also important to note that in both studies the viability was based on culturability of the cells (growth of the cells on solid growth medium) and not necessarily the difference in the integrity of the cellular membrane as was the case in the current study. Therefore, further work is needed in order to elucidate the nature of these PI$^-$ cells (see section 5.5).

Culturable Cells

Compared to PBS, washing cells with chlorine, acidified chlorine or surfactant-containing chlorine solutions caused a significant reduction in the culturability of the cells pre-inoculation in OJ [Figure 5.5(A)]. Compared to the results previously reported in [Figure 5.1(D)] in the current experiment, the mean $\log_{10}$ number of culturable cells after being washed with 200 ppm available chlorine was 1.10 $\log_{10}$ lower. Nevertheless, in both cases, the level of reduction in culturability was within the range reported by Bagci & Temiz (2011). Upon inoculation of the cells in OJ, there was a marked decline in the culturability of the cells particularly in case of AC-OJ and TC-OJ samples. However, from day 1 onward the culturability of the cells in OJ samples remained relatively constant in all samples.

VBNC Cells

The number of VBNC cells were calculated by subtracting the $\log_{10}$ number of culturable cells from that of viable cell ([Figure 5.5(A)] and [Figure 5.4(B)] respectively). Since the population of viable cells remained relatively constant during the course of the study, the total number of VBNC cells (Figure 5.5(B)) closely mirrored the results of the culturability experiment.

Contrary to expectations, in the case of PBS-OJ samples, the number of culturable cells was greater than that of viable cells between day 2 and day 7. Individual colonies grown on the plates were confirmed to be *E. coli* by performing a Gram-stain of the colonies as well as streaking each colony on two *E. coli* selective media of MacConkey Agar (MAC) and Eosin-Methylene Blue

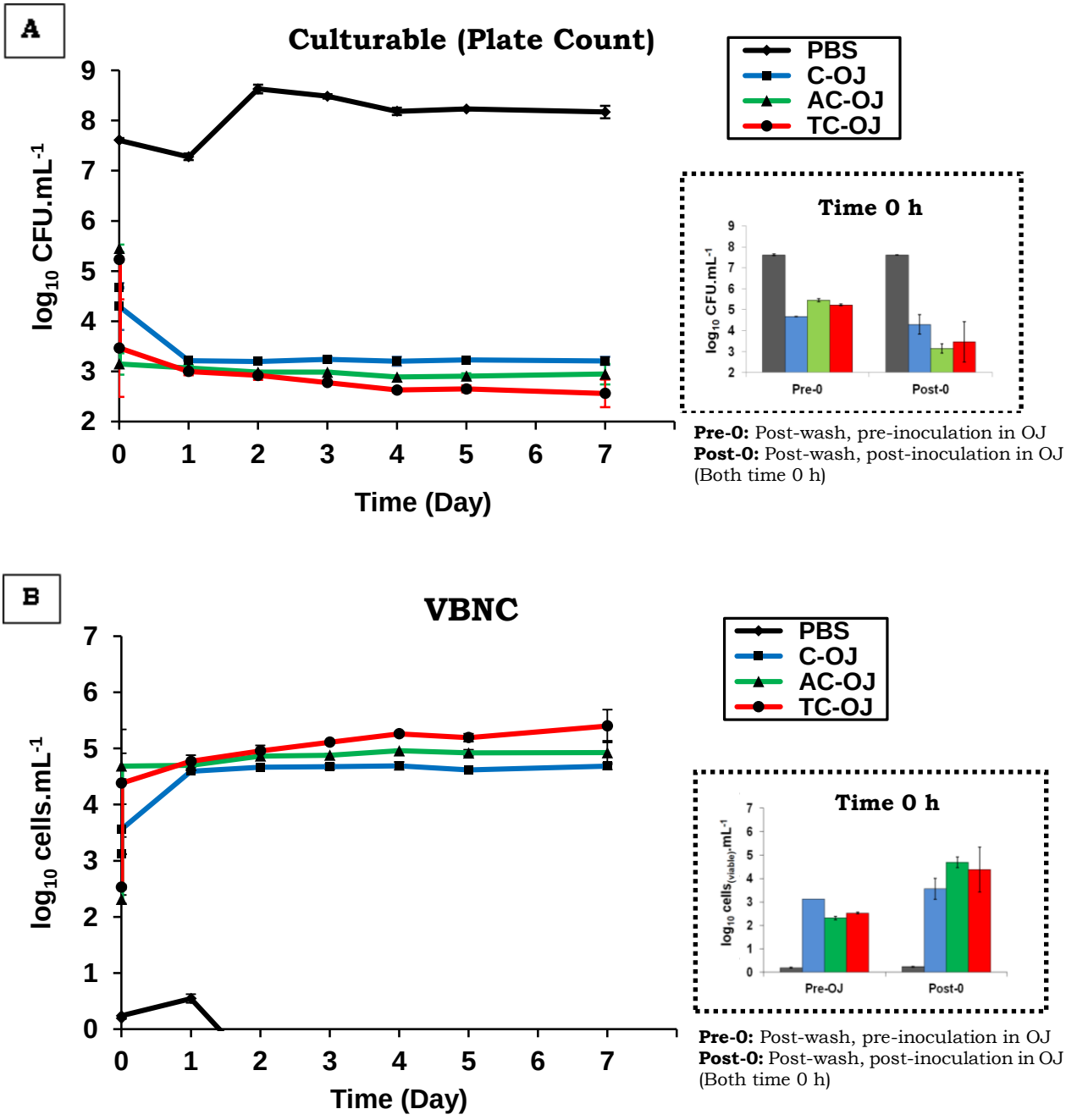


Figure 5.5: The effect of pre-inoculation washing of *E. coli* K-12 SCC1 with PBS, chlorine, acidified chlorine and surfactant-containing chlorine on its culturability during 12 days incubation of OJ samples at 4 °C.

Graph **(A)** and **(B)** show the total number of culturable and VBNC *E. coli* cells for samples already described in Figure 5.4. The number of culturable cells was calculated based on the number of colonies formed on nutrient agar plates after 48 h incubation at 37 °C. The number of VBNC cells was calculated by subtracting the number of culturable cells from the total number of viable cells shown in Figure 5.4(B). The experiment was repeated twice each with a duplicate. The reported values are the mean ± standard deviation values of duplicate samples obtained at each time point for a representative experiment.

Agar (EMBA) (data not shown). Since it is inconceivable to assume a culturable cell as non-viable, therefore the most likely reason for greater number of culturable cells in PBS-OJ samples was assumed to be the under-estimation of the viable cells by FCM. The main source of error is believed to be using a slightly higher threshold at the time of the experiment due to unusual excessive background electronic signal (20,000-25,000 on FSC-H instead of the typical 10,000-12,000 used for all the other experiments). Consequently, this could have led to exclusion of the very small viable cells from the defined gating region, which had been set based on the light scatter properties of the cells (i.e. FSC-A/SSC-A).

Relationship between FSC-A/SSC-A of the Cells and Their Viability

So far, in this section, focus has been made on the overall viability and/or culturability of the sanitizer-washed cells inoculated in OJ during long-term storage at 4 °C. However, the most significant change in viability of the cells occurred immediately after the inoculation of the cells in OJ. As it can be seen from [Figure 5.6(A)], at time 0 h, after washing the cells with PBS or available chlorine-containing sanitizers, the majority of the cells were GFP^+ and presumed to be healthy. Upon inoculation of the cells and particularly the acidified chlorine-washed cells in OJ, there was a significant increase in the percentage of GFP^- (presumed stressed; $p < 0.01$ compared to PBS-OJ). However, since cells were not stained with Bis-oxonol (BOX) which stains the cells with depolarized membrane, it was not possible to determine if the greater reduction in percentage of GFP^- cells was associated with greater number of

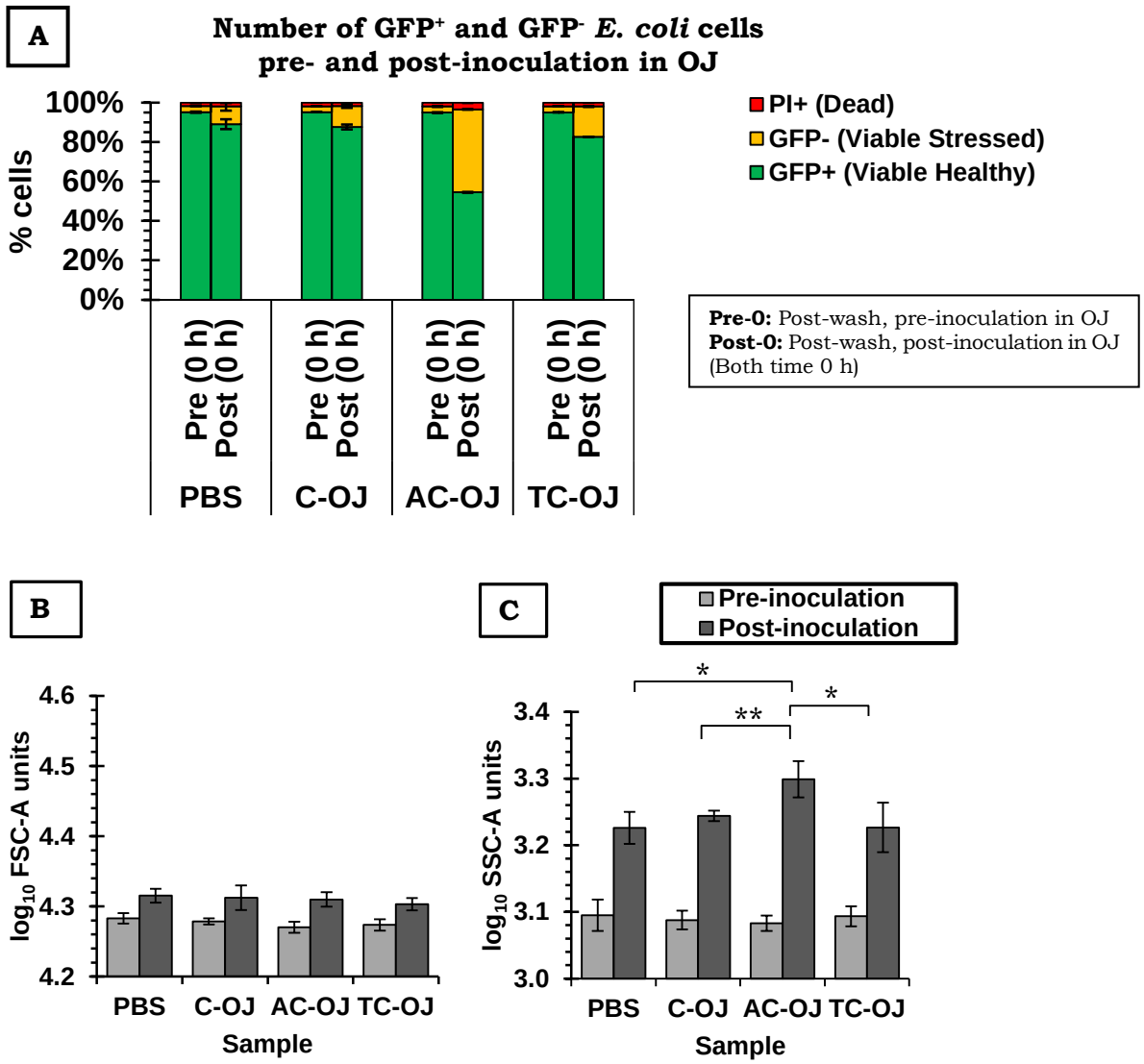
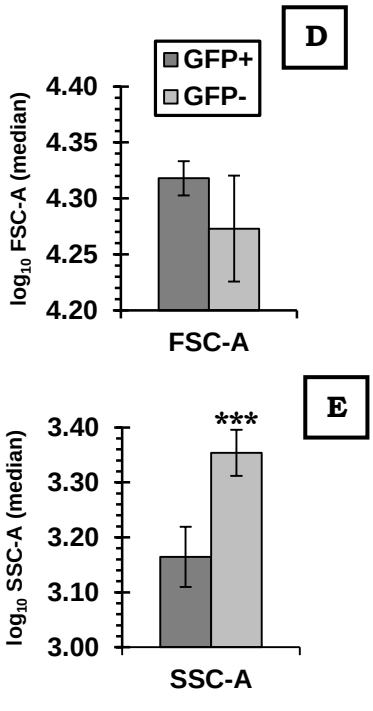


Figure 5.6: The effect of washing *E. coli* K-12 SCC1 with PBS, chlorine, acidified chlorine and surfactant-containing chlorine on its viability in OJ, pre- and post-inoculation in OJ (time 0 h).



(A) The figure shows the pre-inoculation and post-inoculation percentage of GFP+ and GFP- viable cells (PI-) washed with either of PBS (control), 200 ppm available chlorine (C-OJ), 200 ppm available chlorine + 2% citric acid (AC-OJ) or 200 ppm available chlorine + 100 ppm Tween 80 (TC-OJ) as described in Figure 5.4 before and immediately after their inoculation in OJ. The experiment was repeated twice each with a duplicate. The reported values are the mean ± standard deviation values of duplicate samples obtained at each time point for a representative experiment.

(B) and **(C)** respectively show the change in FSC-A and SSC-A of the cells (both GFP- and GFP+) washed with different the aforementioned sanitizers before and immediately after their inoculation in OJ. The reported values are the mean ± standard deviation values of all samples (n = 4) obtained at each time point. (*:$p < 0.05$; **: $p < 0.01$) **(D)** and **(E)** show the difference between the FSC-A and SSC-A of GFP+ and GFP- cells respectively (n = 8; ***: $p < 0.001$)

injured cells.

[Figure 5.6(B)] and [Figure 5.6(C)] show the change in FSC-A and SSC-A of the cells pre- and post-inoculation in OJ respectively. Compared to PBS-washed cells, washing the cells with available chlorine-based sanitizers had no clear effect on the median FSC-A of the cells both pre- and post-inoculation in OJ. On the other hand, a significantly greater increase in median SSC-A was observed in the case of the cells in AC-OJ samples compared to other samples. The results also showed that while there was no significant difference between the median FSC-A of GFP^+ and GFP^- cells [Figure 5.6(D)], the median SSC-A of the GFP^- cells was significantly greater than that of GFP^+ cells ($p < 0.001$; [Figure 5.6(E)]). As was previously shown in Chapter 3, there was a close negative correlation between the percentage of healthy cells and the median SSC-A of the cell population. Therefore, it is possible that the increase in median SSC-A of the cell population in AC-OJ samples was due to greater presence of injured GFP^- cells.

Real-time Analysis of the Available Chlorine-Based Samples

It is important to note that, this rapid change in GFP^+ population occurred during the first 12 seconds of its inoculation in OJ (i.e. during the dispersion of the cells in OJ at time 0 h post-inoculation). Therefore, in order to study the effects of available chlorine-based sanitizers on the viability of *E. coli* during this time period, it was decided to perform a FCM real-time study. In order to achieve this aim, 2×10^{10} late-stationary-phase cells were washed with either of

PBS (control) or 200 ppm available chlorine (with or without 2% citric acid or 100 ppm Tween-80) for 2 min. After washing and harvesting the cells, they were dispersed in PBS to achieve a final concentration of 2×10^7 cells.mL^{-1}. From this suspension, 100 µL was placed in a 3.5 mL polypropylene (PP) test tube and analyzed on the flow cytometer for 45 seconds, before adding 900 µL of OJ (filtered with 1.2 µm filter paper). The overall change in fluorescence intensity (FI) of the cells (Figure 5.7) was studied for a total of 30 min. Reduction in GFP FI was assumed to be due to reduction in pH_i of the cells leading to protonation and conformational changes in GFP and the generation of stressed GFP$^-$ (pH_i < 5) cells (Kneen *et al.*, 1998).

[Figure 5.7(A)] shows the density plots of the change in green FI of the cells (FL1-A) during the first 20 minutes of the study while [Figure 5.7(B)] shows the percentage change in median $\log_{10}$ FL1-A of the GFP$^+$ cells up to time 30 min post-inoculation. With regard to the PBS-washed cells (PBS-OJ), addition of OJ led to 0.39 $\log_{10}$ reduction in the median FL1-A of GFP$^+$ cells by time 2 min post-inoculation. Nevertheless, after the initial reduction, the median FI values for GFP$^+$ cells remained relatively constant throughout the experiment. A significantly greater rate of decrease in $\log_{10}$ median FL1-A was observed in the case of cells washed with 200 ppm available chlorine (with or without acid or surfactant) following their inoculation in OJ. Supplementation of the available chlorine solution with Tween-80 did not affect the FI of the GFP$^+$ cells in OJ compared to cells washed with non-supplemented available chlorine. On the other hand, acidification of the latter with 2% citric acid increased not only the

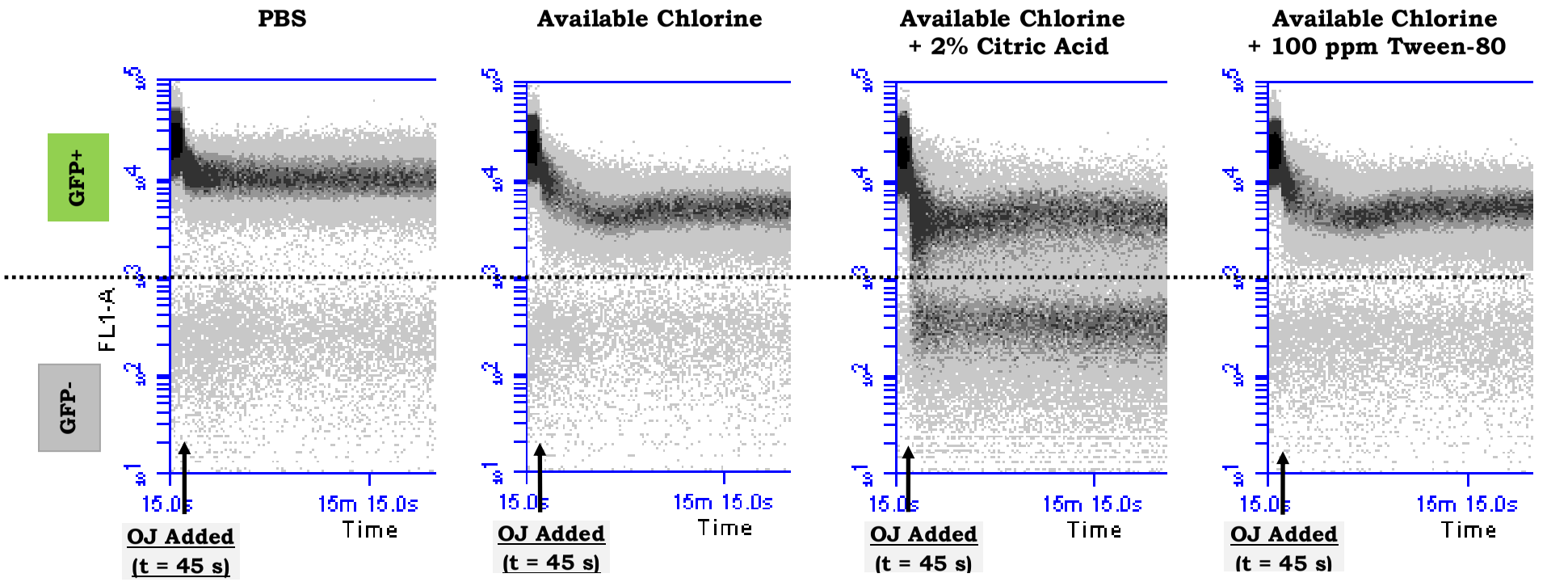
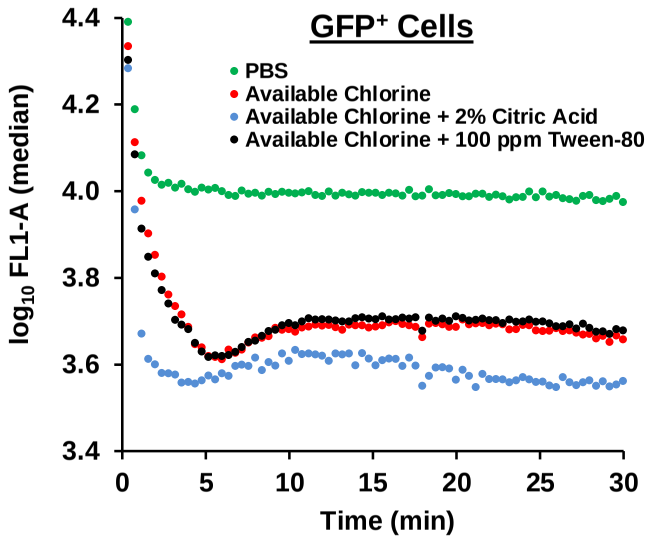


Figure 5.7: The real-time study of the effect of available chlorine-based sanitizers on the GFP fluorescence (FL1-A) of *E. coli* K-12 SCC1 up to time 30 min post-inoculation in OJ.

(A) 2×10^{10} stationary-phase cells were washed with 1 mL of PBS (control), 200 ppm NaOCl, 200 ppm NaOCl + 2% citric acid or 200 ppm NaOCl + 100 ppm Tween 80 for 2 minutes. Cells were harvested by centrifugation, washed with PBS and diluted in fresh PBS to achieve a cell suspension with concentration of 2×10^{7} cells.mL^{-1}. 100 µL of the resultant cell suspension was placed in a tube and analyzed on the flow cytometer for 30 seconds (data collection started from time 15 seconds). At 45 seconds, 900 µL of OJ filtered with 1.2 µm filter paper was added to the cell suspension (indicated by arrow below the plot), gently mixed for 15 seconds and analyzed for another 29 minutes. Here, only the data for the first 20 min of the experiment has been shown.



(B) The median FI of the GFP⁺ cells shown in the density plots above from time 0–30 min (post-inoculation in OJ). Dot represents the median FI of the GFP⁺ cells at 25 seconds intervals.

scale but also the rate of reduction in FI of the GFP^+ cells in OJ.

Unlike PBS-washed cells, the initial reduction in FI of the GFP^+ cells washed with 200 ppm available chlorine (regardless of the supplementation with acid or surfactant) was followed by an increase in FI values up to time 10 min post-inoculation. However, in the case of acidified chlorine-washed GFP^+ cells, the increase was followed by a gradual decrease in FI of the cells during the remaining course of the study. Therefore, it could be hypothesized that washing the cells with available chlorine-based sanitizers led to a greater susceptibility of the cells to OJ and therefore, causing a significant reduction in pH_i of the cells. Consequently, this could have induced a stronger acid stress response in GFP^+ cells leading to an increase in pH_i of the cells, hence their FI. However, in the case of cells washed with acidified available chlorine, the treatment could have adversely affected the homeostasis of the cells. The results of the real-time study was in agreement with the results of time-point study [Figure 5.4(A)] which showed a greater reduction in the percentage of presumed healthy GFP^+ cells in AC-OJ samples.

In summary, based on the finding of the real-time study it could be suggested that washing the *E. coli* cells with available chlorine-based sanitizers, primarily with acidified chlorine adversely affects the pH homeostasis of the cells. Consequently, the lower pH_i of the chlorine-treated cells leads to mis-folding and changes in the conformational structure of the GFP. This subsequently causes a decrease in the FI of the cells as well as an increase in their size and

granularity.

Considering the relationship between the SSC-A of the cells and their granularity, the low pH_i-induced mis-folding of GFP and the possible formation of inclusion bodies (Shapiro, 2003; Lewis *et al.*, 2004) could explain the observed significant increase in the median SSC-A of the GFP^- cells.

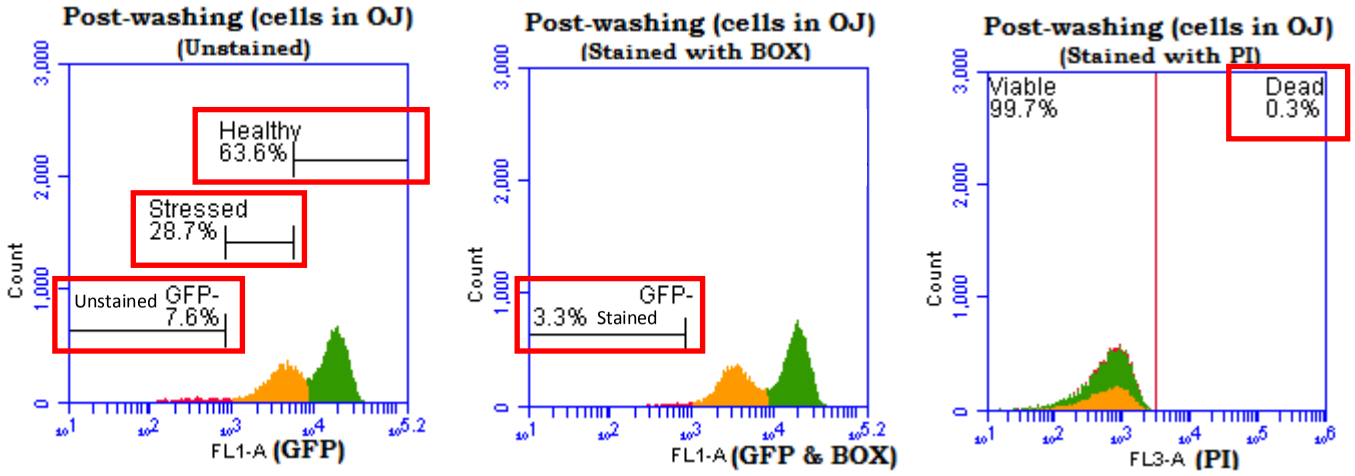
5.3.4. Organic Acids (Citric, Lactic and Acetic)

As was shown above, washing the cells with acidified chlorine had a significant effect on the subsequent stress response of the cells in OJ. However, these results raised the question of whether this effect was due to the combined antimicrobial effects of the citric acid and chlorine or simply due to a lowering the pH of the alkaline chlorine solution. The antimicrobial activity of organic acid such as citric acid, lactic acid and acetic acid against various food pathogens and also their effectiveness in produce sanitation has been extensively studied (reviewed by Parish *et al.*, 2003). It has even been suggested that organic acids such as 2% or 4% lactic acid are more effective sanitizers than both the acidified and alkaline 200 ppm available chlorine for the purpose of washing the surface of fresh produce including orange fruits (Martínez-González *et al.*, 2011). However, the primary aim of these studies was to investigate the efficacy of these sanitizers in reducing the initial number of the cells on the surface of the fruits and not the subsequent survival of sanitizer-treated cells in OJ. Moreover, these studies have generally focused solely on the culturability of the cells and not their overall viability. Therefore, it was decided

to investigate the effects of 2% or 4% citric acid (2CA and 4CA respectively), 4% lactic acid (4LA) and 4% acetic acid (4AA) on both the viability and culturability of the cell pre- and post-inoculation in OJ. The results were compared to acidified chlorine-washed cells.

Physiology of GFP⁻/PI⁻ Cells

In addition, the physiology of the viable GFP^-/PI^- cells was further investigated by the use of bis-oxonol (BOX) which enters cells with a collapsed membrane potential [Figure 5.8]. As GFP and BOX both emit green fluorescence, exploratory experiments were performed. Addition of BOX did not affect the green FI of GFP^+ cells. *E. coli* K-12 SCC1 was treated with 200 ppm available chlorine then inoculated into OJ as described above. FCM of unstained cells revealed three populations on green fluorescence histograms similar to those observed in [Figure 5.7]: A highly fluorescent population, thought to be healthy cells; a medium-fluorescence population, thought to be stressed cells; and a GFP^- population thought to comprise stressed and injured cells. In addition to BOX, GFP^- cells with a collapsed membrane potential became fluorescent; the low fluorescence population decreased from 7.6% to 3.3% indicating that 4.4% of total cells were GFP^- and had no membrane potential. Finally PI was added, which stained 0.3% of cells red; this is the dead population. By subtracting the percentage dead cells from the total percentage of GFP^- cells with no membrane potential it was possible to determine the percentage of injured cells (4.1%).



Presumed Viability State	Population
Healthy (Viable GFP^+)	GFP+ unstained cells in OJ
Stressed (Viable with Medium GFP)	Low GFP unstained cells in OJ
GFP^- (Viable GFP^-/BOX^-)	GFP^- BOX-stained cells in OJ
Injured (Viable BOX^+/PI^-)	[(Unstained GFP^- – Stained GFP^-) – Dead]
Dead (BOX^+/PI^-)	Dead

Figure 5.8: Simultaneous use of two green fluorophores of GFP and bis-oxonol (BOX) along with red fluorescent viability dye of propidium iodide (PI) in order to study the number of healthy, stressed, injured and dead *E. coli* K-12 SCC1 cells inoculated in OJ.

The histograms show the change in green (FL1-A) or red (FL3-A) fluorescence of cells before and after inoculation in OJ. The method of experiment was similar to what was described in Figure 5.1, with the difference of using BOX in addition to PI. **(A)** The GFP fluorescence of cell washed with 200 ppm pre-inoculation in OJ. **(B)** Inoculation of cells in OJ resulted in an increase in the number of low GFP and/or GFP^- cells. Reduction in GFP fluorescence was presumed to be due to change in internal pH and subsequent denaturation of GFP. Therefore, GFP^+ and low GFP cells were considered to be healthy and stressed cells respectively. **(C)** Cells were also stained with BOX in order to determine the percentage of injured and/or dead cells. Addition of BOX caused a reduction in the number of GFP^- cells (compared to histogram B), indicating the staining of GFP^- cells with BOX. **(D)** Staining the cells with PI, on the other hand showed the percentage of dead cells. Consequently, it was possible to calculate the percentage of injured cells by subtracting the number of dead cells (histogram D) and viable stressed GFP^- cells (histogram C) from pre- BOX staining percentage of GFP^- cells.

VIABILITY

Compared to acidified chlorine solutions, washing the cells with organic acids had a significantly greater adverse effects on the viability of *E. coli* K-12 SSC-1 cells at time 0 h pre-inoculation [Figure 5.9]. Washing the cells with acidified chlorine had no significant effect on the percentage of healthy cells and therefore more than 98% of the cells were healthy [Figure 5.9(A)]. In contrast, the cells washed with 2CA, 4CA, 4LA and 4AA had far lower healthy populations [Figure 5.9(B), (C), (D) & (E) respectively] ($p < 0.0001$ for all). While only 0.02% of the acidified chlorine-washed cells were injured (BOX^+/PI^-), a far higher number of cells washed with 2CA, 4CA, 4LA and 4AA were in this populations. It could be suggested that washing the cells with 2% or 4% acidic solutions, particularly the 4AA, led to a significant decrease in pH_i of the cells ($pH_i < 5$) as well as cell injury. Subsequent inoculation of the acidified chlorine-washed cells into OJ led to a significant reduction in the number of healthy cells during the first 24 h of the experiment, followed by a significant increase at time 48 h ($p < 0.05$). These results were similar to those of the previous study [Figure 5.4(A)] which pointed to a possible role of acidified chlorine in inducing cross-protection of *E. coli* to acidic condition of OJ.

With regard to the organic acid-washed cells, 4AA-washed cells exhibited the least resistance to acidic condition of OJ immediately after their inoculation in OJ (37% increase in injured population). On the other hand, in the case of 4LA washed-cells no change in overall viability of the cells was observed upon their inoculation in OJ (time Pre-0). Doubling the concentration of citric acid from 2%

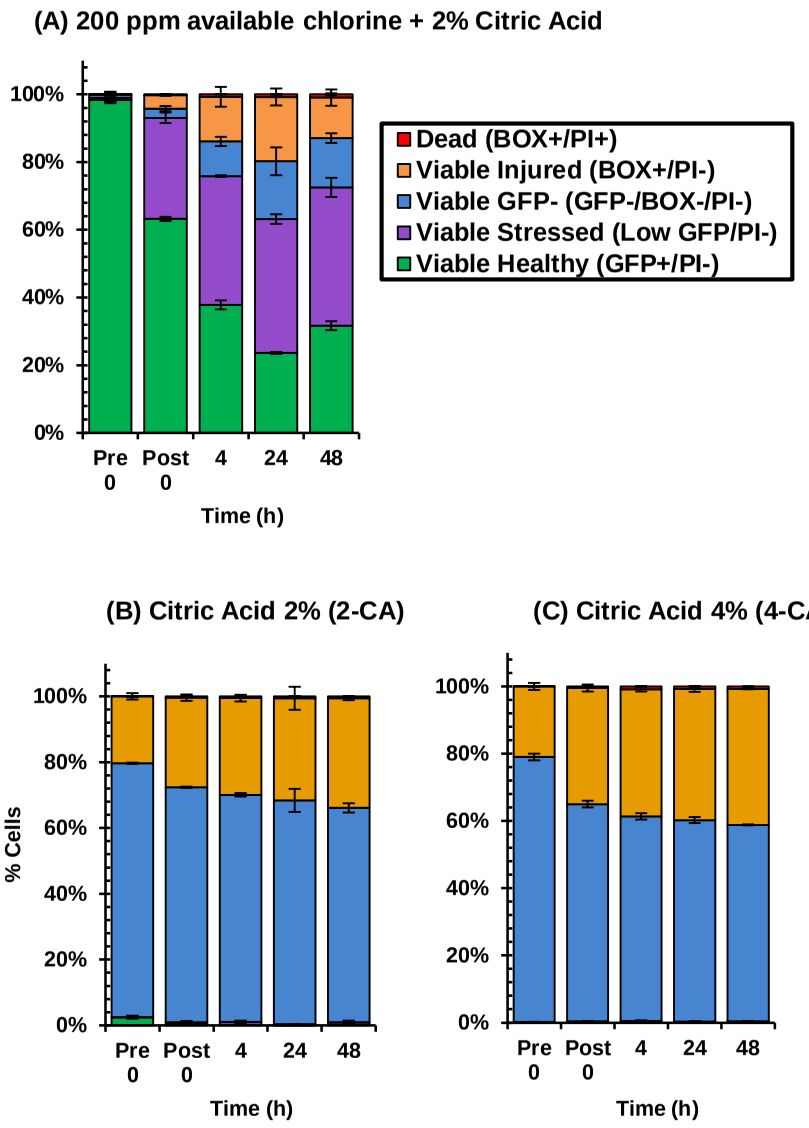


Figure 5.9: The effects of washing *E. coli* K-12 SCC1 with organic acid on its subsequent viability in OJ during 48 h incubation at 4 °C compared to acidified chlorine-washed cells

The method used for this experiment was similar to what was described in Figure 5.1; however, in this case, cells were washed with either of **(A)** 2% citric acid (pH 2.1) **(B)** 4% citric acid (pH 1.9), **(C)** 4% lactic acid (pH 2.1), **(D)** 4% acetic acid (pH 2.5) or **(E)** acidified chlorine (200 ppm NaOCl with 2% citric acid, pH 6.0). Samples were incubated at 4 °C for 2 days. "Pre 0" and "Post 0" refer to the pre-inoculation and post-inoculation in OJ at time 0 h.

Error bars are the ± standard deviation of the mean value obtained at each time point for each type of population (e.g., healthy GFP$^+$, injured, etc.) and not the total percentage of the cells.

to 4%, resulted in 2-fold increase in the rate of increase in the percentage of injured cells. By time 48 h, the overall viability in OJ was similar for cells washed with lactic acid or citric acid (regardless of the concentration). Nevertheless, although organic acids appeared to exert a greater adverse effect on viability of the cells pre- and post-inoculation in OJ, the total percentage of viable cells (PI^-) remained relatively constant during the course of the study, similar to that observed for acidified chlorine-washed cells.

CULTURABILITY

The effects of washing the cells with organic acids on the culturability of the cells were also investigated and the results were compared to that of acidified chlorine-washed cells pre and post-inoculation in OJ. Regardless of the type of organic acid used, washing the cells with organic acids had a significantly greater adverse effect on the culturability of the cells, compared to that of acidified chlorine-washed cells [Figure 5.10(A)]. In comparison to the cells washed with acidified chlorine, the $\log_{10}$ reductions in culturability of the cells at time 0 h post-washing was significantly higher for cells washed with 2CA, 4CA (both $p < 0.05$), 4LA and 4AA (both $p < 0.01$). 4AA was found to be more effective than other sanitizers in reducing the culturability of *E. coli* subsequent to its inoculation in OJ. Compared to acidified chlorine-washed cells, only 4AA-washed cells had a significantly lower culturability in OJ at time 0h and 48 h post-inoculation. Moreover, in agreement with the FCM viability results, 4LA was the least effective sanitizer in reducing the culturability of the cells post-inoculation in OJ.

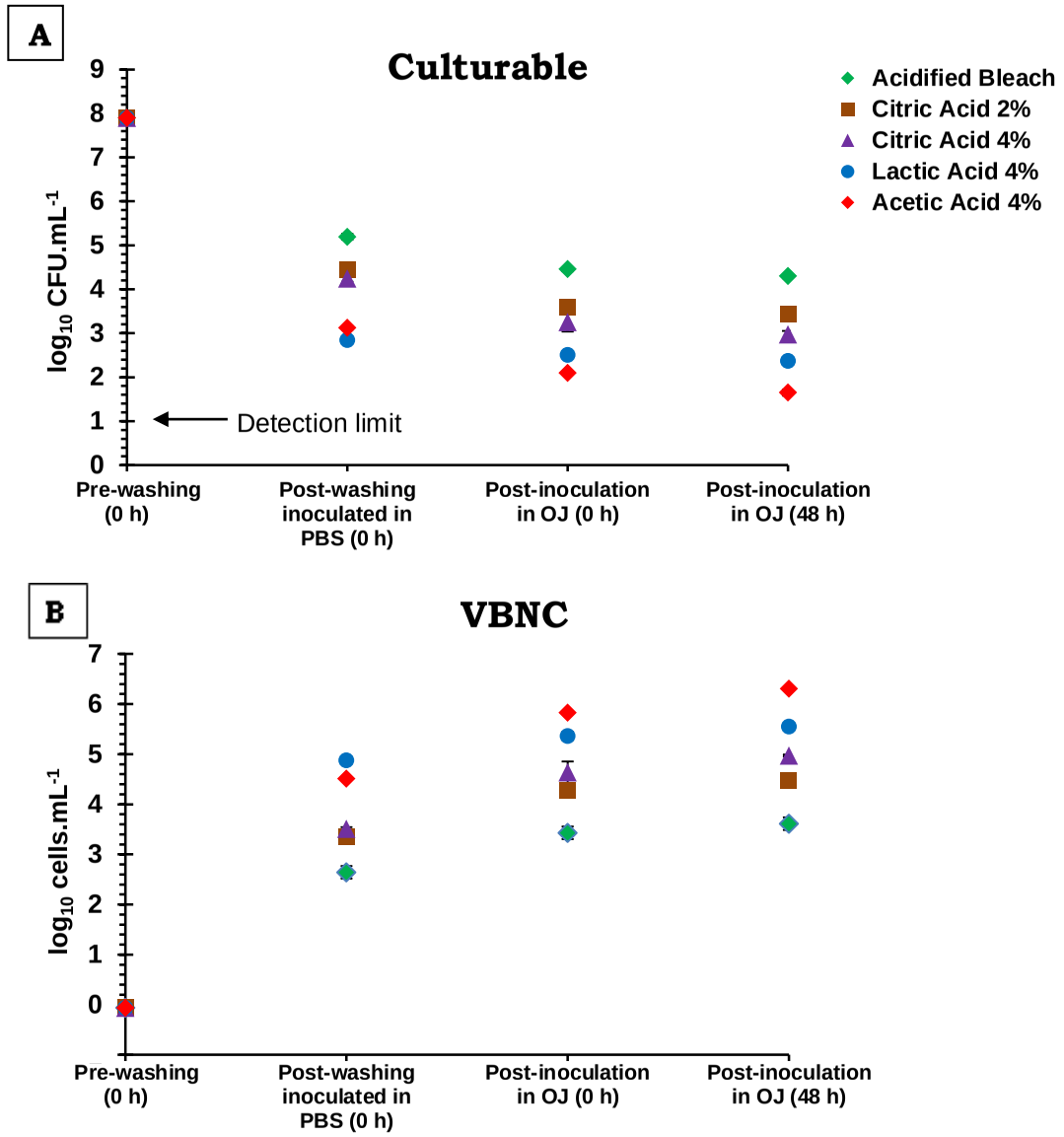


Figure 5.10: The effects of washing *E. coli* K-12 SCC1 with organic acid on its subsequent viability in OJ during 48 h incubation at 4 °C compared to available chlorine-washed cells.

(A) The number of culturable cells was determined at various time points, by decimal serial dilution of the samples in maximum recovery diluent (MRD) and plating 100 µL of the appropriate dilution (or neat samples) on nutrient agar plates. Plates were incubated at 37 °C for 48 h. Error bars are the ± standard deviation of the mean value obtained at each time point.

(B) The number of VBNC cells was calculated by subtracting the $\log_{10}$ CFU.mL^{-1} of culturable cells from the $\log_{10}$ number of viable cells. The latter itself was calculated by first subtracting the percentage of dead cells (Figure 5.9) from 100 and multiplying this number by the dilution rate of the sample analyzed with FCM. Error bars are the ± standard deviation of the mean value obtained at each time point.

VBNC Cells

With regard to the number of VBNC cells [Figure 5.10(B)], the results closely resembled that of the culturability results, mainly because the overall number of viable cells as determined by FCM remained relatively constant throughout the experiment for all samples (7.05 ± 0.08 $\log_{10}$ cells.mL^{-1}). At time 0 h post-washing (pre-inoculation in OJ), compared to acidified chlorine, the increase in VBNC population in case of cells washed with 2CA, 4CA, 4LA and 4AA was significantly higher ($p < 0.01$ for 2CA and 4CA; $p < 0.001$ for 4AA and 4LA). Moreover, 4AA was the only sanitizer capable generating a greater $\log_{10}$ number of VBNC *E. coli* cells than acidified chlorine between time 0 h and 48 h post-inoculation in OJ ($p < 0.05$ compared to time 0 h post-inoculation).

Effects of pH Change on Antimicrobial Efficacy of Acetic Acid

In general, it could be suggested that compared to acidified chlorine, using organic acids and reduction in the pH of the washing solution appeared to exert a significantly greater adverse effect on both the viability and culturability of the cells. However, the greater antimicrobial efficacy of the organic acids was not thought to be solely due to their low pH. For instance, despite possessing the highest pH among the organic acid solutions used in this study, 4AA (pH 2.5) was shown to be a more effective antimicrobial against *E. coli* than 2CA, 4LA (both with pH 2.1) and 4CA (pH 1.9). This was assumed to be due to different antimicrobial mechanism of acetic acid compared to citric acid and lactic acid.

Undissociated acetic acid is capable of diffusing through the membrane and causing intracellular damage. On the other hand citric and lactic acids exert antimicrobial effect by acting as acidulant reducing the pH of the environment (Stratford & Eklund, 2003).

Therefore, it was decided to investigate the role of pH (hence the change in the percentage of undissociated acid) of 4% acetic acid solution on post-inoculation viability of *E. coli* in OJ. For this experiment, pH-adjusted 4% acetic acid solutions (pH 3, 4, 5, 6 or 7 adjusted with 1 M NaOH) as well as unadjusted 4% solution (pH 2.5) and PBS (pH 7.2) were used for washing the cells. Cells were subsequently inoculated in OJ and their viability (time 0 h) was studied by staining the cells with BOX and PI viability dyes and analyzing the samples with a flow cytometer as described above.

As can be seen from [Figure 5.11(A)] increasing the pH of 4% acetic acid washing solution from 2.5 to pH of 7 (NaOH pH-adjusted), caused a gradual reduction in the percentage of injured *E. coli* cells. There was also a negative correlation (R^2 = 0.86) between the percentage of non-BOX^+ cells (sum of percentage healthy, stressed and GFP^- cells) and the percentage of undissociated acetic acid in the solution [Figure 5.11(B)]. Similar FCM viability results were observed for OJ samples which had been inoculated with NaOH pH-adjusted acetic acid solutions of pH 6 or pH 7. This was assumed to be due to the very low percentage of undissociated acids present in the pH-adjusted acetic acid solutions of pH 6 and 7 (4% and 0.4% undissociated acid

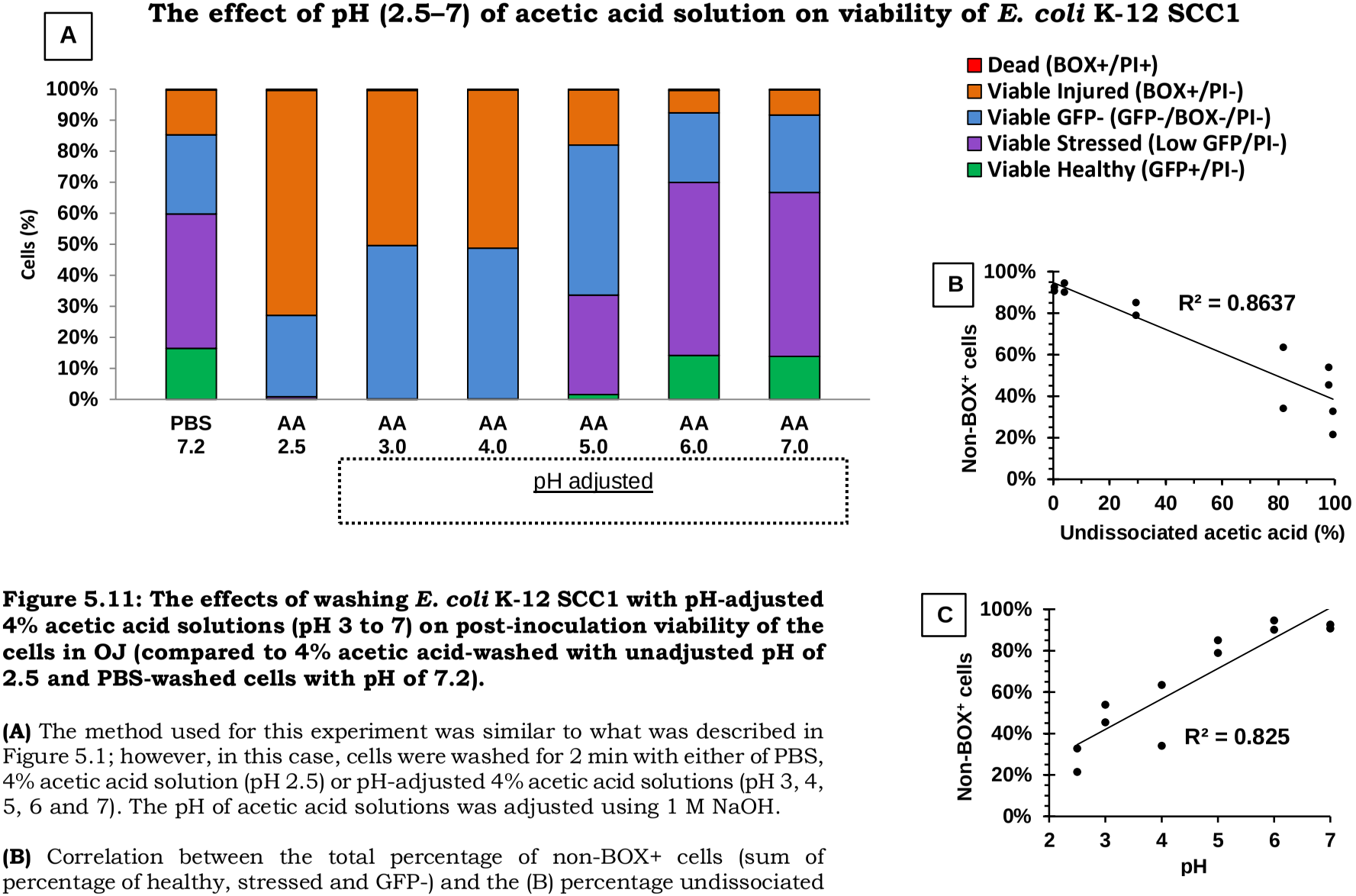


Figure 5.11: The effects of washing *E. coli* K-12 SCC1 with pH-adjusted 4% acetic acid solutions (pH 3 to 7) on post-inoculation viability of the cells in OJ (compared to 4% acetic acid-washed with unadjusted pH of 2.5 and PBS-washed cells with pH of 7.2).

(A) The method used for this experiment was similar to what was described in Figure 5.1; however, in this case, cells were washed for 2 min with either of PBS, 4% acetic acid solution (pH 2.5) or pH-adjusted 4% acetic acid solutions (pH 3, 4, 5, 6 and 7). The pH of acetic acid solutions was adjusted using 1 M NaOH.

(B) Correlation between the total percentage of non-BOX+ cells (sum of percentage of healthy, stressed and GFP-) and the (B) percentage undissociated acetic acid and **(C)** pH of NaOH-pH-adjusted acetic acid solution

respectively) [Figure 5.11 (B) & (C)].

5.4. Discussion

5.4.1. Available Chlorine

Numerous studies have been conducted in order to investigate the effectiveness of the available chlorine-based sanitizers in reducing the microbial load on the surface of the fruits, however the results have been conflicting (reviewed by Parish *et al.*, 2003). In general, it was difficult to compare the results presented here to those reported in the literature, primarily because of the differences in the experimental methods used including the temperature and pH of the sanitizing solutions as well as the duration of the treatment (Pao & Davis, 1999; Sapers *et al.*, 1999; Pao *et al.*, 2000; Bagci & Temiz, 2011; Neo *et al.*, 2013). These parameters could significantly influence the antimicrobial efficacy of the available chlorine. For instance, the reduction in pH from alkaline to neutral could change the equilibrium between OCl^- and HOCl resulting in greater concentration of the latter which has been shown to exhibit stronger antimicrobial effects (McGlynn, 2004; Fukuzaki *et al.*, 2006). Moreover, in some studies the terms available chlorine, chlorine, hypochlorite, NaOCl and bleach have been used interchangeably, making it difficult to determine the exact concentration of free chlorine used (Chung *et al.*, 2011; Narciso & Plotto, 2005; McGlynn, 2004). In addition, unlike the current study in which the cells were suspended in PBS and washed with chlorine before their inoculation in OJ, in these studies cells were artificially inoculated on the surface of the produce before being immersed in or sprayed with available chlorine-based solutions.

In general, washing the cells with a solution of 50 ppm available chlorine [Figure 5.1(B)] was no more effective than ddH_2O [Figure 5.1(A)] in reducing the number of viable or culturable cells in *E. coli* pre- or post-inoculation in OJ. Moreover, while compared to ddH_2O, washing the cells with 100 ppm available chlorine [Figure 5.1(C)] was only effective in reducing the number of healthy and/or culturable cells post-inoculation in OJ, washing the cells with 200 ppm available chlorine [Figure 5.1(D)] resulted in a significant decrease in healthy and/or culturable population pre- and post-inoculation in OJ. Chung *et al.*, (2011) investigated the effectiveness of washing the surface of six fruits and vegetables with 50, 100 and 200 ppm NaOCl for 20 min at ambient temperature. They reported that the increase in concentration of NaOCl from 50 to 200 ppm resulted in a greater decrease in the total microbial, coliform and *E. coli* counts. The range of reduction in the number of culturable *E. coli* cells observed in the current study for cells washed with 200 ppm available chlorine was within the ranges reported in the studies cited above, regardless of the experimental conditions used.

Washing the cells with 200 ppm available chlorine also resulted in a significant increase in the number of VBNC cells. The induction of VBNC in chlorine-stressed cells have previously been reported by Singh *et al.*, (1986), Kolling & Matthews, 2001; Oliver *et al.*, (2005) and Dukan *et al.*, (1997). According to Dukan & Toutati (1996), challenging *E. coli* with HOCl could result in generation of ROS species (hydroxyl radicals) due to occurrence of a Fenton-

type reaction (see Section 1.3.4). In turn, the generation of ROS has been shown to induce VBNC state in *E. coli* (Oliver, 2005). Therefore, it could be proposed that the HOCl-induced generation of ROS was responsible for the observed increase in the number of VBNC cells in OJ subsequent to washing the cells with 100 and 200 ppm available chlorine.

Dukan & Toutati (1996) also showed that the mutation in *rpoS* gene of *E. coli* could make the cell hypersensitive to the HOCl stress. Likewise, as was mentioned in Section 1.3.2, RpoS regulon plays an important role in general stress response of the cells to various stresses including acid and oxidative stresses (Hengge-Aronis, 2002; Battesti *et al.*, 2011). Furthermore, Wang *et al.*, (2009) demonstrated that the treatment of the *E. coli* cells with available chlorine could up-regulate multiple genes responsible for oxidative stress response of the cells. Consequently, it could be hypothesized that the increase in available chlorine concentration of the washing solutions from 100 ppm to 200 ppm, led to a greater oxidative stress response in *E. coli* leading to enhanced acid resistance of the cells. This could also explain why compared to 100 ppm washed cells, the rate of reduction in culturability of the 200 ppm available chlorine washed cells was slower. Greater oxidative stress response could have enhanced the capability of the cells in reducing the high level of generated ROS leading to a better culturability of the cells.

5.4.2. Hydrogen Peroxide

As was mentioned before, available chlorine is the most common sanitizer used

in food industry for the purpose of washing the surface of the fruits. However, its main disadvantage is that it is generally not capable of reducing the microbial load on the surface of the fruit in excess of 2 $\log_{10}$ cells.mL^{-1} (Parish *et al.*, 2003). As a result, it has been suggested that the use of available chlorine within the permitted range (maximum 200 ppm) does not ensure the safe elimination of food pathogens from the surface of fresh produce (Sapers, 2001). This is particularly the case in fresh produce such as orange fruit with porous and hydrophobic surfaces (e.g., due to the presence of wax and essential oils) which can potentially protect the bacteria against available chlorine action by reducing the accessibility of the aqueous available chlorine-based sanitizer to the bacteria (Adams *et al.*, 1989; Beuchat & Ryu, 1997, Martinez-Gonzales *et al.*, 2011). It has been suggested that H_2O_2 is a more effective sanitizer against *E. coli* (Sapers *et al.*, 1999). Therefore, it was decided to study the effects of H_2O_2 on physiology of *E. coli* pre- and post-inoculation in OJ.

In common with the results observed for chlorine-washed cells, increasing the concentration of H_2O_2, resulted in a greater decrease in the number of healthy GFP$^+$ and culturable cells (Figure 5.2). However, except for 5% H_2O_2 [Figure 5.2(D)] washing cells with 1% and 2.5% H_2O_2 [Figure 5.2(C) & (C) respectively] was not as effective as 200 ppm available chlorine [Figure 5.1(D)] in reducing the population of healthy or culturable cells. Sapers *et al.*, (1999) and Sapers & Sites (2003) studied the efficacy of 1%, 2.5% and 5% H_2O_2 in reducing the *E. coli* population on the surface of apples and compared them to that of 200 ppm available chlorine. An increase in concentration of H_2O_2 from 1% to 2.5% or 5%

was not significantly different. Moreover, all concentrations were found to be as effective as 200 ppm available chlorine in reducing the *E. coli* population. In other studies conducted by these researchers similar results were observed (Sapers *et al.*, 2000, 2002; Sapers, 2001). The discrepancy between the results of these studies and those reported in the current study is believed to be due to combined effects of using a different strain of *E. coli*, incubation of the stationary phase cells at 4 °C for 24 h before H_2O_2 treatment as well as using different temperature for the H_2O_2 solution (50 °C instead of 30 °C in the current study) by these researchers.

With regard to viability and culturability of H_2O_2-treated cells post-inoculation in OJ, with the exception of 5% H_2O_2-washed cells, there was a close agreement between the total number of healthy and culturable cells. Even in case of 5% H_2O_2-washed cells, the rate of reduction in the number of healthy cells was relatively similar to that of culturability throughout the experiment.

This could be explained by the mechanisms of action of available chlorine and H_2O_2. Both available chlorine and H_2O_2 share a common mechanism based on a Fenton-like chemistry which involve the diffusion of HOCl or H_2O_2 through the microbial cell membrane, leading to the oxidation of Fe^{2+} to Fe^{3+} and the formation hydroxyl radicals (·OH). Subsequently, these radicals damage the cell by inhibiting the essential enzymatic activity as well as damaging the membrane and DNA (Fukuzaki, 2006; Finnegan *et al.*, 2010). However, the important point here is that the available chlorine-based sanitizing solutions

used in this part of the study were not pH-adjusted and therefore had basic pH. This meant that the majority of the available chlorine were in OCl^- form which unlike the electrically-neutral HOCl is unable to diffuse through the cell membrane. On the other hand, similar to HOCl, H_2O_2 is a small neutral molecule which can easily diffuse through the membrane. As a consequence, while HOCl and H_2O_2 can damage the cells both from inside and outside, OCl- is only able to exert an oxidizing effect on the outer membrane of the cell (Fukuzaki, 2006). Therefore, it could be hypothesized that the slower rate of reduction in the number of GFP^+ healthy cells in chlorine-treated cells after their inoculation in OJ was most likely due to inability of the OCl- in diffusing into the cells, leading to lower damage to the essential enzymes and DNA of the cells.

5.4.3. Effects of Available Chlorine and H_2O_2 on Viable Populations

Despite the reduction in the number of healthy GFP^+ or culturable cells, the number of viable cells in OJ samples increased regardless of the sanitizing regime used [Figure 5.3]. Moreover, while the magnitude of increase in the number of viable cells was dependant on the concentration of H_2O_2 [Figure 5.3(B)], the chlorine concentration [Figure 5.3(A)] did not seem to have any effect on the total number of viable cells in OJ. The overall increase in the number of viable cells in OJ was assumed to be due to oxidative stress-induced cross-protection against acid stress.

One gene which could have played a role in the observed cross-protection is

ycfr. YcfR is a multiple stress response protein which has been shown to play an important role in resistance of *E. coli* to extreme change in pH (Maurer *et al.*, 2005). Five-fold induction of *ycfr* has also been reported in an acidic model apple juice (Bergholz *et al.*, 2001). However, what is important is that the genes have also been shown to be strongly induced under H_2O_2 stress (Zheng *et al.*, 2001). Therefore, it is possible that the treatment of the cells with increasing concentrations of H_2O_2 resulted in a greater expression of *ycfr*, hence improved viability of the cells in OJ. The YcfR-induced aggregation of the cells and protection of the cells by cloud particles settled at the bottom of the universal bottles, could also have facilitated the increase in viable population. The up-regulation of this gene in *E. coli* has also been reported for chlorine-stressed cells. However this up-regulation was found to strain-dependant (Deng *et al.*, 2011). Therefore it is possible that the treatment of SCC1 strain with available chlorine affected the expression of *ycfr*. The observed formation of biofilm in OJ [Table 5.1] further supports this theory. This is mainly because *ycfr* is one of the primary regulators of the biofilm formation and its expression in *E. coli* cells in biofilms is strongly affected in response to stress conditions (Beloin *et al.*, 2004; Zheng *et al.*, 2007).

5.4.4. Acidified and surfactant-containing Chlorine

The results of the study [Figure 5.4(A)] showed that, compared to other samples, washing the cells with acidified chlorine led to a significantly lower $\log_{10}$ number of healthy GFP^+ cells in OJ up to time 24 h post-inoculation. However, from this time onward, the mean $\log_{10}$ of healthy cells in OJ samples

washed with acidified chlorine was greater than the population of healthy cells in other OJ samples. Therefore, it appeared that the initial acid stress with acidified chlorine increased the capability of the survivors to resist the very low pH of OJ. Nevertheless, acidification of 200 ppm available chlorine with citric acid or its supplementation with Tween 80, did not affect the overall number of viable cells [Figure 5.4(B)]. This could further support the aforementioned hypothesis that the available chlorine within the ranges used in the current study was not capable of inducing the *ycfr*-dependant stress response regardless of the type of available chlorine (e.g., HOCl or OCl^-).

Numerous studies have focused on the use of available chlorine adjusted to mild acidic pH of approximately 6–6.5 using various organic or inorganic acids (Adams *et al.*, 1989; Sapers *et al.*, 2002; Martínez-González *et al.*, 2011). For instance, Adams *et al.*, (1989) showed that the acidification of 100 ppm available chlorine from pH 8.8 to 4 with H_2SO_4 increased its efficacy for removing the microbial load on the surface of the lettuce by approximately 0.8 $\log_{10}$ (washing time of 5 min). Similar range of reduction was observed when various organic acids (acetic, lactic, citric and propionic acids) were used instead of H_2SO_4. They also reported that the supplementation of the 100 ppm available chlorine with 100 ppm Tween-80 decreased the microbial load by 34% compared to un-supplemented solution. Martínez-González *et al.*, (2011) reported that the acidification of a 200 ppm available chlorine solution with HCl and therefore reducing the pH of the solution from 10 to 6 was effective in significant reduction in the $\log_{10}$ population of *S.* Typhimurium and *L.*

monocytogenes but not *E. coli* O157:H7 on the surface of orange fruits (washing time of 15 s).

Due to varying treatment conditions and concentrations of available chlorine, it was difficult to compare the results of these two studies with that of the current study. Nevertheless, no significant difference was observed between the total number of culturable cells post-inoculation in OJ regardless of the supplementation with acid or surfactant. With regard to Tween-80 supplemented solution, a possible reason for the discrepancy between the results of the current study and that of Adams *et al.*, (1989) could be due to the difference in the experimental methods. While in case of the latter, Tween-80 could have improved the efficacy of available chlorine by reducing the hydrophobicity of the surface of the lettuce, in the current study, *E. coli* cells were washed with the surfactant-containing solution in a microcentrifuge tube and therefore no physical removal of bacteria could have occurred.

However, the FCM study of the change in the percentage of GFP^+ cells provided a better picture of the effects of acidified chlorine on the physiology of the cells. The time-point [(Figures 5.4(A) & 5.6(A)] and real-time study [(Figure 5.7)] of the effects of chlorine-based solutions on the population of GFP^+ cells indicated that washing the cells with acidified chlorine led to a significant increase in number of GFP^- cells. This could be attributed to the greater concentration of HOCl in the acidified chlorine and lower antimicrobial efficacy of OCl^- (McGlynn, 2004; Fukuzaki *et al.*, 2006). It is known that OCl- exerts an antimicrobial effect by

rupturing the cell wall inactivation of the functional proteins in the plasma membrane. On the other hand, HOCl can damage the cells not only from the outside but also by oxidizing the highly nucleophilic sites of the enzymes and DNA as well as uncoupling the membrane proton gradient (Fukuzaki *et al.*, 2006). This could have rendered the cells incapable of maintaining the membrane proton gradient (ΔpH), leading to greater reduction in pH_i of the cells when inoculated in OJ. In turn, this could have led to greater decrease in FI of the GFP due to denaturation/mis-folding of the GFP which has been shown to be sensitive to pH of less than 5 (Kneen *et al.*, 1998). It is known that mis-folding of the proteins and the formation of inclusion bodies can increase the SSC-A in *E. coli* (Lewis *et al.*, 2004). This could also explain the significant increase in the SSC-A of acidified chlorine-washed cells following their inoculation in OJ, compared to other samples [(Figure 5.6(E)].

5.4.5. Organic Acids

Organic acids are known to exhibit antimicrobial activity against a wide range of microorganisms. As was previously discussed in Chapter 3, although both the dissociated and undissociated forms of organic acid can exert antimicrobial effects. However, the undissociated form has been shown to be more effective. When undissociated organic acids enter the cell, they dissociate, leading to generation of excess protons, hence reducing the pH_i of the cells. In order to prevent the adverse effects of low pH_i on enzymatic activity and nucleic acids, cells actively employ ATP in order to extrude the excess proton and this eventually leads to cell death (Salmond *et al.*, 1984; Davidson & Harrison,

2003; Lu *et al.*, 2011). This mechanism is also known as the theory of "weak-acid preservative".

Based on the Henderson-Hasselbalch equation, the "percent dissociation" in increasing order was 0.55%, 1.71%, 5.56% and 8.54% for acetic acid (4%), lactic acid (4%) and citric acid (4% and 2%) respectively. In agreement with aforementioned theory, the lowest percentage of healthy cells and greatest percentage of injured and/or dead cells (BOX^+) at time 0 h pre- and post-inoculation in OJ was observed in 4% acetic acid [Figure 5.9(D)] which contained the greatest percentage of undissociated acid.

Nevertheless, no significant difference was observed between the post-washing percentage of healthy, injured or dead cells among the other three conditions (Figure 5.9(A), (B) and (C)]. This could be because other factors other than the pK_a (hence dissociate percent) of acids might have played a role in overall viability of the cells. For instance, Stratford *et al.*, (2009) showed that sorbic acid and acetic acid exerted different antimicrobial effects against various fungi despite having a near identical pK_a. Both acids were capable of reducing the pH_i of the *Aspergillus niger* conidia; however, the pH_i of acetic acid-washed cells was significantly lower than those washed with sorbic acid-washed cells (pH_i 4.73 and 6.38 respectively). They proposed that while acetic acid conforms to the acidification of cytoplasm theory, other acids including sorbic acid employ a different antimicrobial mechanism of action such as damaging the membrane. Hsiao & Siebert (1999) also showed

that the antimicrobial efficacy of organic acids against microorganisms depends on many factors including the polarity, chemical bonds, molecular weight and solubility in ether and ethanol.

As previously discussed in Chapter 3, in order for the undissociated weak acid to lower the pH_i and exert antimicrobial effects, it needs to be either small (less than 3 carbons) or lipophilic in order to be able to diffuse through the plasma membrane (Stratford & Eklund, 2003). Citric acid is not only a large weak acid (six carbons) but also hydrophilic with a partition coefficient or $\log_{10}$ *P*oct of -0.172. Lactic acid on the other hand is a small organic acid (3 carbons) which can pass through the plasma membrane. However because of its high hydrophilicity ($\log_{10}$ *P*oct of -0.62), its diffusion is very slow. Therefore, both acids are believed to exert antimicrobial effects by acting as an acidulant reducing the pH of the environment. On the other hand, despite being a hydrophilic acid ($\log_{10}$ *P*oct of -0.319), acetic acid can pass through the membrane in undissociated form due to its small size (3 carbons) and therefore reducing the pH_i and causing intracellular damage (Stratford & Eklund, 2003). The diffusion of undissociated acetic acid and to some extend the lactic acid through the membrane could explain the greater antimicrobial efficacy of these acids against *E. coli* compared to hydrophilic citric acid.

The induction of VBNC in various microorganisms including *E. coli* as a result of treatment with weak acids such as lactic (0.1%) and acetic acids (0.1% and

1%) has previously been reported by Li *et al.*, (2005). The exact mechanism for induction of VBNC state with weak acids is not clear. However it has been suggested that the lactic acid could increase the intracellular concentration of Fe^{2+} by increasing the rate of dissociation of iron from protein. The free Fe^{2+} is known to react with H_2O_2, leading to generation of hydroxyl radicals (HO^-) via Fenton chemistry or Haber-Weiss reactions (Bruno-Bárcena *et al.*, 2010). As previously described in Chapter 3, the generation of ROS could induce VBNC state in bacteria (Kong *et al.*, 2004; Oliver, 2005). Therefore, it is possible that in the current study a similar mechanism was also behind the observed significant induction of VBNC state in acetic acid-treated cells.

5.5. Conclusions and Further Work

In summary, the data presented in this study demonstrated the successful application of FCM for monitoring the efficacy of washing procedures. The results confirmed the hypothesis that consecutive subjection of *E. coli* to maximum legally permitted concentrations of sanitizers (200 ppm available chlorine and 1% H_2O_2) and OJ induces VBNC state. More importantly, instead of reducing the total $\log_{10}$ number of viable cells, inoculation of stationary-phase *E. coli* resulted in an increase in PI^- population of *E. coli* in OJ. Treatment with H_2O_2 but not available chlorine led to further increase in the number of PI^- cells, particularly after day 5 post-inoculation. As previously mentioned, there is little support in the literature for the growth of *E. coli* in acidic environments at 4 °C. Considering the fact that throughout this study PI^- cells have been presumed to be viable, further work is needed to confirm that the observed

increase in PI^- cells is indeed due to growth of *E. coli* in OJ samples at 4 °C. This could be achieved by, for instance, staining the cells with fluorescent dyes such as Alexa Fluor® 633 (Molecular Probes, Eugene, USA) conjugated to anti-*E. coli* polyclonal antibodies (Abcam plc, Cambridge, UK) before their analysis with FCM. This would facilitate the simultaneous isolation of the cells (based on their far-red fluorescence) and investigation of their viability (based on their green and red fluorescence using GFP/BOX and PI respectively).

The increase in the number of PI^- cells was also presumed to be due to oxidative stress-induced cross protection against acid stress in OJ presumably due to up-regulation of *ycfr* gene, however this hypothesis was not investigated further. Furthermore, it was hypothesized that the oxidative stress-induced over-expression of *ycfR* could have played a role in improved viability of the cells in OJ by inducing the aggregation of the cells and therefore protecting them from acidic environment of OJ by cloud particles. These hypotheses could be investigated further by using *ycfR* mutant cells and comparing the viability of wild-type and mutant strains in OJ similar to the study conducted by Bergholz *et al.,* (2001) using apple juice.

Acidified chlorine led to a greater reduction in the number of GFP^+ cells up to time 24 h post-inoculation compared to other surfactant containing and un-supplemented available chlorine solution. Nevertheless, from this time-point onwards, it also resulted in a greater population of GFP^+ cells. It was hypothesized that the initial mild-acid (pH 6) stress with acidified chlorine

might have improved the capability of the survivors to resist the acidic low pH of OJ. Nonetheless, acidification or supplementation of available chlorine with surfactant did not affect the total number of viable or culturable cells in OJ.

Compared to available chlorine however, organic acid had significantly greater adverse effects on both the viability and culturability of the cells. 4% acetic acid was found to be more effective than 4% citric or lactic acid in reducing the population of healthy (BOX^-) cells. The greater efficacy of acetic acid was presumed to be due to greater concentration of undissociated acid in the sanitizing solution.

In this study the antimicrobial efficacy of only a limited number of sanitizers at a single temperature/time setting (30 °C/2 min) was investigated. Further work is needed to study the combined effects of the change in treatment temperature and/or time on the viability of the cells and the induction of VBNC state in *E. coli*. Moreover, it would be interesting to use FCM in order study the efficacy of other fruit cleaning chemicals such as acidified sodium chlorite (ASC), tri-sodium phosphate (TSP), peroxyacetic acid (PAA) and chlorine dioxide (ClO_2) on subsequent viability of *E. coli* in OJ.

In the current study, cells were washed in a microcentrifuge tube instead of washing them on the surface of the orange fruit. It is known that hydrophobic properties of the peel of orange fruit could affect the antimicrobial efficacy of the chlorine-based solutions (Adams *et al.*, 1989). Therefore it would be useful to

investigate the role that these compounds could play on the physiological response of the cells post-washing with sanitizer and post-inoculation in OJ. Another limitation of this study was that, although it was possible to calculate the percentage of different populations (e.g., healthy, stressed, GFP^-, injured or dead), however it is not possible to investigate the contribution of each population to the total VBNC population. Using a cell sorter FCM, it should be possible to collect each population in a separate container and make it easier to plate each type of population separately. Moreover, the possibility of resuscitating the VBNC population with various ROS scavengers such as catalase and sodium pyruvate deserves further investigation.

Chapter 6

General Conclusions

The results of this body of work demonstrated a successful application of FCM for rapid time-point and real-time monitoring of the physiology of *E. coli* in OJ and MOJ. As well as rapid determination of the cell count within the sample, FCM was found to be a valuable technique for studying the viability of *E. coli* in OJ and MOJ. Moreover, studying the change in FCM light scatter characteristics of the cells provided valuable information about the morphology of the cells. Finally, comparing the results of the CFU plate counts with the FCM cell count provided the data needed for determining the number of VBNC cells in the samples.

With regard to the effect of the OJ composition (Chapter 3) on physiology of *E. coli*, change in sugar concentration of the MOJ within the ranges naturally found in OJ affected neither the viability nor the culturability of the *E. coli* in MOJ. Organic acids were only effective in reducing the culturability or viability of *E. coli* when their concentration in MOJ was increased to the maximal levels naturally found in OJ. On the other hand, supplementation of MOJ with ascorbic acid and amino acids within the ranges naturally found in OJ resulted in a significant improvement in both the viability and culturability of the *E. coli* cells. Similar results were also observed when OJ was supplemented with amino acids. With regard to the morphology of the

cells in MOJ and OJ, significantly higher FSC-A and SSC-A values were observed for injured and dead cells indicating a possible OJ-induced increase in cell size and granularity respectively. Although the results of this study showed a significant increase in VBNC population in OJ and MOJ, however, the possibility of resuscitating this cells was not investigated. Further work could involve using various ROS scavengers such as catalase and sodium pyruvate for their potential in resuscitating OJ- and MOJ-induced VBNC cells.

In this study a single strain of *E. coli* (K-12 MG1655) was used. However, the behaviour of pathogenic *E. coli* such as *E. coli* O157:H7 in OJ could be significantly different from those observed in this study. Therefore, further studies are needed in order repeat some of the results of this study using EHEC strains particularly for the findings which were found to be significant in order to validate the results for pathogenic strains. Furthermore, some of the results reported in Chapter 3 regarding the effects of different components of OJ on the viability of *E. coli* were based on using exponentially growing cells instead of stationary-phase cells. Considering that the stationary-phase cells were shown to be more resistant than exponential-phase cells both in MOJ and OJ, further work is also needed to repeat some of these results using stationary-phase cells.

In general, the clarification of OJ (Chapter 4) and the removal of the pulp and cloud particles resulted in an improvement in culturability of *E. coli* in

OJ. FCM results also showed that cloud particles of between 0.22 μm and 0.7 μm (presumed to be hesperidin crystals) exerted a greater adverse effect on the viability of *E. coli* when larger cloud particles were removed from the sample. This was believed to be due to greater interaction of hesperidin crystals with *E. coli*.

Further exploration of the role of each component of OJ on the viability and culturability of *E. coli* is needed especially regarding the role of hesperidin crystals and the interaction of OJ cloud particle with both hesperidin and *E. coli* cells. This could be achieved by using scanning electron microscopy and transmission electron microscopy for microscopic observation of the particles as well as measuring the ζ-potential of the different types of cloud particles as previously described in Chapter 4.

In Chapter 5, it was shown that washing the *E. coli* cells with increasing concentrations of available chlorine or H_2O_2 resulted in a significant reduction in the population of healthy or culturable *E. coli* cells following their inoculation in OJ. However, neither the available chlorine nor H_2O_2 were effective in reducing the total number of viable cells. Washing the cells with organic acids was found to be more effective than both the H_2O_2 and available chlorine in reducing the healthy population regardless of the type of organic acid used.

In case of H_2O_2-washed cells, the increase in the concentration of H_2O_2 solution

led to a greater number of PI^- *E. coli* cells post-inoculation in OJ. Based on the results of the current study, it was difficult to make a definitive conclusion on the nature of these cells and further work is needed to confirm that the observed increase in PI^- cells is indeed due to possible growth of *E. coli* in OJ samples at 4 °C.

Furthermore, in this study it was hypothesized that the oxidative stress-induced over-expression of *ycfR* could have played a role in improved viability of the cells in OJ by inducing the aggregation of the cells and therefore protecting them from acidic environment of OJ by cloud particles. This hypothesis could be investigated further by using *ycfR* mutant cells and comparing the viability of wild-type and mutant strains in OJ similar to the study conducted by Bergholz *et al.,* (2001) in case of apple juice.

The most important limitation of this study was the detection limit of the flow cytometer used. As a result, FCM study of the physiology of *E. coli* in OJ or MOJ was only possible in sample with high cell density. Moreover, although FCM made it possible to identify distinct viable sub-populations, it was not possible to study the culturability of each sub-population separately. Using a cell sorting flow cytometer could facilitate investigating the culturability of each population separately.

With regard to the wider implications of this study for food industry, it is important to take into consideration the current risk assessments and

pathogen reduction targets (5 $\log_{10}$ reduction in pathogen of concern to public health) for the production of unpasteurized fruit juices (Vojdani *et al.*, 2008). With this regard, none of the interventions discussed in this study (e.g., change in the composition, filtration and washing condition) led to more than 5 $\log_{10}$ reduction in the population of *E. coli* when compared to the control conditions. This is especially critical taking into consideration the widely reported greater resistance of EHEC strains compared to compromised laboratory strain of K-12 strain.

Although this issue does not in any way affect the scientific validity of the results reported throughout this body of work, nevertheless, the readers are advised to take caution in interpreting the results reported here. In other words, the observation of a scientifically and statistically significant reduction in population of *E. coli* K-12 in OJ through application of the interventions and/or treatments described here, might not necessarily lead to industrially significant reduction in the population of EHEC in OJ if the same treatments were used for the production of OJ.

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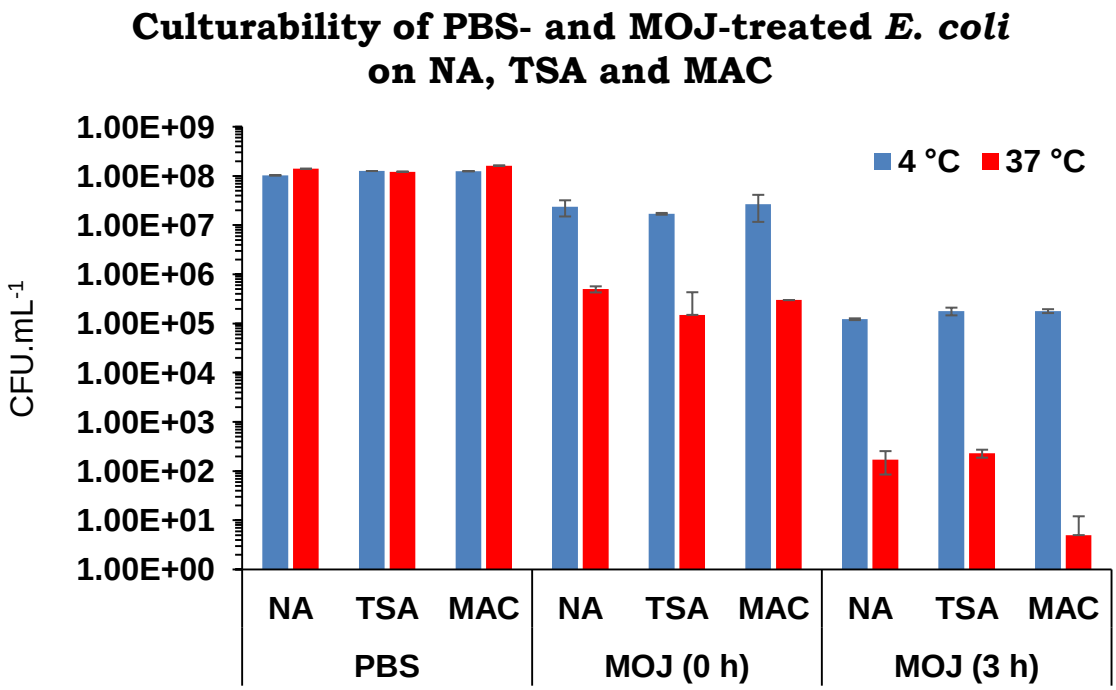
Appendix 1



Appendix 1: The culturability of *E. coli* K-12 MG1655 on nutrient agar (NA), tryptic soy agar (TSA) and MacConkey agar (MAC) following its inoculation in PBS (Control) and MOJ (0 h and 3 h post-inoculation).

Mid-log-phase *E. coli* K-12 MG1655 cells were inoculated in PBS or MOJ as described in Section 3.2. Samples were incubated at 4 °C and 37 °C and subsequently plated on NA, TSA and MAC at time 0 h and 3 h post-inoculation. No significant difference observed between the recovery rates of the cells at time 0 h post-inoculation regardless of the incubation temperature and/or type of plate used. However, at time 3 h post-inoculation, significantly lower number of cells were recovered on MAC plates for samples incubated at 37 °C ($p < 0.05$).

Appendix 2

List of electronic files ((DVD Enclosed) containing the raw data

Results of Chapter 3 (Figures 3.11-3.17)

1. Fig 3.1-3.17.xls

Results of Chapter 4 (Figures 4.1-4.14)

1. Fig 4.1-4.14.xls

Results of Chapter 5 (Figures 5.1-5.11)

1. Fig 5.1 & 5.3(A).xls
2. Fig 5.2 & 5.3(B).xlsx
3. Fig 5.4-5.6(A).xlsx
4. Fig 5.1 & 5.3(A).xlsx
5. Fig 5.6(B-C).xlsx
6. Fig 5.6(D-E).xlsx
7. Fig 5.7.xlsx
8. Fig 5.9-5.10.xlsx
9. Fig 5.11.xlsx