Cellular Interactions of Probiotic Bacteria with Porcine Intestinal and Progenitor Immune Cells

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A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

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STATEMENT OF AUTHENTICATION

The work presented in this thesis is, to the best of my knowledge and belief, original except as acknowledged in the text. I hereby declare that I have not submitted this material, either in whole or in part, for a degree at any other institution.

Signed: ………………………………………………………..

Date: …………………………………………………………..
DEDICATION AND ACKNOWLEDGEMENTS

This thesis is dedicated to the loving memory of my father, James Moore, who taught me the value of hard work.

Thank you to my supervisors, Dr Mark Jones, Dr Michael Phillips and Associate Professor Kasipathy Kailasapathy for their advice, assistance, guidance, encouragement and for often going beyond their role as Supervisors throughout the completion of this thesis. I am indebted to Ben Sainsbury and Sebastian Bowman for providing the fluorescent proteins used in this thesis. Thank you to Laura Andrews, Stephen Mieruszynski and Jodie Newman for their assistance with the confocal and RICS components and to Stephanie Pritchard, Junus Salampessy and Mariam Farhad for their assistance and companionship.

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**ABSTRACT**

Probiotic bacteria have been shown to convey health benefits to hosts and are included in a large number of food and beverage products. The enhancement of immunological activity is one of the proposed health benefits conveyed by probiotic bacteria. At the molecular level, the mechanisms that mediate the immune cell responses to probiotic bacteria remain undefined. Studying the intracellular responses of immune cells to probiotic stimulation may provide insights into molecular events associated with enhanced immunological activity. This thesis utilises the expression of fluorescent proteins genes and subsequent generation of fluorescent proteins as a bio-indicator of immune intracellular responses to probiotic bacteria. Real-time analysis of fluorescent protein expression models has been performed using advanced fluorescence microscopy systems such as laser scanning confocal microscopy (LSCM). Coupling LSCM data with a relatively novel image analysis technique, raster image correlation spectroscopy (RICS), provides the ability to measure intracellular dynamics.

The viability of probiotics in food and beverages must be maintained to ensure the health promoting activities in hosts. Food processing and storage procedures have been found to compromise the viability of probiotics. Microencapsulation of probiotic bacterial strains has been suggested to protect fragile probiotic strains. Microencapsulation has also been associated with a higher viability when compared to free cells in simulated gastrointestinal conditions. The effects of the microencapsulated environment on the probiotic bacterial behaviour has not been described. This thesis utilises the fluorescent protein expression immune cell model and fluorescent microscopy analysis as a downstream indicator of stress-related responses exhibited by the microencapsulated probiotic cells.
In this study, the ability of microencapsulated probiotic bacteria to survive confinement was investigated. Microencapsulated probiotics were observed to survive and proliferate following 24 h incubation. A viability standard curve was developed for microencapsulated bacteria using the probiotic strains *Lactobacillus acidophilus* LAFTI® L10 bacteria, *Bifidobacterium lactis* HN019 (DR10™) and the pathogenic strain, *Streptococcus pyogenes*, the LIVE/DEAD BacLight™ bacterial viability kit, LSCM and the image analysis software, Bitplane Imaris. The microencapsulated bacteria were co-cultured with adaptive immune cells to determine stress-related changes by the confined bacteria cells.

The fluorescent proteins, monster green® (phMGFP) and DsRed2 (pClneo-DsRed2), were utilised as intracellular bio-indicators of gene expression in porcine progenitor B- (L23) and T- (L45) immune cells treated with probiotic strains. Both transfected immune cell lines exhibited stable fluorescence expression of monster green® and DsRed2 plasmids within 24 hours of transfection and 2 years post-transfection. Transfected cells exhibited fluorescence throughout the entire cell during apoptosis (programmed cell-death). Further experimentation was conducted using cells expressing almost entire fluorescence as negative controls during probiotic treatments.

The fluorescence-expressing progenitor immune cells were exposed to non-microencapsulated and microencapsulated bacterial-produced soluble factors. Probiotic strain-related effects on the proliferation of the immune cells were observed. Increased proliferation was exhibited for the L45hMGFP, L45pClneo-DsRed2 and L23hMGFP cells treated with non-microencapsulated *L. acidophilus*
LAFTI® L10 produced soluble factors; opposite results for the microencapsulated *L. acidophilus* LAFTI® L10 treatments were observed. The non-microencapsulated and microencapsulated *B. lactis* HN019 (DR10™) treated L45hMGFP and L45pClneo-DsRed2 exhibited decreased proliferation. The treated B-cell lines exhibited similar proliferation responses to the respective control. The downstream responses of the immune cells to the microencapsulated bacteria indicate that the confined environment influences probiotic bacterial activity.

To determine if similar effects would be observed in a model *in vitro* gastrointestinal system, porcine fibroblast intestinal cells (IPI-1) were co-cultured with the non-microencapsulated and microencapsulated probiotic bacteria; the soluble factors produced by the co-cultures were applied to the transfected immune cells. The proliferation of the immune cells in response to IPI-1/free and microencapsulated co-cultures exhibited relationships between the immune cell type, if the bacteria were microencapsulated, the bacteria strain and the fluorescent protein, unlike the free and microencapsulated probiotics treated immune cells. These results show that the downstream immune cellular responses to the intestinal cell co-cultures with free and microencapsulated probiotics are regulated by intestinal cellular activities, more specifically, soluble factors produced by the intestinal cells.

Cytokine gene expression was used to determine the type of immunological response to the free and microencapsulated probiotics. Similar to the proliferation studies, cytokine gene expression appeared dependent on the immune cell type, fluorescent protein and bacteria strain. Pro-inflammatory cytokine gene expression was observed for the immune cells treated with microencapsulated probiotic bacteria.
conditioned media. The probiotic bacterial-conditioned media treated cells exhibited both pro-inflammatory and anti-inflammatory cytokine responses. Similarly, the immune cells treated with the IPI-1/free and microencapsulated probiotic co-cultured conditioned media exhibited gene expression for regulatory, pro-inflammatory and anti-inflammatory cytokines.

Using the novel image analysis technique, RICS, the intracellular fluorescent protein diffusion coefficients were shown to be dependent on the bacteria strain, immune cell types, presence of intestinal cells and if the bacteria were free or microencapsulated. Significant differences were observed for free and microencapsulated \textit{S. pyogenes} treated L45hMGFP and L23pCIneo-DsRed2 (respectively) cells compared to the control.

Probiotic bacterial affects on adaptive immune cells were shown to be dependent on the immune cell type (i.e. T and B cell), probiotic strain, presence of intestinal cells and if the probiotic strain was microencapsulated. Observing changes in the intracellular milieu of the immune cells treated with the free and microencapsulated bacteria is possible using fluorescent protein bio-indicator systems. Future advancements in fluorescence microscopy and image analysis software will provide further in-depth information of intracellular environmental responses to probiotic bacteria treatments.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>2D</td>
<td>2 Dimensional</td>
</tr>
<tr>
<td>3D</td>
<td>3 Dimensional</td>
</tr>
<tr>
<td>4D</td>
<td>4 Dimensional</td>
</tr>
<tr>
<td>APCs</td>
<td>Antigen presenting cells</td>
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<tr>
<td>BHI</td>
<td>Brain Heart Infusion</td>
</tr>
<tr>
<td>CD marker</td>
<td>Cluster of differentiation marker</td>
</tr>
<tr>
<td>cDMEM-AF</td>
<td>Complete DMEM-antibiotic free</td>
</tr>
<tr>
<td>CM</td>
<td>Conditioned mammalian cell culture media by bacteria</td>
</tr>
<tr>
<td>Con A</td>
<td>Concanavalin A</td>
</tr>
<tr>
<td>DCs</td>
<td>Dendritic cells</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleotide acid</td>
</tr>
<tr>
<td>DR10™</td>
<td>Bifidobacterium lactis</td>
</tr>
<tr>
<td>FAO</td>
<td>Food and Agricultural Organisation of the United Nations</td>
</tr>
<tr>
<td>FBS</td>
<td>Foetal bovine serum</td>
</tr>
<tr>
<td>FCS</td>
<td>Fluorescence correlation spectroscopy</td>
</tr>
<tr>
<td>FP</td>
<td>Fluorescent Protein</td>
</tr>
<tr>
<td>GALT</td>
<td>Gut-associated lymphoid tissue</td>
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<td>GFP</td>
<td>Green fluorescence protein</td>
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<tr>
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<td>Gastrointestinal</td>
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<tr>
<td>hMGFP</td>
<td>Humanised Monster Green® Fluorescent Protein</td>
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<td>Image Correlation Spectroscopy</td>
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<td>Interferon gamma</td>
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<td>L45</td>
<td>Porcine Progenitor T cell line</td>
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<td>Laser Scanning Confocal Microscopy</td>
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<tr>
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<td>Major histocompatibility complex</td>
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<tr>
<td>MLNs</td>
<td>Mesenteric lymph nodes</td>
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<tr>
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<td>deMan, Rogosa and Sharpe</td>
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<td>Non-Essential Amino Acids</td>
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<td>pCIneo vector with DsRed2 fluorescent protein</td>
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<td>Photon Multiplier Tube</td>
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<td>Pathogen Recognition Receptors</td>
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<td>Point spread function</td>
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1. CHAPTER ONE

Literature Review

1.1 PROJECT INTRODUCTION

The health benefits associated with probiotics, including immunomodulation, drive the worldwide multi-billion dollar consumer market for probiotic food and drink products (Sanders, 1998). Numerous studies have evaluated the immunomodulatory properties of probiotics to identify the type of immunological response including anti-inflammatory, regulatory and maturation of immune cells (Iwasaki and Kelsall, 1999; Matsuzaki and Chin, 2000; Paturi et al., 2007; Takeda and Okumura 2007). The interactions between probiotics and intestinal cells have also been investigated by researchers, primarily the alleviation of intestinal inflammation by probiotics to ameliorate the symptoms of disease states such as Crohn’s Disease (Boudeau et al., 2003).

A better understanding of the immune cell molecular behaviour and responses to soluble factors produced by probiotics and intestinal/probiotic co-cultures will benefit future research. Such understanding will allow for the identification of new probiotic strains with immunomodulatory effects, ascertain minimal probiotic dose requirements, distinguish the type of immunological response to probiotics and identify potential probiotic soluble factors that induce immunological reactions.

This project involved the use of adaptive immune cells with intracellular-expressed fluorescent proteins acting as bio-indicators of responses to soluble factors produced by probiotic strains and intestinal/probiotic co-cultures. Real-time fluorescence microscopy and image analysis software (Raster Image Correlation
Spectorscopy (RICS) was applied to determine the diffusion rates of the fluorescent proteins in response to the probiotic treatments. Immune cell proliferation and gene expression of cytokines and CD markers were also investigated. In addition, immune cell responses to microencapsulated probiotic bacteria were explored. Microencapsulation has been proposed to protect probiotic bacteria from food processing and harsh gastrointestinal environments. However, the behaviour of probiotics in the confined environment has not been described. The immune cell response to the microencapsulated probiotics was used as a downstream indicator to determine changes in the behaviour of the confined probiotics.

1.2 History & Development of Probiotic Bacteria

Approximately 2,500 years ago, Hippocrates (460 – 377 BC) suggested that food could be of medicinal use advocating “let food be thy medicine and medicine be thy food”. For thousands of years, microorganisms have been incorporated into food and beverages. The concept of microorganisms conveying beneficial effects to hosts was proposed over a hundred years ago. Metchnikoff, in 1907, suggested that “the dependence of the intestinal microbes on the food makes it possible to adopt measures to modify the flora in our bodies and to replace the harmful microbes by useful microbes”. He also noted the long life of Bulgarian peasants who consumed fermented milk products and suggested that the ingestion of yogurts containing *Lactobacillus* sp. can replace harmful intestinal bacteria. Metchnikoff hence introduced the concept of functional foods containing beneficial microbes. Currently, increasing consumer awareness of personal health has led to health-promoting/functional foods becoming more popular. Contemporary consumers are looking beyond immediate nutritional requirements from food products and seeking
those that promote a number of health-enhancing, as well as disease preventing characteristics.

In the past 50 years, considerable research has been conducted into the beneficial properties conveyed by probiotic bacteria to hosts; the health-promoting characteristics associated with probiotic bacteria include immunological enhancement, re-establishment of intestinal microbial balance following antibiotic treatments, and the potential to treat gastrointestinal (GI) disorders (Gomes and Malcata, 1999; Shah, 2007; Prado et al., 2008). However, the underlying mechanisms of probiotic health-conveying activities, in particular immune cell responses to probiotics, have not been addressed. The health-promoting characteristics of probiotic bacteria have led to the incorporation of these microorganisms into many foods and beverages, in particular, but not limited to dairy products. The most recognised probiotic bacteria are gram-positive lactic acid bacteria (LAB), in particular the strains of *Lactobacillus* and *Bifidobacterium*, with both genera found in the human intestinal microbiota populations. Other probiotic microorganisms include *Bacillus cereus* (animal probiotic), *Streptococcus salivarius* sp. *thermophilus* and *Saccharomyces boulardii* (Prado et al., 2008). To be classified as probiotic, a strain must possess a number of desirable characteristics and activities such as validation of health benefits and strain identification.

1.2.1 Definition of Probiotics and Probiotic-Active Substances

Many proposed definitions attempt to reflect the role and characteristics of probiotic bacteria. The investigation of the health benefiting effects conveyed by probiotic bacteria to consumers has resulted in a change to past definitions.
Subsequent to Metchnikoff’s proposal in 1907 (to replace harmful microorganisms with beneficial microorganisms in the intestinal tract), the term probiotic was first used by Kollath (1953) to describe organic and inorganic substances in food that alleviated malnutrition in patients (Hamilton-Miller et al., 2003). Later, the term probiotic was used by Lilly & Stillwell (1965) and Sperti (1971) to describe substances produced by microorganisms that encouraged the growth of other microorganisms. The term probiotic was also used to identify substances that improved resistance to infections (Fujii and Cook, 1973). The definition for probiotics was further developed to encompass microorganisms as well as substances. In 1974, Parker defined probiotics as “organisms and substances which contribute to intestinal microbial balance” in reference to advancing animal health using feed supplements. Currently, the most referenced definition was stated by Fuller in 1989 describing probiotics as “a live microbial feed supplement which beneficially affects the host animal by improving its intestinal balance”. Fuller emphasised that such microorganisms should be viable. At present, accepted definitions for probiotic bacteria portray viable microorganisms with the ability to convey beneficial health effects to the host. The World Health Organisation (WHO) and Food and Agricultural Organisation (FAO) of the United Nations (2001) proposed probiotic bacteria as ‘live microorganisms which, when administered in adequate amounts, confer a health benefit on the host’.

Current definitions of probiotics describe these microorganisms as viable, whole cells. Probiotic cellular structure fragments, such as nucleic acids and cell wall elements, have been reported to influence the intestinal ecosystem. Interactions between these cellular components and the intestinal environment were described
including induced immunomodulatory activities (Naidu et al., 1999). The potential use of non-viable microorganisms or cellular components in nutritional pharmabiotics was outlined by Shanahan (2004) who also declared that the concept of current definitions limit probiotic microorganisms to being viable and of human origin. Such bacterial components and non-viable cells have been termed probiotic-active substances (Naidu et al., 1999). The concept of stimulation by probiotic-active substances has been supported by studies using nucleic acids, cell wall structures and non-viable bacterial cells, among other probiotic bacterial components, that reportedly enhance immunological activities in mice (Lin et al., 2007) and humans (Lammers et al., 2003), and attenuated experimental colitis in mice (Rachmilewitz et al., 2004). Further, various probiotic strains in non-viable states were proposed to be capable of stimulating immunological effects (Maldonado Galdeano et al., 2007).

As outlined, the present definitions for probiotic microorganisms detail the need for viable probiotic cells to convey beneficial effects to the host. Probiotic bacterial metabolising activities within the GI tract are believed to form the underlying mechanisms to the subsequent health-promoting events conveyed to hosts. The evidence supporting probiotic-active substances suggests a number of physiological interactions with components of the gastrointestinal system; this includes communications with intestinal epithelial cells and subsequently with the gut-associated lymphoid tissues (GALT). Therefore, via a number of interactive events within the GI tract, cellular components and metabolic activities of probiotic bacteria may contribute in a synergistic manner to the overall health benefitting effects conveyed to the host by these microorganisms (Boirivant and Strober, 2007).
1.2.2 Probiotic Properties and Classification

The underlying principle for a microorganism to be classified as probiotic is the ability of that microorganism to interact with, and to survive within the GI environment. Many selection criteria have been proposed to identify probiotic microorganisms. Due to a great deal of variation in experimental results from experiments with probiotic strains (Berg, 1998), the establishment of a classification system for probiotic microorganisms is considered essential. This classification will be particularly relevant for microorganisms incorporated into food products. A criterion encompassing “bio-safety, production, administration, survival and/or colonisation in the host” is described by Havenaar et al. (1992) for the selection of probiotic strains. Properties considered for the classification of probiotic bacteria include acid and bile resistance, attachment abilities by the probiotic cells to intestinal epithelial cells, human intestinal colonisation, antimicrobial substance production and the ability to convey beneficial effects that promote the health of the host (Havenaar et al., 1992; Collins et al., 1998; Holzapfel et al., 1998; Kailasapathy and Chin, 2000; Dunne et al., 2001; Tuomola et al., 2001; Morelli, 2007; Pineiro and Stanton, 2007; Prado et al., 2008). Potential health benefits of the genera *Lactobacilli* and *Bifidobacteria* include immune enhancement that protects against intestinal infections, colon cancer and hypercholesterolaemia, improved lactose utilisation, prevention of GI tract diseases and gut mucosal barrier stabilisation (Kailasapathy and Chin, 2000). The non-pathogenicity of probiotic microorganisms is also considered an important classification requirement with much emphasis placed on the Generally Regarded As Safe (GRAS) characteristics (Havenaar et al., 1992; Collins et al., 1998; Liong, 2008; Prado et al., 2008) especially for those probiotic microorganisms in commercial applications. Collins et al. (1998) also
outlined characteristic of “good probiotic strains” including being of human origin, the ability to undergo activities within the GI tract such as immunostimulation, antimitagenic and anticarcinogenic (Table 1.1). Considering the environment of the GI tract, Fuller (1992) suggested the following mechanisms for probiotic effects conveyed to the host: 1) compete against other microorganisms for nutrients, 2) compete for adhesion sites on the gut epithelium to establish colonisation, 3) produce antimicrobial substances, and 4) immunological stimulation. However, the properties of strains in vitro and in vivo have been reported to differ, for instance, the ability to persistently colonise the intestinal tract (Bujalance et al., 2007). In addition, Fuller (1992) outlined the following characteristics as a selection criterion for animal probiotic strains: 1) the ability to be prepared at an industrial level as a viable product, 2) the ability to remain stable and viable for long durations in storage and field conditions, 3) the ability to survive in the intestine (not necessarily to grow), and 4) the ability to produce a beneficial effect in the host animal. Further guidelines were outlined to classify probiotic bacteria by the FAO and WHO (2001) and included details of potential treatment of diseases by probiotics, safety considerations and a number of regulatory measures for marketed products.
Table 1.1. Properties considered to be classified as a ‘good’ probiotic strain.

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<td>Human origin</td>
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<td>2.</td>
<td>Possession of generally regarded as safe (GRAS) status</td>
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<td>3.</td>
<td>Possession of a desirable antibiogram profiles e.g. metronidazole resistance with desirable sensitivities</td>
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<td>4.</td>
<td>Production of antibacterial factors antagonistic for potentially pathogenic microorganisms, particularly invasive Gram negative pathogens</td>
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<td>5.</td>
<td>Desirable metabolic activity</td>
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<td>6.</td>
<td>Technological suitability</td>
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<td>7.</td>
<td>Non-pathogenic even in immunocompromised hosts</td>
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<td>8.</td>
<td>Non-inflammatory-promoting microorganisms</td>
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<td>9.</td>
<td>Survival in association with the adult mucosal immune system</td>
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<td>10.</td>
<td>Immunostimulatory for the mucosal immune system with appropriate cytokine stimulation</td>
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<td>11.</td>
<td>Antimutagenic and anticarcinogenic properties (protection against genotoxic agents)</td>
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<td>12.</td>
<td>Potential vehicle for the delivery of recombinant proteins and peptides in a site specific fashion to the human GIT</td>
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<td>13.</td>
<td>Lactose hydrolysis to improve lactose tolerance</td>
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Adapted from Collins et al. (1998)

The FAO and WHO Consultation in 2001 outlined the significance of linking strain-specific activity, not the source of the microorganism, to the health promoting effects passed onto the host. To further classify a strain as being probiotic, in vivo evaluations using animal models and subsequent clinical trials in humans are considered essential to screen and validate potential probiotic strains (Havenaar et al., 1992; Berg, 1998; Guarner and Schaafsma, 1998; Dunne et al., 2001). However, due to animal welfare concerns, there has been a shift towards cell culture models that emulate the intestinal system for probiotic experimentations (Cencic, 2010). Probiotic treatments of cell cultures allow for molecular mechanisms to be investigated at the individual cell level. Further, the availability of intestinal and immune cell lines from human and many different animals provides a vast array of models to investigate mammalian cell responses to probiotics.
1.3 MICROENCAPSULATION – MECHANISMS TO PROTECT SENSITIVE PROBIOTIC STRAINS

Probiotics are incorporated into many food and beverage products for human consumption. Indeed, the most researched strains, such as *L. casei* strain Shirota and *L. acidophilus*, are found in dairy products, fermented beverages and foods. As outlined previously, probiotic bacterial viability is considered important for the health promoting effects conveyed to hosts (Fuller, 1989). However, a high proportion of cell viability is lost during food processing and long periods of storage. Other factors have also been proposed to contribute to low probiotic bacterial viability within commercial probiotic foods and beverages including high acidity in yogurt (Shah et al., 1995). Further, the survival of probiotic bacteria during transit through the GI tract can be compromised by the GI environment. The FAO and WHO (2001) outlined assessment guidelines for probiotic bacteria in food noting the properties considered favourable including survival and proliferation abilities within the GI tract. The administration of high concentrations of probiotic bacterial cells (10^8 CFU g⁻¹ of product) to balance cellular losses during GI transit have been suggested to overcome low viability cell numbers (Kailasapathy et al., 2008). In addition, other suggestions to improve bacterial viability during food processing and storage include reducing storage temperatures (Sakai et al., 1987), the use of whey protein concentrate to increase buffering capacity and hence, minimise pH decreases in yoghurt during processing and storage (Kailasapathy et al., 1996), two step-fermentation and stress adaptation (Shah, 2000). Delivery tools, such as microencapsulation, are also being investigated to improve the survival rate of probiotic strains.
The microencapsulation of probiotic bacteria offers the advantage of cellular protection against the harsh GI conditions; however, the ability to release these cells at sites of interest within the GI tract is also an important consideration for microencapsulated strains. A desired feature for microencapsulating polymers is the ability to protect the core material, such as probiotic cells, from the acidic conditions of the stomach and to release the cells under the alkaline conditions of the small intestine (Kailasapathy, 2008). The release of probiotic bacteria cells in the small intestine is desired due to the location of the Peyer’s patches; this would ensure increased interactions between probiotics and immunological tissue in this area (Kailasapathy, 2008). Calcium alginate and pectinate beads were shown to maintain their structure during in vitro simulated acidic conditions of the stomach with subsequent release of the contents in colonic-like conditions (Mandal et al., 2006b).

In a similar manner, the attributes for the release of L. casei strain Shirota from CCAS capsules were investigated in different regions of porcine GI contents ex vivo including the ileum, colon, duodenal, jejunal and stomach contents; most cells were reportedly released during incubation in the contents of the ileum at 8 h whereas 12 h was required for complete release in the colon, partial release occurred in the duodenal and jejunal contents with no significant release of cells in the stomach contents at 24 h (Iyer et al., 2005).

### 1.3.1 Microencapsulation of Probiotic Bacteria

Microencapsulated probiotic bacteria have been applied to a number of dairy foods including yoghurts, cheese, ice cream and milk powders (Anal and Singh, 2007). The protection provided by the capsule assists in maintaining cell viability during food processing. Alginate microencapsulation of L. acidophilus CSCC 2400
was shown to increase viability compared to non-microencapsulated cells in simulated gastric conditions (Chandramouli et al., 2004). Similar results were found by Kim et al. (2008) with increased viability of sodium alginate microencapsulated \textit{L. acidophilus} ATCC 43121. The authors also reported that bacterial activities including cholesterol reduction and intestinal cell adhesion were not affected (Kim, 2008). Microencapsulation of \textit{B. bifidum} was described to increase IgA production in BALB/c mice (Park et al., 2002). However, culture supernatant from \textit{B. bifidum} growth (conditioned medium) was shown not to induce IgA production and the authors suggested the cellular components of the microencapsulated bacteria were the activating elements for the observed immunological responses (Park et al., 2002).

To date, very little research has described the effects of the microencapsulated environment on probiotic bacteria activities. In addition, further investigations are required to determine the bacteria cellular distribution within the capsule, potential release of bacteria cells from the capsule and duration of maintaining high levels of cellular viability. Changes in the behaviour of probiotics due to the microencapsulated environment may potentially affect downstream applications including immunological responses to probiotic bacterial-produced soluble factors. This thesis investigated immune cell responses to the soluble factors produced by microencapsulated probiotics and used the response of the immune cells to free probiotic bacterial-produced soluble factors as a comparison.

1.4 GASTROINTESTINAL (GI) TRACT

The metabolism of food by the GI tract provides energy for the host. Numerous mechanical and biochemical processes utilise essential nutrients, water
and electrolytes to aid in the regeneration of body tissues and to provide energy for
cells. Essentially, complex molecules from consumed food are broken-down into
simple molecules that are utilised and processed by the cells of the GI system. These
simple molecules are absorbed from the GI lumen, across the layers of epithelial
cells lining the GI tract and into the blood and lymph vessels for circulation
throughout the host’s body.

The GI tract forms part of the GI system and includes the mouth (mastication
of food), pharynx, oesophagus, stomach (breaks down food), small intestine
(duodenum, jejunum and ileum – digests molecules and nutrients) and large intestine
(cecum and colon). Further assistance, for the processing and digestion of food, is
provided by ‘accessory organs’ that secrete substances into the GI tract such as
enzymes, mucus, bile salts and acid. The accessory organs form part of the GI
system (not the GI tract) and include salivary glands, liver, gallbladder and pancreas
(Fig. 1.1). These secretions occur at specific locations within the GI tract and are
relative to the function of that particular site e.g. hydrochloric acid in the stomach
assists in the breakdown of food and maintaining the populations of pathogenic
microorganisms from food products and the external environment.

The effectiveness of food digestion is influenced by various factors such as
the health of the host, the external environment and the type of food. Food is
processed essentially by hydrolysis and fermentation in the GI tract forming suitable
molecules for absorption (Ewing and Cole, 1994). Hydrolytic reactions are
conducted by numerous enzymes throughout the GI tract and gut fermentation
involves a large number of microorganisms that assist in the breakdown of
indigestible substances. However, the extent to which a host’s microbiota assists with this process is dependent on the host. The lumen of the GI tract is continuously in contact with the external environment via numerous encounters with microorganisms in consumed food and water, as well as the antigens of digested food. The GI tract has evolved mechanisms to not only contain these microorganisms and antigens within the GI tract, but also to form a symbiotic relationship that benefits the host.

The GI tract differs from species to species and is dependent on the individual’s metabolic requirements. Furthermore, each area of the GI tract differs relative to the activity and role of that site. For example, pre-gastric fermenters, such as ruminant animals (cows and sheep) conduct the microbial fermentation of indigestible substances in the rumen, therefore prior to the digesta being exposed to the small and large intestine; whereas, the microbiota population in hindgut fermenters (i.e. non-ruminant, simple stomached animals such as man, pigs and poultry) performs fermentation further down the GI tract, in the large intestine (Ewing and Cole, 1994). The symbiotic relationship between the host and GI tract microbiota is essential to the homeostatic state of the body. This is achieved through numerous interactions such as assisting with digestion and protection against pathogens; this will be discussed further in the following sections.

1.4.1 Functions of Microbiota in the GI Tract

A symbiotic relationship is formed between a host and their GI microbiota (commensal microbiota). Fundamentally, commensal microbiota participates in food digestion. Microbiota colonisation of the GI tract commences at birth, prior to which
The GI tract is sterile (O’Grady and Gibson, 2005; Bjorksten, 2006; Kligler et al., 2007; Parracho et al., 2007), and continues throughout an individual’s lifetime. The type of birthing (caesarean or natural births) and feeding practices (formula or breast-milk) is believed to influence the variety of microbiota colonisation within newborns/infants GI tract (Holzapfel et al., 1998; Salminen et al., 1998b; O’Grady and Gibson, 2005). An adult’s GI tract contains a diverse range of microbiota (Fig. 1.1), the majority of which are non-cultivable due to their fastidious growth requirements. Sequence analysis of human microbiota ribosomal RNA (rRNA) by Eckburg et al. (2005) identified at least 500 different species, the majority being bacteria, as well as a few Archaea and unicellular eukaryotes.

The microbiota of the GI tract participates in numerous activities. These include the fermentation of indigestible substances (Ewing and Cole, 1994; Guarner and Malagelada, 2003; Guarner, 2006), associations with the epithelial cells of the GI tract (Salminen et al., 1998b; Guarner and Malagelada, 2003; Guarner, 2006; O’Hara and Shanahan, 2006), protection against pathogenic microorganisms, and interactions with the gut-associated lymphoid tissue (GALT) resulting in subsequent immunological roles (Guarner and Malagelada, 2003; Guarner, 2006). It is thought that immunological functioning is influenced by the interactions of the gut microbiota with the organised lymphoid structures of the small intestinal mucosa (Guarner, 2006). The function and activities of the GI tract influence the quantity and type of microbiota within that particular region. As shown in Figure 1.1, low numbers of microorganisms reside in the stomach area due to the high acidity content, whereas the colon contains high quantities of microbiota due to the slow transition of contents. This allows the microbiota to proliferate and survive by
fermenting indigestible material as well as endogenous secretions (O'Grady and Gibson, 2005; Guarner, 2006). The number of bacterial cells in the adult human GI tract is approximately ten times more than the amount of eukaryotic cells in the adult human body (Tancrede, 1992; Palmer et al., 2007).

The microbiota of an individual is distinct and contains dominant and subdominant species (Guarner, 2006) as influenced by environmental conditions, diet, antibiotic use and health status, among other factors (Table 1.2). Furthermore, categorisation of the colonisation pattern by microbiota of the GI tract has been used to describe the nature of the microorganisms. Autochthonous microorganisms will remain permanent residents of the GI tract in the area where they originally colonised, whereas, allochthonous microorganisms will have a transient nature inhabiting regions other than the area they originally colonised (Tannock, 1999; O'Grady and Gibson, 2005). However, for both types, these microorganisms will inhabit areas of the GI tract based on suitable conditions for their survival.
Figure 1.1: The anatomy and microbiota composition of the adult human gastrointestinal tract.

**Oral cavity**
- $10^{11}$ cells/g of dental plaque
- *Streptococci, Lactobacilli, Actinomyces, Fusobacteria, Neisseria & Veillonella*
- **pH**: Neutral pH, high bacterial growth

**Colon**
- $10^9 - 10^{12}$ CFU/ml
- *Bacteroides, Bifidobacteria, Streptococci, Fusobacteria, Enterobacteriaceae, Lactobacilli, Clostridia, Veillonella, Proteus, Staphylococci, Pseudomonas, yeasts & protozoa*

**Proximal colon** (cecum, ascending & transverse colon) **pH**: 5.0 – 6.0, high bacterial growth

**Distal colon** (descending colon) **pH**: Neutral pH, low bacterial growth

**Stomach & Duodenum**
- $10^4 - 10^5$ CFU/ml
- *Lactobacilli, Streptococci & Yeasts*
- **Stomach pH**: 2.0 – 3.5, low bacterial growth
- **Duodenum pH**: 4.0 – 5.0, low bacterial growth

**Jejunum & ileum**
- $10^4 - 10^7$ CFU/ml
- *Lactobacilli, Streptococci, Enterobacteriaceae, Bacteroides, Bifidobacteria & Fusobacteria*
- **Jejunum & ileum pH**: 4.0 – 5.0, moderate bacterial growth
Table 1.2. Factors and influences that potentially affect the microbiota of the GI tract

<table>
<thead>
<tr>
<th>Function</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Host mediated factors</td>
<td>pH, secretions such as immunoglobulins, bile, salts, enzymes</td>
</tr>
<tr>
<td></td>
<td>Motility e.g. speed, peristalsis</td>
</tr>
<tr>
<td></td>
<td>Physiology e.g. compartmentalisation and available adhesion sites</td>
</tr>
<tr>
<td></td>
<td>Exfoliated cells, mucins, tissue exudates</td>
</tr>
<tr>
<td></td>
<td>Interactions with the immune system</td>
</tr>
<tr>
<td></td>
<td>Transit time in the intestine and peristals</td>
</tr>
<tr>
<td></td>
<td>Age of the host</td>
</tr>
<tr>
<td></td>
<td>Antibiotic use</td>
</tr>
<tr>
<td>Microbial factors</td>
<td>Adhesion</td>
</tr>
<tr>
<td></td>
<td>Motility</td>
</tr>
<tr>
<td></td>
<td>Nutritional flexibility</td>
</tr>
<tr>
<td></td>
<td>Spores, capsules, enzymes, antimicrobial components</td>
</tr>
<tr>
<td></td>
<td>Generation time</td>
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<tr>
<td></td>
<td>Bacterial metabolite production</td>
</tr>
<tr>
<td></td>
<td>Antimicrobial substance production</td>
</tr>
<tr>
<td>Microbial interactions</td>
<td>Synergy</td>
</tr>
<tr>
<td></td>
<td>Metabolic cooperation</td>
</tr>
<tr>
<td></td>
<td>Growth factors and vitamin excretion</td>
</tr>
<tr>
<td></td>
<td>Changes to Eh, pH, O₂ tension</td>
</tr>
<tr>
<td></td>
<td>Antagonism/stimulation</td>
</tr>
<tr>
<td></td>
<td>Short-chain fatty acids, amines</td>
</tr>
<tr>
<td></td>
<td>Changes to Eh, pH, O₂ tension</td>
</tr>
<tr>
<td></td>
<td>Antimicrobial components</td>
</tr>
<tr>
<td></td>
<td>Nutritional requirements, etc.</td>
</tr>
<tr>
<td>Diet</td>
<td>Composition, non-digestible fibres, drugs, etc.</td>
</tr>
</tbody>
</table>

Modified after Holzapfel et al. (1998)

The microbiota of the GI tract forms a symbiotic relationship with the host through a number of interactions with intestinal and immunological cells, and metabolic activities (including the production of soluble factors and antimicrobial
substances) that contributes to the host’s health. Such a microbiota profile is resultant from the acquiring and persistence of microorganisms during an individual’s lifetime in response to dietary, environment and other factors (O’Grady and Gibson, 2005). It is possible that the microbiota of the human GI tract have adapted to processed foods in addition to natural occurring food such as fruits and vegetables.

The activity of the microbiota is influenced by the environment of the GI tract with particular areas providing more nutritional support for the growth of certain microorganisms. For instance, the ascending and transverse (proximal) colon contains high levels of bacterial growth due to the high quantity of substrates from digested food, a low pH due to the production of acid from bacterial fermentation activities and a rapid transit; whereas, the descending (distal) colon has slower bacterial growth due to factors such as lower production and availability of substrates, a near neutral pH and slower transition period (Salminen et al., 1998a; Guarner, 2006). One of the primary activities of the GI tract microbiota is the fermentation of undigested materials from the upper digestive tract including dietary carbohydrates, resistant starches that are metabolised to short-chain fatty acids (SCFA), non-starch proteins (NSP) such as hemicellulose, pectins and gums (Salminen et al., 1998a; O'Grady and Gibson, 2005; Ramakrishna, 2007) and endogenous mucus (Guarner, 2006). The production of SCFA by saccharolysis produces butyrate, acetate, propionate and lactate that is utilised as energy for metabolism in the large intestinal mucosa, as well as for the growth of intestinal epithelial cells (O'Grady and Gibson, 2005; Guarner, 2006). In addition, the microbiota of the GI tract is also associated with the stimulation of the immune
system, metabolism of potential carcinogenic products, and the production of digestive and protective enzymes (Holzapfel et al., 1998; Ramakrishna, 2007).

The microbiota provides physical protection (the “barrier effect”) for the GI tract by competing with pathogenic microorganisms for nutritional metabolites and adhesion sites on the intestinal epithelium (Holzapfel et al., 1998; Salminen et al., 1998b; Blum et al., 2002; O’Grady and Gibson, 2005; Corthesy et al., 2007). Furthermore, the microbiota produces antimicrobial substances, such as bacteriocins, that inhibit the growth of other bacteria preventing overgrowth and potential pathogenic states (Guarner, 2006). However, in the event where the balance of microbiota is disrupted, commensal microorganisms can become pathogenic causing diseased states. For example, Candida spp. and Clostridium spp. are non-pathogenic to the host in low quantities; however, can become harmful in situations that favour their abundance in growth (O’Grady and Gibson, 2005). Immunological tolerance to microbiota was proposed to be generated by the mucosal immune system upon exposure to commensal microbiota with the immune system recognising the microorganisms’ antigens and subsequently the host’s immunological system can distinguish between resident microbiota and pathogens (Turroni et al., 1998).

The ability of microorganisms with non-pathogenic characteristics to permeate the intestinal epithelial layer and induce an immune response has been suggested and related to immune tolerance of those particular microorganism’s antigens. Immunoglobulin A (IgA) secretion, an adaptive immune response, also assists in protecting the mucosal surfaces from pathogenic microorganisms (Cerutti, 2008). The secretion of interleukins and other chemical signals by intestinal and
immunological cells stimulates the production of IgA molecules that subsequently covers the antigen and initiates a cascade of immunological events. The importance of microbiota in assisting the intestinal tract to defend against pathogenic microorganisms was demonstrated by the colonisation of germ-free mice with microbiota. Reports outlined the development of an immune system in these mice whereas germ-free mice without microbiota were found to have a defective immune system (Macpherson and Harris, 2004). Isolauri et al. (2001) described increased antigen transport across the gut mucosa in the absence of intestinal microbiota suggesting that such transportation of antigens assists in the development of the intestine’s defence barrier. It is believed that the interaction of commensal microbiota with the immune components of the GALT results in an induction of tolerance allowing both mucosal and systematic immune systems to differentiate between commensal and pathogenic microorganisms (Isolauri et al., 2001). Subsequently, a response is induced for microorganisms recognised as being pathogenic or no response for those recognised as being commensal microbiota. Tolerance induction by microbiota colonisation of the intestinal tract has been found to stimulate GALT maturation due to the antigenic exposure and resulted in the increase of IgA producing cells (Isolauri et al., 2001). In addition, decreased bacterial cell translocation across the intestinal epithelial was reported and associated with the development of the intestine’s immunologic defence and IgA recognising specific antigens (Isolauri et al., 2001).

1.5 **GUT-ASSOCIATED IMMUNOLOGICAL TISSUE (GALT)**

The gastrointestinal tract is continuously in contact with antigens from food and microorganisms. In addition to digesting and absorbing nutrients for the host’s
metabolic requirements, the intestinal tract provides a protective barrier for the host’s underlying connective tissue from potentially pathogenic antigens. The mucosal-associated lymphoid tissue (MALT) provides immunological protection for mucosal surfaces and is composed of both innate (non-specific response) and adaptive (immunological memory) immunological components. The major functions of the MALT include (Simpson and Wetzler, 2004):

- Primary lymphoid (immune cell) development,
- Generation and intensification of mucosal immune responses, and
- Production of local mucosal immunological responses.

The MALT protects mucosal sites by IgA production, specific activated lymphocytes (T and B cells) and mucous membranes. Forming part of the MALT, the GALT provides immunological protection at the intestinal level. Components of the GALT include tonsils, adenoids, appendix (small intestine) and Peyer’s patches (PPs). Innate immunological defences (gastric acid, bile, saliva and mucous membranes) assist the GALT in defending the GI system from pathogens. The GALT also functions to reduce hypersensitive reactions to food proteins and normal flora (Britti et al., 2006) by inducing immunological tolerance (mediated by adaptive immunological cells, T and B lymphocytes) to commonly encountered, non-pathogenic antigens. To gain an understanding of the immunological events occurring at the GALT level, the structure and interactions of the GALT, immune tissues and cells are described in the following sections.
1.5.1 Structure and function of the GALT

All mucosal epithelial surfaces in the body are comprised of a single layer of gut epithelial cells that forms the first line of defence – this also applies to the intestinal mucosa. The gut epithelial contains many different cell types including:

- Goblet cells that produce mucous and antimicrobial molecules such as lysozyme (breaks down bacterial cell wall components) (Mak and Saunders, 2008),
- Enterocytes that secrete hormone-like molecules to stimulate surrounding cells and function in nutrient absorption (Mak and Saunders, 2008), and
- Microfold cells (M cells, also known as membranous cells) that uptake intact microorganisms and antigens from the intestinal lumen and deliver them to the underlying PPs for adaptive immunological processing (Parham, 2009).

Tight junctions between enterocytes assist in pathogen containment by inhibiting pathogen, peptide and macromolecular movement across these cells. Furthermore, the apical surface of enterocytes possesses microvilli projections covered in the negative-charged glycocalyx, a layer of membrane-anchored glycoproteins. This forms the brush border and functions in impeding pathogenic access to microvilli. Shedding of the microvilli in enterocyte vesicles (epithelial shedding) also assists in pathogen removal. The follicle-associated epithelium (FAE) separates the intestinal lumen from the underlying connective and lymphoid tissue (Mowat, 2003). The FAE consists of M cells, enterocytes and expresses the chemokine CCL20 (Rumbo et al., 2004) that is involved in DC mobilization in the subepithelial of PPs (Iwasaki and Kelsall, 2000). Underneath the intestinal epithelial layer and FAE is the lamina propria (underlying connective tissue). The lamina propria consists of the
aggregated tissues, Peyer’s patches (PPs), and single, lymphoid follicles (with B-cell germinal centres), and the non-aggregated tissues, mesenteric lymph nodes (MLN) and lymphoid tissues (Schley and Field, 2002). Figures 1.2 and 1.3 show the organisation and immunological components of the GALT, respectively. Adaptive immune cells, T and B cells, are found within the PPs, non-aggregated lymphoid tissue, intraepithelial and MLN. These cells interact with innate immune components including dendritic cells.

Figure 1.2. Gut-associated lymphoid tissue. Source: Macpherson & Harris (2004)
Antigens are transversed from the intestinal lumen to the underlying lymphoid tissues by M cells. Internalisation of antigens occurs by macropinocytosis, clathrin-mediated endocytosis or phagocytosis. The antigens are transcytosed from the intestinal lumen to the underlying PPs consequently initiating a mucosal immunological reaction incorporating innate (i.e. dendritic cells) and adaptive immune cells (T and B cells) (Mak and Saunders, 2008). It is believed that since M cells do not express major histocompatibility complex (MHC) class II molecules, intact antigens are passed onto antigen-presenting cells (APCs) within the PPs and underlying regions (Mowat, 2003). Dendritic cells (present in the MLNs, PPs and lamina propria) also sample antigens and GI microorganisms. These cells are able to directly sample GI luminal antigens by forming tight-junction like structures with intestinal epithelial cells (IECs) (Rescigno et al., 2001) and projecting dendrites into the GI lumen (Coombes and Powrie, 2008). Upon the recognition of pathogen-associated molecular patterns (PAMPs), DCs internalizes microbial components,
mature and present microbial peptides on MHC molecules (Kindt et al., 2007). Whole bacteria taken up by dendritic cells are able to survive for several days (Macpherson and Uhr, 2004). Dendritic cells then migrate through lymphatic vessels to nearby lymph nodes, where they present the antigens to T cells. Subsequent interactions between dendritic cells and adaptive immune cells allows for the differentiation between pathogenic and non-pathogenic antigens. This results in the activation of T and B cells in the PPs and the migration of intact bacteria to the MLNs for further antigenic presentation to T cells. Within the PPs, activated B cells undergo immunoglobulin class switching from IgM to IgA (Strober et al., 2005). However, B cellular differentiation and class-switching to IgA-production is believed to be influenced by T cell interactions (Strober et al., 2005). Nonetheless, B cell class-switching also occurs via T cell independent pathways (Cerutti, 2008). Activated T and B cells are able to travel throughout the bloodstream via the thoracic duct and back to the intestinal mucosa (Macpherson and Harris, 2004); priming of other immunological tissues occurs throughout the body. Therefore mucosal and systemic activation is believed to occur from microbiota exposure in the GALT.

1.5.2 Probiotics and the GALT

Immunological tolerance is believed to be one of the mechanisms by which probiotics convey immunomodulatory activities. As outlined in Section 1.4.1, development of the immune system and GALT is influenced by the microbiota population within the intestinal tract. This concept can be demonstrated by the colonisation of germ-free mice with associated immunological development (Macpherson and Harris, 2004), the strengthening of intestinal defence barriers and increased production of IgA molecules that recognise specific antigens (Isolauri et
al., 2001). It has been suggested that microorganisms without pathogenic characteristics are able to permeate the intestinal epithelial layer and induce an immunological response. This notion has been related to the immune tolerance of that particular microorganism’s antigens (Boirivant and Strober, 2007).

The ability of probiotics to adhere to intestinal epithelial cells is proposed as an ideal property for colonisation purposes and subsequent health benefits. The adhesion abilities of probiotics also allows for physiological interactions with intestinal epithelial cells. The dependency of intestinal epithelial cell activation and subsequent immunological activation by bacteria (including probiotics) is proposed to require direct contact between the cell-surface molecules on microorganisms and intestinal cells (Herick and Levkut, 2002). Intestinal epithelial cells express pathogen recognition receptors (PRRs) that recognise microbial motifs or PAMPs. In respect to evolution, PAMPs are highly conserved and constant in microorganisms of the same class (Winkler et al., 2007); therefore mammalian cells are able to recognise different types of microorganisms and initiate appropriate immunological responses. Uematsu and Akira (2004) suggested that PAMPs should be referred to as microorganisms-associated molecular patterns (MAMPs) in consideration that all microorganisms are not necessarily pathogenic. Mammalian cells produce two types of PRRs to detect PAMPs: Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain receptors (NODs). Upon interactions between TLRs and PAMPs, a signal cascade is initiated with associated protein-protein interactions and conformation changes, phosphorylation, activation of transcription factors, and cytokine and chemokine secretion that induces innate and adaptive immune responses (Medzhitov et al., 1997). The production of cytokines, chemokines and
inflammatory effector molecules depends on the dose and type of PAMPs interacting with the TLRs (Winkler et al., 2007). The stimulation of intestinal epithelial cells via TLRs and probiotic PAMPs interactions has the potential to indirectly induce the immune system.

Antigen processing via intestinal epithelial cells is proposed as one of many interaction mechanisms between probiotic bacteria and immunomodulatory activities of the GALT (Herick and Levkut, 2002). The transportation of antigens from the intestinal lumen to underlying connective tissue is proposed as a necessary event to initiate specific T cells and local immune responses (Herick and Levkut, 2002). Processed bacterial products (antigens) by innate immune cells, such as dendritic cells, are presented to naïve T and B cells for further immunological attention.

1.6 IMMUNE SYSTEM

The immune system is divided into two components: innate and adaptive. Innate immunity is a non-specific response to pathogens and hence, does not acquire an immunological memory. Natural killer cells (cytotoxic cells) and phagocytes such as macrophages, monocytes, neutrophils, dendritic cells and granulocytes, are cells of the innate immune system. Known as the first line of defence, the innate immune system also includes anatomical barriers (e.g. skin and mucous membranes) and biochemical defences such as complement system and lysozyme secretion. The adaptive (acquired) immune system is a specific immunological response to pathogens. Adaptive immune cells recognise specific physiological components (antigens), such as bacterial cell wall molecules. Upon interacting with a pathogenic antigen, an immunological memory is created and stored for future encounters. The
The adaptive immune system consists of T and B cells and is further divided into two components: humoral and cell-mediated immunity (Fig. 1.4). Humoral immunity interacts with cell-mediated immunity during responses to infections (Elgert, 1996) and involves an antibody-mediated response initiated by B cells. This provides protection against circulating extracellular antigens e.g. bacteria, microbial exotoxins and viruses in extracellular phages. Cell-mediated immunity involves T cells that direct antigen-specific responses and protects against intracellular parasites e.g. viruses. Two types of T cell subpopulations assist with such responses: helper T (T\textsubscript{H}) cells and cytotoxic T (T\textsubscript{C}) cells. The T\textsubscript{H} cells are further divided into T\textsubscript{H}1 and T\textsubscript{H}2 cells based on cytokine secretion patterns and functional activities – T\textsubscript{H}1 and T\textsubscript{H}2 cells direct cellular and humoral immunity, respectively (Fig. 1.4). Regulatory T (T\textsubscript{reg} or T\textsubscript{H}3) cells are another subset of T cells that assist in regulating T\textsubscript{H} cellular functions and activities to maintain homeostasis within the intestinal environment (Gillingham and Lescheid, 2009). A balance in T\textsubscript{H}1/T\textsubscript{H}2 activities is important in maintaining homeostasis. In the event of an imbalance, hypersensitivity occurs from the overproduction of either T\textsubscript{H}1 or T\textsubscript{H}2 cells (Fig. 1.5).

Polarization of naïve T cells initially involves interactions with APCs, predominately DCs. Upon interacting, the release of cytokines directs either T\textsubscript{H}1 or T\textsubscript{H}2 production. Cell-mediated immunity (T\textsubscript{H}1-directed) is associated with immunological responses to intracellular pathogens such as viruses, certain bacteria, yeast, fungi and protozoans; also prevents tumour cell development (Gillingham and Lescheid, 2009). Inflammation is resultant from cell-mediated/T\textsubscript{H}1 directed immunity with the production of pro-inflammatory cytokines such as IL-12, IFN-\gamma and TNF-\alpha. These cytokines direct macrophages to produce further pro-
inflammatory cytokines. In the event of persistent T<sub>H1</sub>-mediated inflammation, diseases such as Crohn’s disease in the GI tract occur (Neurath et al., 2002). Humoral immunity (T<sub>H2</sub>-directed) results in the activation of specific B cells promoting antibody class-switching based on the type of stimulus/antigen initiating the immunological response. Cytokines released by T<sub>H2</sub> cells include IL-4, IL-5, IL-10 and IL-13. In the events of uncontrolled T<sub>H2</sub> activity, diseases such as allergies, eczema, asthma and ulcerative colitis occurs (Gillingham and Lescheid, 2009). Regulatory activities occur from the production of IL-10 by Treg cells that down-regulates T<sub>H1</sub> activities and hence associated inflammatory responses. Also, Treg cells produce transforming growth factor β (TGF-β), a cytokine involved in modulation of T<sub>H1</sub> and T<sub>H2</sub> activities (Kidd, 2003).

**Figure 1.4.** Humoral and cell-mediated immune responses. Modified after BiondVax Pharmaceuticals Ltd. (2007)
Innate immunity is the primitive form of immunological defence for vertebrates. The non-specific mechanism of innate immunity provides a rapid, short-term protection against infections. Most innate immune components are present at sites in the body prior to the onset of infections; reactions are initiated by the recognition of antigenic PAMPs found on pathogens (Kindt et al., 2007). Pathogen recognition receptors (PRRS) on mammalian cells, e.g. TLRs, are able to recognise PAMPs belonging to bacteria, yeasts, viruses or parasites. In such an event, immunological
responses are initiated accordingly to the pathogen. However, the evolution of vertebrate and mammalian innate immune defences is linked with the evolution of microbial evasion mechanisms. Pathogens induce infections by overcoming innate defences. Disruptions to physical barriers e.g. animal bites can facilitate the proliferation of infectious states. The body responds to pathogenic damage by inducing inflammation. Blood flow increases to the infected area with associated localisation of phagocytic cells, fibroblasts/tissue cells (contains infections and repairs damage) and extracellular matrix components involved in cellular and tissue regeneration. Phagocytic cells ingest extracellular materials, including microorganisms, by endocytosis, receptor-mediated endocytosis or pinocytosis. Upon secreting signalling molecules, these cells initiate an immunological response by alerting other innate cells and cells of the adaptive immune system. In addition, antimicrobial peptides and biochemicals are produced.

The non-specific protection provided by the innate immune system encompasses the following functions:

- Recruitment of innate immune components e.g. macrophages, complement system, phagocytes, etc. to the site of infection by secreting chemical signals;
- Immune cellular identification of pathogenic PAMPs with PRRs;
- Activates the complement cascade,
- Promotes the clearance of dead cells and/or antibody complexes,
- Activates the adaptive immune system by innate antigen presenting cells (APCs) such as dendritic cells, and
- Interacts with the adaptive immune system to initiate the removal of foreign substances in organs, tissues, blood and lymph.
Close interactions between the innate immune components, dendritic cells, and adaptive immune cells provides the basis of immunological reactions at the GALT.

### 1.6.2 Adaptive immunological responses

The acquired immune system responses are mediated by lymphocytes (T and B cells) and initiated by interactions with the innate immune system. The specific targeting of pathogens by lymphocytes encompasses the following features:

- Recognition of specific antigens: lymphocytes are able to distinguish slight dissimilarities between antigens such as protein molecules that differ by one amino acid,

- Diverse recognition abilities: lymphocytes can recognise a broad range of organisms based on intracellular and extracellular components and discriminate between subtle differences,

- Immunological memory generation: upon second and further encounters, an immunological memory is generated with the same antigen that ensures a heightened state of immune reactivity, and

- Differentiation between self and non-self cells: this mechanism allows for the avoidance of adaptive immunological responses to self-cells. The failure to discriminate between self and non-self cells could lead to serious and fatal outcomes.

Consequently, adaptive immunological responses occur after the host is exposed to a pathogen. The importance of discrimination between self and non-self cells by the adaptive immune system has been outlined above. This mechanism is possible due to the expression of the major histocompatibility complex (MHC) on the host’s
cells which assists the adaptive immune system in identifying self-cells and antigens (potentially of pathogenic descent). There are two types of MHC proteins: all host cells express class I proteins (MHC class I) and only APCs (macrophages, dendritic cells and B cells) express class II proteins (MHC class II). The MHC plays a significant role in T cell immunity and B cell selection processes. The development, differentiation and maturation stages of T and B cells are able to be distinguished by the extracellular expression of CD markers. The production of cytokines and expression of CD markers are used to indicate the stage of T and B cell maturation, and the type of adaptive immunological reaction being performed.

1.7 PROBIOTIC IMMUNOMODULATORY ACTIVITIES

Immunomodulatory activities have been attributed to the strain level of probiotics. The activation of mucosal and systematic immune responses has been described for probiotics. To date, most research describes innate immunological enhancement by probiotics, in particular dendritic cellular reactions, as opposed to adaptive immune enhancement. Physiological interactions between probiotics and cells of the GALT system influence subsequent immunological responses. As mentioned previously, interactions between cells of the intestinal tract and microflora reinforce the intestinal barrier and mediate immunological development. Local and systemic immunological enhancement is believed to occur from the ingestion of probiotics (outlined below).

It has been proposed that LAB utilise distinct pathways of gut internalization to interact with immunocytes in underlying tissues and the type of internalization mechanisms are different for all strains (Winkler et al., 2007). The different
responses can be attributed to the recognition of PAMPs by the intestinal epithelial cell TLRs. Subsequent cellular activities are conducted relative to the particular PAMPs detected. However, further understanding is required to determine the molecular events behind these effects (Herick and Levkut, 2002). Probiotic bacteria have been proposed to regulate the balance between necessary and excessive immune responses, in particular, assisting the immune system’s response to infections by enhancing Th1 cellular activities with the production of proinflammatory cytokines, enhanced phagocytic activities and macrophage activation (Winkler et al., 2007). Maldonado Galdeano et al. (2007) suggested that probiotic strains selected for inclusion in probiotic products should consider the ability of strains to maintain intestinal homeostasis while improving gut immune responses. The authors outlined properties considered to contribute to this concept including high cell viability and resistance to bile and low pH, persistence within the intestinal tract, adherence to intestinal epithelial cells and the ability to interact or convey signals to gut immune cells (Maldonado Galdeano et al., 2007).

Although interactions and activities associated with probiotic bacteria and the immune system have been recognised (outlined below), further research is required to determine specific effects between strains and the host’s immune system. Differences between in vitro and in vivo outcomes have been described for two probiotic strains, L. johnsonni NCC 533 and L. paracasei NCC 2461, including different colonisation efficacy of the intestinal lumen by L. johnsonni (colonising at high levels) and L. paracasei (rendering low levels); however, similar growth, adherence and survival in vitro were reported for the two strains (Ibnou-Zekri et al., 2003). For characterisation of effects by specific probiotic strains, Delcenserie et al.
(2008) suggested establishing profiles of cytokine secretion by lymphocytes, enterocytes or dendritic cells for new probiotic strains and to also use models emulating the mucosa to further ascertain the interactions between probiotic bacteria and this environment.

### 1.7.1 Probiotic effects on the innate immune system

The health benefits associated with probiotics and the innate immune system encompasses enhanced immunological responses and activities. Many investigations research the role of probiotic bacteria in innate immunomodulatory activities against pathogens and the conditioning of the gastrointestinal tract environment by probiotic strains and microbiota. In particular, the focus of innate immunological response research is based on dendritic and natural killer (NK) cellular responses due to the role of these cells.

The ability to enhance cancer cell targeting by NK cells is driving the interest in the effects of probiotics on these innate immune components. Natural killer (NK) cells are large, granular cytotoxic lymphocytes without T or B cell receptors that target and kill tumour cells and some cells infected with viruses; NK cells also participate in antibody-dependent cell-mediated cytotoxicity (Kindt et al., 2007). *Lactobacillus casei* strain Shirota (LcS) has been reported to enhance effects on NK cellular activity and proliferation. Matsuzaki and Chin (2000) described increases in murine NK cellular activities by the LcS following oral administration. The authors stated that these observations confirmed that oral administration of this strain enhanced innate immunological responses (Matsuzaki and Chin, 2000). Similar results were obtained by Takeda and Okumura (2007) for the treatment of middle-
aged and elderly volunteers who ingested fermented milk containing viable LcS. Increased activity levels for NK cells were described and the authors suggested “that daily intake of LcS provides a positive effect on NK-cell activity” (Takeda and Okumura, 2007). A model using induced carcinogenesis reportedly exhibited delayed onset of tumour development and reduced incidence in mice fed LcS (Takagi et al., 2001). In addition, NK cell cytotoxicity was found to be higher in mice fed with LcS compared to the control group (Takagi et al., 2001). These examples demonstrate the influence of ingested probiotics at a systemic immunological level.

Dendritic cells form the bridge between innate and adaptive immune systems being one of the main innate cells to interact with adaptive immunological cells in the GALT. At the GALT level, DCs have been implicated in the maintenance of tolerance towards commensal microflora and generation of protective immunological responses to pathogens (Coombes and Powrie, 2008). It has been reported that the location of DCs determines their functional properties. For instance, activated DCs within the PPs reportedly produced higher levels of IL-10 compared to DCs in the spleen as well as exhibiting higher levels of MHC class II molecules and induced T_{H}2 cell development, IL-4 and IL-10 production (Iwasaki and Kelsall, 1999). The authors also reported that DCs of the PPs primed T cells to produce reduced levels of IFN-γ compared to spleen DCs (Iwasaki and Kelsall, 1999). In addition, the location within the intestinal tract, i.e. PPs, MLNs, small intestine lamina propria and colonic lamina propria, also influences the type of activities DCs perform (Coombes and Powrie, 2008). Therefore, the DC population have adapted to particular areas within the body to convey suitable immunological responses upon stimulation.
The influence of probiotics on DCs is being explored to elucidate the effects on dendritic cellular regulatory functions. In particular, much research is conducted to determine the effects of such dendritic cellular regulatory activities on adaptive immune cells following probiotic treatments. Foligne et al. (2007) reported LAB stimulation encouraged dendritic cellular regulatory functions by targeting specific pattern-recognition receptors and pathways. In addition to the regulatory effects on dendritic cells, LAB treatment resulted in the reduced expression of pro-inflammatory genes in mice with induced colitis (Foligne et al., 2007). Dendritic cells play an important role in adaptive immune responses, in particular mediating T cell activities. As mentioned previously, DCs have the ability to retain live microorganisms up to several days allowing for the selective induction of IgA production providing protection for mucosal surfaces (Macpherson and Uhr, 2004). Such interactions occur from dendritic cellular sampling and presentation of live microorganisms to T and B cells. Dendritic cells treated with Lactobacilli were reported to regulate T cell responses favouring TH1 and Te polarization (Mohamadzadeh et al., 2005). Further, DCs exposed to L. acidophilus strain L-92 induced apoptosis in antigen-stimulated CD4+ T cells in vivo and in vitro (Kanzato et al., 2008). The authors suggested that this effect was due to the modulation of costimulatory molecular expression on DCs by L-92 that subsequently inhibited the T cell response (Kanzato et al., 2008). Such activities by DCs have potential treatment roles in immunological diseases, in particular those with hyperactive T cell activities.

Stimulation of macrophages by probiotics has also been reported. Gill et al. (2000) described enhanced phagocytosis by peripheral blood leucocytes and
peritoneal macrophages in mice treated orally with *L. rhamnosus* HN001 (DR20™), *L. acidophilus* HN017 and *B. lactis* HN019 (DR10™) in comparison to control mice. The authors also described enhanced adaptive immunological responses to these species including antibody reactions to both oral and systemically administered cells (Gill et al., 2000). Both innate and acquired immune responses to *L. casei* were investigated in BALB/c mice. The authors expressed the absence of specific antibodies against this strain; however, described enhanced innate immunological responses including the increased expression of cluster-of-differentiation (CD)-206 (roles in innate immune responses) and TLR-2 on innate immune cells including macrophages and DCs isolated from the PPs (Maldonado Galdeano and Perdigon, 2006). Recently, oral ingestion of non-probiotic *L. acidophilus* strains was described to sustain immune cell activation at normal levels throughout the innate immune system in BALB/c mice (Dogi et al., 2008). Comparisons were made with the probiotic strain *L. casei* CRL 431 that showed more evident immunological responses than the non-probiotic strains. The authors suggested that such effects could be attributed to the cell wall chemical structure or cytoplasmic antigenic molecules (Dogi et al., 2008).

Increased phagocytic activity of granulocytes and monocytes were exhibited in human volunteers consuming *L. acidophilus* 74-2 and *B. animalis* subsp *lactis* DGCC 420 indicating modulation of unspecific cellular immune responses (Klein et al., 2008). Macrophage responses to probiotic gram-positive and gram-negative bacteria were compared. *L. casei* strain Shirota and *E. coli* Nissle 1917 reportedly induced IL-12 and TNFα secretion by the murine monocyte/macrophage cell line J774A.1, however only *E. coli* Nissle 1917 induced IL-10 production (Cross et al.,
In addition, live bacteria stimulated higher levels of IL-10, IL-12 and TNFα secretions than heat-killed bacteria and only live *E. coli* Nissle 1917 induced IFN-α (Cross et al., 2004). Similarly, increases in TNFα and IFNγ levels (but not IL-10) in the PPs of BALB/c mice were also reported following the ingestion of non-pathogenic gram-positive, gram-negative and probiotic gram-positive strains (Dogi et al., 2010).

The majority of investigations have described the enhancement of innate immunological responses to probiotic bacteria. Links between innate and adaptive immune responses to probiotic bacteria have been made (Mohamadzadeh et al., 2005; Maldonado Galdeano and Perdigon, 2006; Kanzato et al., 2008). This suggests that probiotic bacterial interactions with the immune system involve multiple mechanisms interacting with both innate and adaptive immunological components. These interactions have the potential to convey health benefits to hosts.

### 1.7.2 Probiotic effects on the adaptive immune system

Adaptive immunomodulatory effects induced by probiotic bacteria include interactions with B-cells subsequently influencing IgA production, cell-mediated immunological responses by T-cells directing antigen-specific effects, as well as the immune cellular production of cytokines and surface CD marker expression (Delcenserie et al., 2008).

Probiotic bacterial effects on the adaptive immune response have included reports of increased levels of IgA-, IL-10- and IFN-γ-producing cells in BALB/c mice fed with both *Lactobacillus acidophilus* LAFTI L10 and *Lactobacillus*
paracasei LAFTI L26 (Paturi et al., 2007). Increased secretions of IL-10 and IFN-γ were also described by the authors who suggested that L. acidophilus and L. paracasei strains improved specific gut and systemic immune responses in mice as a consequence of ingesting the probiotic bacterial strains (Paturi et al., 2007). Long-term consumption of fermented milk (contained L. delbrueckii subsp. bulgaricus, Streptococcus thermophilus and L. casei) by BALB/c mice reportedly enhanced intestinal mucosa immunity through mediation by IgA⁺ cells and increased cytokine production of IL-10; enhancement of TNF-α, IFN-γ and IL-2 cells were also described (de Moreno de LeBlanc et al., 2008). These results suggest that probiotics improve the protection of the intestinal mucosal by stimulating cells of the intestinal and immune systems to establish a balance between T_H1 (inflammation) and T_H2 (allergy) activities. In an event of imbalances in T_H1/T_H2 activities, gastroenteritis develops due to the overstimulated and unregulated activities of T cell populations. To further support this, probiotic Lactobacilli and bifidobacteria, and their genomic DNA, were reported to modulate T_H1/T_H2 cytokine responses of peripheral blood mononuclear cells (PBMCs) to some allergens (house dust mite, Staphylococcus enterotoxin A and others) in a dose-dependent manner (Ghadimi et al., 2008). B. animalis and B. longum treatment of PBMCs induced the secretion of IFN-γ, TNF-α (T_H1 profile) and regulatory IL-17 production; the authors suggested profiling cytokine production to indicate the type of T_H responses to probiotic treatments (Lopez et al., 2010). Interleukin-10 is an anti-inflammatory cytokine found to enhance B cell survival, proliferation and antibody production amongst other effects (Male et al., 2006). Improved gut immunity was maintained and prevented gut inflammatory immune responses due to the down-regulation by cytokines such as IL-10 (de Moreno de LeBlanc et al., 2008). Increased CD4⁺ and CD8⁺ cell populations
in the lamina propria of the intestine (compared to a control model) were also observed (de Moreno de LeBlanc et al., 2008) indicating humoral and cell-mediated immunological enhancement. Ibnou-Zekri et al. (2003) described the inducement of germinal centre formation and IgA-bearing lymphocytes in the mucosa of germfree mice by *L. johnsonii* NCC 533 and *L. paracasei* NCC 2461. The authors proposed that activation of mucosal B cell responses occurred as a result of colonisation by these strains (Ibnou-Zekri et al., 2003). A dog model was employed to determine the effects of LAB on faecal IgA, circulating IgG and IgA levels, and lymphoid cell subset proportions (Benyacoub et al., 2003). The authors reported higher levels of faecal IgA and canine distemper virus (CDV) vaccine-specific circulating IgG and IgA in puppies treated with *Enterococcus faecium* (SF68) compared to the control (Benyacoub et al., 2003). However, no differences were observed in CD4⁺ and CD8⁺ T cell subsets in the treated and control puppies, but proportions of mature B cells (CD21⁺/MHC class II⁺) were higher in probiotic-treated puppies (Benyacoub et al., 2003). A concept rarely investigated is the treatment using host-specific probiotic strains. Commensal and non-commensal probiotic strains of BALB/c mice were investigated to comparatively determine the effects of host-specific and host non-specific strains on IgA cells, proinflammatory cytokine and regulatory cytokine production (Dogi and Perdigon, 2006). The authors reported no differences in the number of IgA secreting cells, however, non-commensal probiotics increased TNF-α production whereas commensal probiotics increased IFNγ production; both strains induced different patterns for IL-4 and IL-10 production (Dogi and Perdigon, 2006). Internalisation pathways were also evaluated with no differences observed between the commensal and non-commensal strains (Dogi and Perdigon, 2006). The authors concluded that both commensal and non-commensal probiotics interact with the
intestine in the same way and were able to reinforce the gut mucosal immune system
associating commensals with intestinal homeostasis regulation and non-commensals
with immune activation (Dogi and Perdigon, 2006).

The effects from probiotic ingestion have been proposed to extend beyond
mucosal tissues of the intestinal system to other tissues of the body. Systemic
immunological priming from ingested probiotics has been described. The
immunomodulatory properties of seven probiotic strains were investigated in a pilot
study involving human volunteers; serum levels of IgA, IgG and IgM were
monitored. The authors reported immunoglobulin level increases (compared to
controls) from treatments with six probiotic strains (Paineau et al., 2008). Ingested
LAB reportedly increased bronchial IgA⁺ B cells with the authors suggesting that
interactions between LAB and intestinal epithelial cells/M cells within the PPs were
capable of inducing IgA production (Perdigon et al., 1999). The authors also noted
that IgM⁺ cells increased when the stimulus did not induce IgM to IgA class-
switching; increases in CD4⁺ T cell populations were suggested to result from
interactions of the LAB with PPs and the subsequent enhancement of B and T cell
migration (Perdigon et al., 1999). Systemic and intestinal immunological
enhancement was reported in piglets treated with *L. fermentum* I5007. Increases in
peripheral blood CD4⁺ T cells and intestinal mucosa IFN-γ and TNF-α levels were
described; the authors concluded that *L. fermentum* I5007 can improve mucosal
immunity (Wang et al., 2009).

There is much interest in the immunomodulatory activities and mucosal
tolerance induced by probiotics. *L. reuteri* and *L. casei* primed monocyte-derived
DCs were reported to induce regulatory T cell development, these cells subsequently produced IL-10 inhibiting T\textsubscript{H}1 activities (Smits et al., 2005). Probiotic priming of Treg cell development presents potential treatment options for gastroenteritis and mucosal tolerance. This may be achieved by downregulating T cell-associated inflammatory and allergy responses to antigens. Suppression of BALB/c CD4\textsuperscript{+} cellular proliferation \textit{in vitro} was described by von der Weid et al. (2001) following treatment with \textit{L. paracasei} NCC2461, this effect was reported to be dose-dependent and does not appear to affect cell viability. They noted a decrease in T\textsubscript{H}1 and T\textsubscript{H}2 effector cytokines while IL-10 production was maintained and TGF-β production was induced in a dose-dependent manner. In addition, CD8\textsuperscript{+} T cell-mediated colitis was down-regulated by increased suppressive activities of Foxp3\textsuperscript{+}CD4\textsuperscript{+} T-reg cells in the colon lamina propria of immunocompetent mice following \textit{L. casei} DN-114 001 treatment (Hacini-Rachinel et al., 2009). Further, Hacini-Rachinel et al. (2009) suggested that protection against acute and relapsing colitis by effector/memory CD8\textsuperscript{+} (respectively) was provided by CD4\textsuperscript{+} T-reg cells.

Published research results have described enhanced immunological responses to probiotic bacteria. The establishment of cytokine and CD marker profiles for individual probiotic strains would provide an indication of the type of immunological responses to strains. Furthermore, such profiles by different immunological cells, i.e. adaptive and innate immune cells, would allow for the elucidation of individual responses to probiotics and lead to further understanding of interactions between adaptive and innate immune systems at the GALT and systemic levels. However, the underlying mechanisms at the individual immune cellular level have rarely been described, in particular intracellular responses to probiotic bacteria exposure. Such
information could assist with identifying immunological intracellular pathways involved and determine if the immunological intracellular milieu is affected by the probiotic bacteria. For instance, at the DNA, RNA and protein level, this could subsequently reveal the probiotic bacterial dose requirements to induce an immunological response. In the past, limitations in technology have restricted the opportunity to study in-depth immunological cellular responses to probiotic bacteria exposure. Real-time imaging using confocal microscopy allows for changes in DNA, RNA and protein synthesis to be determined. In addition, advanced confocal computer programs are being developed that can potentially study individual molecular movements within cells. Therefore, this technology could be used to elucidate the real-time responses of immune cells to probiotic treatments and hence increase the understanding of immunological intracellular responses to probiotic bacteria.

1.8 CONFOCAL MICROSCOPY

Imaging real-time events in biological systems is made possible using confocal microscopy techniques. The advantages of confocal microscopy over conventional microscopy include depth of field control, reducing background noise whilst improving image quality, and the ability to collect optical sections from thick samples (Claxton et al., 2006). The measurement of fluorescence intensity from either fluorophores or internally expressed fluorescent proteins provides a bioindicator of intracellular events/responses to stimuli/external factors. This is made possible through specific labels for individual components of cells, such as DNA, RNA, actin, etc. Both living and fixed cells can be analysed using confocal microscopy. Three dimensional (3D) rendering is also possible through the use of
confocal microscopy and optical sectioning. Furthermore, monitoring cellular activities by fluorescence expression over time allows for 4D imaging. Therefore, real-time live cell analysis is possible and can potentially provide information regarding the complex nature of intracellular processes over time. Multiple components can be analysed using fluorescent probes that label specific molecules. Most confocal microscopy systems employ multiple photomultiplier channels with filters that specifically split the emission signals into certain wavelengths; this allows for more than one fluorescence signal to be detected. Improved resolution is achieved by the spatial filtering techniques of confocal microscopy that eliminates out-of-focus light from large specimens exceeding the immediate focus plane (Claxton et al., 2006) by using a pinhole (Paddock, 2000).

## 1.8.1 Fluorescent Dyes

Many analytical applications utilise fluorescent dyes (i.e. fluorophores or fluorochromes) to visualise and analyse nucleic acids, proteins and cellular organelles; therefore to characterise intracellular activities. Fluorophores are created from synthetic aromatic organic chemicals with the purpose of either interacting and binding biological molecules such as DNA, proteins, etc. or localising within organelles/intracellular areas such as the nucleus or mitochondria (Haugland, 2005). Further applications include the ability to examine cellular processes (e.g. calcium fluxing), structural integrity (e.g. actin filaments) or cellular viability (e.g. apoptosis versus necrosis) (Johnson, 1998).

Advancements in analytical tools, particularly in the past ten years, have provided the opportunity for real-time analysis using live cells (as opposed to fixed
cells). However, careful consideration is required when labelling live cells with a fluorescent compound/s, in particular, to avoid disrupting cellular processes or causing cytotoxicity. For instance, in live cells, the staining of a cellular component will, in some way, disrupt the normal functioning of the cell; however, excessive dye concentrations can cause phototoxicity and/or cytotoxicity as well as high excitation intensities can cause irreversible damage to the cell resulting in either necrosis (mechanical damage to the cell) or induce apoptosis (programmed cell-death). Fluorescent dyes specific for DNA interacts via non-covalent mechanisms, either binding the minor groove of DNA or intercalating between the bases of the DNA strands (Sibirtse, 2007). The type of binding mechanism can be dependent on concentration of the fluorescent dye (Zipper et al., 2004). Fluorescent dyes bind via different modes to different DNA base-pair sequences (Pilch et al., 1995) with intercalating dyes binding between complementary bases (Tomosaka et al., 1997). Such mechanisms involve either hydrogen bonding between extra nitrogen or oxygen atoms of the fluorescent dye and the nitrogen bases of the DNA strands (Sibirtse, 2007) or via other electrostatic interactions (Zimmer and Wahnert, 1986). Potential carcinogenic and mutagenic effects can occur from fluorescent dye intercalation into DNA as observed from frameshift mutations in bacteria and bacteriophages (Tomosaka et al., 1997; Ferguson and Denny, 2007). However, such effects on mammalian cells have not been reported.

1.8.2 Fluorescent Proteins

The first fluorescent protein (FP) was discovered by Osamu Shimomura and Frank Johnson in 1961. They isolated a calcium-dependent bioluminescent protein (aequorin) from the Aequorea-victoria jellyfish and a second protein with the ability
to produce green fluorescence (green fluorescent protein or GFP). Genetic manipulation of FPs have led to the development of far-red, red, green, yellow, orange, cyan and blue fluorescence emitting proteins. Fusion FPs were developed to target specific cellular proteins or enzymes in living cells (eukaryotes and prokaryotes), tissues, organs or whole organisms (e.g. mice) to examine gene expression and intracellular protein movements. Associated advancements in microscopy technologies have allowed for the detailed analysis of cellular processes. In addition, the development of such technologies has provided the opportunity to visualise and analyse disease-states within whole organisms such as cancer (McElroy et al., 2008).

Fluorescent proteins are transfected into cells as exogenous genes that can be fused to a specific cellular component, for example actin, or can be non-specifically expressed. The cell processes the resulting RNA and subsequent protein to ideally form a functional FP. Despite the originating species or genetic manipulation, the size of all FPs is approximately 25 kilo Dalton (kD) and is quite large compared to fluorescent dyes that average approximately 1 kD (Kremers et al., 2011). The function and fluorescence properties of an FP is relative to the complete protein structure and the formation mechanism is similar regardless of the source (coral or jellyfish) with four amino acid residues conserved in all FPs (Remington, 2006). The structure of FPs consists of a rigid 11 stranded β-barrel surrounding a central α-helix, that upon mutation, changes the fluorescence properties such as the excitation and emission profile (colour) (Ormo et al., 1996). The β-barrel protects the chromophore from the outside environment (Tsien, 1998). As mentioned previously, sequence mutations have led to the development of a wide range of FPs with different spectral
emissions. In addition, a number of modifications were performed to FPs for fluorescence expression in mammalian cells, for instance, sequence mutations to improve fluorescence expression at 37 °C (Cormack et al., 1996) and to improve protein folding and chromophore formation effectiveness (Tsien, 1998). The use of fluorescent proteins reportedly has advantages and disadvantages for live cellular quantitative imaging and is dependent on the GFP variant used (Patterson et al., 1997). Nonetheless, fused fluorescent proteins and molecules are expressed at a ratio of 1:1 allowing for quantitative analysis (Patterson et al., 1997).

The expression of GFP genes has been reported as an indicator of cellular activities (Steff et al., 2001; Strebel et al., 2001) including labelling sub-cellular locations such as the mitochondria (Rizzuto et al., 1995). Therefore, the expression of FPs gene potentially provides a bio-read out of intracellular activities and responses to external stimulants such as probiotics. A number of considerations are required for FP use including the brightness, protein stability, pH and temperature stability, and if used as a fusion protein, the potential interference of the FP on the behaviour and dynamics of the molecule being tracked (Kremers et al., 2011). The spectral emission profile of a FP is also a consideration, particularly if used with a fluorescent dye co-stain.

1.8.2.1 Humanized Monster Green® Fluorescent Protein (hMGFP)

The Monster Green® Fluorescent Protein (MGFP) is a synthetic version of the GFP gene from the Montastrea cavernosa great star coral (Almond, 2003). The MGFP gene was produced by random mutagenesis of the native GFP gene with associated resistance to photobleaching and brighter fluorescence; comparatively, the
native GFP gene reportedly photobleaches producing faint fluorescence (Almond, 2003). However, due to the presence of many consensus transcription factor binding sites in the MGFP gene, non-specific transcriptional activation can occur depending on the experimental conditions (Almond, 2003). The consensus transcription factor binding sites in the MGFP gene were reduced on the synthetic humanized MGFP (hMGFP) for improved reliability of protein expression and thus accurate fluorescence reporting (Almond, 2003). In addition, the hMGFP gene contains mammalian codons for improved expression levels in such cell lines and optimised-Kozak sequences for increased translation efficiency (Almond, 2003). The hMGFP gene was cloned into the Promega pCI Mammalian Expression Vector (phMGFP) (Almond, 2003). The phMGFP is commonly used as a fluorescent reporter in neuroblastoma cell lines (Betz and Farfan, 2003). Research employing phMGFP as a fluorescent reporter have included targeted gene therapy to investigate the efficacy of atomic force microscopy in live cells (Afrin et al., 2009), the distribution of a synthetic peptide fusion products in cells (Reggiani et al., 2006) and small interfering RNA (siRNAs) effects on keratinocyte-GFP expression in mouse epidermal tissue (Gonzalez-Gonzalez et al., 2009).

### 1.8.2.2 DsRed2 Fluorescent Protein

Comparatively to GFP, the DsRed protein (drFP583) is resistant to photobleaching, pH insensitive and exhibits higher extinction coefficients and quantum yields; however, the DsRed protein exhibits strong oligomerization and a slower maturation (Baird et al., 2000). An improved variant, the DsRed2 gene is a humanized variant of the *Discosoma* sp. red fluorescent protein, drFP583 (Matz et al., 1999). The DsRed2 gene contains six amino acid substitutions that accelerate
chromophore maturation and decrease protein aggregation; also contains a series of silent base-pair changes for higher expression in mammalian cells and less cytotoxic effects. Similar to the DsRed1 protein, the DsRed2 protein is a tetramer; DsRed2 was referred to as E57-NA prior to being commercially available (Yanushevicha et al., 2002). The DsRed2 protein has been employed as a fluorescence reporter for cancer metastasis models (Lu et al., 2003), plant gene expression (Lin et al., 2011), tracking fungal infections in tomato plants (Nahalkova and Fatehi, 2003) and gene expression in mammalian cells (Miyagishi and Taira, 2002; Yamamoto et al., 2004).

1.8.3 Intracellular Tracking of DNA, RNA and Proteins using Image Analysis Software

Advancements in fluorescence microscopy hardware and associated software have provided the opportunity to analyse cellular interactions over time, therefore providing the ability to perform 4-dimensional (4D) microscopy. Interactions between intracellular molecules and changes in nucleic acid can be monitored using this equipment; essentially live cell bio-readouts of intracellular responses to treatments are possible.

1.8.3.1 Fluorescence Correlation Spectroscopy (FCS)

Fluorescence correlation spectroscopy (FCS) is an application used to analyse diffusing particles in solution or living cells. This method was developed by Madge et al. (1972) to distinguish the difference between ethidium bromide bound to dsDNA and unbounded ethidium bromide. Fluorescence intensity variations within a sample are measured by FCS that determines the spontaneous fluorescence
concentration. Fluorescent molecules and particles move in and out of a limited
detection volume (point-spread function [PSF]) and the photons in this volume are
measured by a focused laser beam over time (Lenset et al., 2010). Fluorescence
intensity fluctuations are due to Brownian particle motion. The autocorrelation of
these fluorescence intensities is calculated over time using FCS (Fig. 1.6). The
number of molecules in an illuminated volume and their time-dependent spontaneous
fluctuations can be obtained from the autocorrelation function (the correlation
coefficient between the initial intensity at $t = 0$ and intensities over time) of the
fluorescence signal as an average of the fluorescent particles. This autocorrelation of
the fluctuation frequency is derived from the correlation of a time-shift replica with
itself at different time values (Lenset et al., 2010). The average fluorescent particle
quantity and diffusion times are analysed due to the measurement of photons by the
FCS equipment that detects and distinguishes the photon’s pulse and the number of
detected photons within a specified time (Digman and Gratton, 2011; Moens et al.,
2011).

Two components are analysed in FCS correlation functions: the fluctuation
temporal spectrum obtained from the autocorrelation function and the amplitude
spectrum (Fig. 1.7a) obtained from a photon-counting histogram (Fig. 1.7b) or
fluctuation intensity distributions (Digman and Gratton, 2011). The particle
concentration and brightness is shown by the fluctuation amplitude with the
frequency representing diffusion times of the fluorescent particles; the
autocorrelation curve provides the diffusion coefficient and concentration of the
diffusing particle (Lenset et al., 2010). Such information can be represented in the
photon counting histogram; this graph can distinguish between species of similar
**Figure 1.6.** Autocorrelation function development. At time 0, the autocorrelation between two signals is equal to 1. As time proceeds, the autocorrelation of the two signal decreases. Source: Schwille and Haustein (2004)

**Figure 1.7.** Photon counting histograms a) The amplitude distribution of photon counts over time b) Photon counting histogram. Source: Malengo et al. (2008)
diffusion coefficients based on the molecular brightness, resolve aggregation by the species brightness levels and differentiate multiple species in a homogenous sample (Chen et al., 1999). Based on the probable photon count distribution in a small volume, the photon counting histogram includes the average photon counts (or concentration) of the fluorescence species and the molecular brightness or average amount of photons per sampling time per molecule (Chen et al., 1999). A number of advantages exist for FCS analysis including the tracking of single molecules (using single-point FCS) and single-particles. However, single-point FCS only provides information about a point measured in a sample therefore limiting its use for monitoring molecules in complex systems such as cells (Digman and Gratton, 2011). Further, large isolated particles are required for single-particle tracking in cells (Digman and Gratton, 2011).

Image correlation spectroscopy (ICS) is a program that mathematically analyses an image or stack of images providing the spatial autocorrelation function (spatial fluorescence fluctuations) using the two-dimensional fast-Fourier transform algorithm (Digman et al., 2005a). Image correlation spectroscopy has the ability to monitor slow fluorophore diffusion (unlike FCS) and can be used to analyse slow receptor molecules, receptor clusters and fixed cells (Kolin and Wiseman, 2007). Therefore, ICS analyses the fluorescence fluctuations in a sample to determine the concentration, and the level and quantity of protein aggregation in a cell membrane (Digman et al., 2005b). The ICS and FCS programs were frequently combined to analyse spatial relationships of particles and their positions within cells. However, FCS acquires high temporal resolution of single-point FCS (microseconds) and ICS a
low temporal resolution (seconds) therefore limited information is provided by the varying timescales (Digman et al., 2005a).

Generally, FCS is used in confocal or two-photon microscopy that focuses light on a sample to measure the fluorescence intensity fluctuation of diffusion, physical or chemical reactions, or aggregation, to evaluate the autocorrelation. Depending on the type of equipment being used, LSCM can be limited in obtaining single-point FCS data (Moens et al., 2011). As mentioned previously, ICS is also limited due to time resolution. To overcome this SimFCS software was developed by Enrico Gratton and can acquire dynamic, spatial and quantitative information from commercial LSCM by detecting with raster image correlation spectroscopy (RICS) and number and brightness analysis. Further variations of FCS include cross-correlation FCS that measures the simultaneous fluctuation events in two or more channels using single-point FCS; fluorescent particles with similar diffusion coefficients can be distinguished providing the two species are labelled by two different fluorescent probes, and excitation is performed with two different laser sources.

1.8.3.2 Raster Image Correlation Spectroscopy (RICS)

Raster-image correlation spectroscopy (RICS) measures the movement of molecules by the variation of fluorescence intensities at any given pixel in a confocal image (Digman et al., 2005b). The diffusion nature of intracellular molecules or particles can be distinguished from those either binding or bound using RICS. This software can be used to obtain spatial and temporal relationships within and between cells (including live cells) from images obtained using commercial LSCM systems.
Therefore, RICS is capable of extracting hidden time information that can be used to analyse fast and slow dynamic events including molecular concentrations within cells (Brown et al., 2008). A space-time pixel matrix is created within the image as the laser undergoes the raster scanning movement (Fig. 1.8a). The laser’s temporal and spatial sampling at each pixel (pixel dwell time) is known as well as the time between images and the line scans; hence, information of the pixel’s horizontal scanning axis (measured in microseconds) and vertical scanning axis (between scan lines measured in milliseconds) can be used to generate spatial-temporal maps of cellular dynamics (Brown et al., 2008). The acquisition of the frame is repeated between 50 to 100 frames using RICS (Fig. 1.8b), this enables the identification and subtraction of immobile fractions from the image stack and the application of the 2D correlation operation to each image of the stack to calculate the average 2D spatial correlation (Digman and Gratton, 2009). The RICS program involves the measurement of two pixels, the first is taken for a short time and then the second neighbouring pixel is sequentially measured. Should the molecule have moved to the next pixel, a correlation will exist in the intensity fluctuations of the two pixels with an associated brief time delay (Digman and Gratton, 2009). Factors that determine this spatial correlation are the rate of diffusion and pixel size, potentially involving several adjacent pixels. Slow pixel fluctuations signify a location-specific bound fluorescent molecule that remains in that site for an extended time period; comparatively, diffusion events will be differently distributed as the fluctuation will not transfer to the next pixel (Digman and Gratton, 2009).
Figure 1.8. Raster image correlation spectroscopy (RICS) a) Raster scan direction and movement of a laser. Source: University of Oregan (2009); b) The line, pixel and frame time scale of a image obtained by confocal microscopy. Source: Digman et al. (2005)

The RICS data (spatial autocorrelation function) is presented graphically and can distinguish between slow diffusing particles and those with more rapid diffusion. Fast diffusing particles are represented by a horizontal band whereas slower moving particles exhibit a circular-like band (Fig. 1.9). These graphic representations are due to the spatial autocorrelation function of the particles diffusion; rapid diffusing particles spatial autocorrelation function decays in the X direction (producing the horizontal band) with the slower diffusing particles decaying in both the X and Y directions producing a band analogous to the morphology of the molecule. The spatial autocorrelation function is calculated by an algorithm from the correlation of an image acquired during a time scan (50 to 100 frames) of the intensity fluctuations in the X and Y direction. The spatial and temporal information of RICS data can be presented in 3D as the fit of the spatial-autocorrelation function. Similar to the 2D representation, rapid diffusing particles decay in the X direction with slower diffusing particles decaying in the X and Y directions (Fig. 1.8). Therefore, the diffusion properties of a molecule or particle can be identified using the autocorrelation function analysis of the fit and presented in 3D. The RICS program
has been applied to study the binding and unbinding nature of proteins and subsequent diffusion rates of molecules in a number of cell biology applications. Research utilising RICS analysis have included analysing the membrane diffusion rates of calmodulin (a protein responsible for cellular maintaining calcium-concentrations) (Sanabria et al., 2008), diffusion rates of cytosolic enhanced GFP (EGFP) (Rossnow et al., 2010) and EGFP tagged-paxillin (an adhesion adaptor molecule) (Brown et al., 2008) as well as the diffusion of GFP tagged-protein 53 (p53 – tumour-suppressor protein) in response to DNA-damaging reagents (Hong et al., 2010).

Figure 1.9. Simulations of fast and slow moving particles in solution represented by the image, 2D and 3D spatial autocorrelation function; a) Image of slow moving particles, b) 2D spatial autocorrelation function graphic of the slow moving particles, c) 3D spatial autocorrelation function graphic of the slow moving particles, d) Image of fast moving particles, e) 2D spatial autocorrelation function graphic of the fast moving particles, e) 3D spatial autocorrelation function graphic of the fast moving particles. Source: Digman et al. (2005a).

1.9 PROJECT OVERVIEW

Many studies have described immunological enhancement from probiotic bacteria treatments including in vivo, in vitro and ex vivo experiments. However, the
immune intracellular mechanisms underlying such responses have rarely been described. In this thesis, the porcine progenitor immune cell lines, L45 and L23 (T- and B-cell) are used to determine intracellular responses to probiotic bacterial-produced soluble factors. Porcine immune cells were chosen for these investigations due to the close relationship with human cells and also are applicable to animal cellular studies.

As mentioned in the literature review, developments in fluorescence microscopy have provided the opportunity to obtain real-time information from viable cells. Fluorescent proteins have been widely used as bio-indicators of cellular responses. The porcine progenitor immune cells were transfected with the fluorescent proteins, phMGFP and pCIneo-DsRed2. Changes in fluorescence and diffusive properties of these fluorescent proteins in response to the probiotic bacterial-produced soluble factors were imaged by LSCM and FCS and analysed using the RICS program. These changes were compared to the immune cellular responses to pathogen-produced soluble factors (*Streptococcus pyogenes*). The intracellular responses of the immune cells to the probiotics and pathogen were compared to determine if the probiotic strains exhibited similar effects on immune cellular responses.

Also mentioned previously, microencapsulation has been investigated as a delivery tool for probiotics to maintain cellular viability and to release the cells at sites of interest within the GI tract. Changes in probiotic bacterial-activities from the microencapsulated environment have rarely been described and were evaluated in this thesis using the transfected porcine progenitor immune cells as a downstream
indicator. To date, very few studies have described any form of immunological responses (innate or adaptive) to microencapsulated probiotic bacteria. These results were compared to the responses of immune cell treated with non-microencapsulated probiotic bacteria.

Initially, mammalian cell culture media was conditioned by free and microencapsulated bacterial-produced soluble factors and applied to the transfected porcine progenitor immune cells. The immune cellular proliferation, cytokine response and intracellular fluorescent protein diffusion rates were analysed. To gain an understanding of the responses of immune cells within the GI tract, porcine fibroblast intestinal cells (IPI-1) were used as an intestinal cell barrier to determine if the porcine immune cells responses to the free and microencapsulated bacterial-produced soluble factors differed. The proliferation, cytokine response and intracellular fluorescent protein diffusion rates were also analysed and compared to the treatments without an intestinal cell barrier.

The aims of this thesis are:

- To investigate the effects of microencapsulation on the viability of probiotic bacteria and develop a standard curve for microencapsulated bacteria using LSCM and image analysis software (Chapter 2).
- To develop a fluorescent immune cell model using porcine progenitor immune cell lines, L45 and L23, and the fluorescent proteins, humanized Monster Green® Fluorescent protein (phMGFP) and pClneo-DsRed2 (Chapter 3).
- To determine the effects on the proliferation of the transfected porcine progenitor immune cell lines by free and microencapsulated probiotic bacterial-produced soluble factors (Chapter 4).

- To determine the effects on the proliferation of the transfected porcine progenitor immune cell lines by free and microencapsulated probiotic bacterial-produced soluble factors that have been co-cultured with the porcine fibroblast intestinal cells (IPI-1) (Chapter 5).

- To investigate cytokine gene expression by the treated porcine progenitor immune cells in response to the free, microencapsulated, IPI-1/free and IPI-1/microencapsulated probiotic bacterial-produced soluble factors (Chapter 6).

- To determine if the porcine progenitor immune intracellular fluorescent protein diffusion rates are influenced by the free, microencapsulated, IPI-1/free and IPI-1/microencapsulated probiotic bacterial-produced soluble factors (Chapter 7).
2. CHAPTER TWO

**Growth of Probiotic Bacteria in Mammalian Cell Culture Conditions & Confocal Microscopy Analysis of Microencapsulated Probiotic Bacteria**

2.1  **INTRODUCTION**

**Microencapsulation of Probiotic Bacteria**

Probiotic bacteria are reported to convey health benefits to hosts upon consumption and are included in a number of food products, such as yoghurt and cheese (Parvez et al., 2006). The viability of probiotic bacteria is considered an important characteristic in the health-conveying benefits to hosts (Fuller, 1992). However, the viability of such bacteria is significantly reduced during food processing procedures and storage; the consumer usually receives less than the recommended amount of viable probiotics at the time of purchase and consumption (Kosin and Rakshit, 2006).

Microencapsulation has been proposed to ‘protect’ probiotics from processing procedures and to assist in maintaining probiotic viability during food storage (Kailasapathy, 2002). Furthermore, microencapsulation has been postulated to protect probiotics during transit through the acidic conditions of the gastrointestinal tract. Ideally, the microencapsulating polymers are resistant to acidic stomach conditions and release the interior probiotic content in the alkaline environment of the small intestine increasing the interactions between viable probiotics and the underlying immunological tissues (Kailasapathy, 2008).
Calcium alginate is the most commonly used microencapsulation ingredient (Kailasapathy, 2002). Calcium alginate beads were shown to convey resistance in *in vitro* simulated acidic stomach conditions and released the probiotic cells in colonic-like conditions; the encapsulated probiotics also had a higher viability than the control free cells (Mandal et al., 2006a). Similar results were reported for microencapsulated probiotics treated in porcine GI contents *ex vivo*, the release of the probiotics occurred at different durations depending on the conditions of the intestinal area (Iyer et al., 2005). The calcium alginate gel can be combined with a number of other components, such as chitosan, to coat and enhance the stability of the capsules in acidic conditions (Chavarri et al., 2010). Although it has been established that the viability of microencapsulated probiotics is comparatively higher than free bacteria in conditions simulating the gastrointestinal tract, further research is required relative to the behaviour of probiotics whilst encapsulated. Currently, no research has explored the real-time viability of microencapsulated probiotics, the distribution of cells or the stress-related changes to the probiotics as a result from the confined environment and capsule composition.

**Probiotic Bacteria & Pathogenic Bacteria Strains**

The two probiotic strains, *Lactobacillus acidophilus* LAFTI® L10 and *Bifidobacterium lactis* HN019 (DR10™), and the pathogenic strain, *Streptococcus pyogenes*, were used in this thesis to investigate their effects on T and B cell immunological responses to free and microencapsulated bacteria cells. This model (the fluorescent immune cells) was also used to determine the effects of microencapsulation on bacterial activities by investigating the downstream responses
of co-cultures with the immune cells; these effects were compared to the immune cellular responses to the free bacteria.

*L. acidophilus* LAFTI® L10 is a non-motile, non-sporing Gram-positive rod ranging from short rods to long filaments. This species is homofermentative that ferments sugars into lactic acid and grows easily in low pH environments with an optimum growth temperature of 37 °C either in or without oxygen. In milk, this species produces acid without apparent gas production (Curran et al., 1932). *L. acidophilus* LAFTI® L10 has been associated with many health beneficial effects on hosts including immunological modulation (Paturi et al., 2007; Paturi et al., 2008) and immunomodulation of allergic responses with inactivated *L. acidophilus* (Rasche et al., 2007). This strain has been incorporated in a number of dairy products including cheese and yogurts.

*B. lactis* HN019 (DR10™) is a probiotic bacterium isolated from a commercial yogurt; the New Zealand Dairy Research Institute (Palmerston North) culture collection maintains this strain. The diary product producers, Fonterra (New Zealand) and Danisco (USA), market this strain as DR10™ and HOWARU Bifido, respectively. The HN019 (DR10™) strain has been described as a Gram-positive rod with an irregular morphology, non-sporing, catalase negative, produces lactic acid from the fermentation of glucose, lactose, raffinose and ribose, and was established as part of the genus *Bifidobacterium* due to the activity of fructose-6-phosphate phosphoketolase (Sanders, 2006). Enhancement of immunological activities has been associated with *B. lactis* HN019 (DR10™) (Prasad et al., 1998) with studies
describing increased innate immune cellular activity in the elderly and consumers with inadequate immune function (Sanders, 2006).

The pathogenic strain, *S. pyogenes*, is a gram-positive spherical bacterium that is the causative agent of Group A streptococcal infections (Ahmad et al., 2010) and contains the streptococcal group A antigen on its cell wall. This bacterium is non-motile, catalase negative, non-sporing coccus that forms chains or pairs of cells, is facultatively anaerobic with an optimum growth temperature at 37 °C and metabolises fermentatively producing mostly lactate without gas (Holt et al., 1994). On blood agar, this strain produces beta-hemolysis and is known as a Group A (beta-hemolytic) strain. Pathogenic features of this bacterium include the surface antigen, M protein, leipoteichoic acid and the exotoxin streptolysin O (Ahmad et al., 2010).

This Chapter initially investigates the ability of the probiotic (*L. acidophilus* LAFTI® L10 and *B. lactis* HN019) and pathogenic (*S. pyogenes*) strains to survive and proliferate in mammalian cell culture conditions for further applications with mammalian co-cultures (Chapters 4 and 5). The real-time viability of microencapsulated probiotics was evaluated using the LIVE/DEAD® BacLight™ bacteria viability kit (Invitrogen, Mulgrave, Australia) and laser-scanning confocal microscopy analysis (LSCM); the distribution of cells were analysed using the Bitplane Imaris program. The LIVE/DEAD® BacLight™ bacteria viability kit employs two nucleic acid stains, SYTO® 9 and propidium iodide (PI). The SYTO® 9 stain is able to cross intact cell membranes and the PI stain enters cells with damaged cell membranes. Upon interacting with nucleic acids, SYTO® 9 produces fluorescence in the green spectrum and PI producing fluorescence in the red
spectrum. When both stains gain access to cellular nucleic acid content, PI displaces and quenches SYTO® 9 by fluorescence resonance energy transfer (Stocks, 2004). Stress-related changes to the bacteria from the confinement of microencapsulation were determined from comparative analysis between the downstream mammalian cellular responses to treatments with non-encapsulated and encapsulated bacteria (Chapters 4 to 7).

2.2 **AIMS & OBJECTIVES**

The aims of this Chapter are:

- To determine if the probiotic bacteria, *Lactobacillus acidophilus* LAFTI® L10 and *Bifidobacterium lactis* HN019 (DR10™), and pathogen *Streptococcus pyogenes* can grow in mammalian cell culture conditions for further experimentation with mammalian progenitor immune cells.

- To investigate the effects of microencapsulation on the viability of the two probiotic strains and the pathogenic bacteria.

- To determine the cellular distribution of the bacteria within the capsules.

The objectives of this Chapter are:

- To perform a growth curve of the three bacteria strains in their respective media and incubation conditions.

- To identify if the three bacteria strains can grow in mammalian cell culture conditions by developing a growth curve.

- To develop a viability standard curve for free bacteria using the LIVE/DEAD® BacLight™ Bacterial Viability and Counting Kit (Invitrogen, Australia).
- To perform LSCM analysis on microencapsulated bacteria to develop a viability standard curve
- To determine the distribution of bacteria cells by using the bacteria viability kit, LIVE/DEAD® BacLight™ Bacterial Viability and Counting Kit (Invitrogen, Australia) and LSCM.

2.3 MATERIALS AND METHODS

2.3.1 Bacteria Growth Curves

2.3.1.1 Bacteria Cells and Growing Media

The probiotic bacterial strains, *Lactobacillus acidophilus* LAFTI® L10 and *Bifidobacterium lactis* HN019 (DR10™), were purchased from DSM Nutritional Products (DSM Food Specialties Ltd., Sydney) and Fonterra Co-operative Group (New Zealand), respectively. The pathogenic strain, *Streptococcus pyogenes*, was received from the Prince of Wales Hospital, Randwick (Rec. 1979, 248 UNSW 026700). The probiotic bacterial strains were grown in de Man, Rogosa and Sharpe (MRS) broth (CM0359, Oxoid, Adelaide, Australia) at 37 °C for 24 h under anaerobic conditions using a anaerobic chamber (AG0025, Oxoid, Adelaide, Australia) and AnaeroGen™ sachet (AN0025, Oxoid, Adelaide, Australia). *S. pyogenes* cultures were grown aerobically at 37 °C for 24 h in Brain Heart Infusion (BHI) broth (CM1135, Oxoid, Adelaide, Australia). All cultures were incubated in the Thermoline Scientific Australia Incubator (Model I60G-290-D).
2.3.1.2 Growth curves for *L. acidophilus*, *B. lactis* and *S. pyogenes*

*L. acidophilus* LAFTI® L10, *B. lactis* HN019 (DR10™) and *S. pyogenes* were grown for 24 h as outlined in Section 2.3.1.1. A 2 mL aliquot of each strain was removed aseptically and placed into the respective broth (equilibrated at 37 °C) at a final volume of 100 mL. The samples were mixed by the C24KC Refrigerated Incubator Shaker (Serial No. 101129215, Model No. C24KC; Edison, New Jersey, USA) at 150 rpm and 37 °C for the duration of the experiment (28.5 h) in aerobic conditions. Absorbance was measured at 550 nm by the Thermo Helios Gamma spectrophotometer (Series UV6 123720; Thermo Electron Corporation, South Australia, Australia) using 1 mL of culture suspension placed into a cuvette (613101, Greiner Bio-One, Interpath Services P/L Heidelberg West, Australia). The absorbance was read initially (0 h) and at half hour increments up to 3 h, and then read every 1 h up to approximately 8 h and again at approximately 28.5 h. Absorbance (log scale) versus time was plotted to determine the bacteria generation time.

2.3.2 Bacteria Growth Curves in Mammalian Cell Culture Conditions

2.3.2.1 Bacteria and Mammalian Cell Culture Reagents

*L. acidophilus* LAFTI® L10, *B. lactis* HN019 (DR10™) and *S. pyogenes* were grown in complete DMEM without antibiotics, supplemented with FBS (10 %) and non-essential amino acids (NEAA) (1 %). This media is referred to as cDMEM-AF from this point forward.
2.3.2.2 Bacteria Growth Curves in Mammalian Cell Culture Conditions

The effects of standard mammalian cell culture media on the growth of *L. acidophilus* LAFTI® L10, *B. lactis* HN019 (DR10™) and *S. pyogenes* were determined using spread plates. *L. acidophilus* LAFTI® L10, *B. lactis* HN019 (DR10™) and *S. pyogenes* were grown as outlined in Section 2.3.1.1, for 24 h at 37 °C in the Thermoline Scientific Australia Incubator (Model I60G-290-D). Streak plates were prepared to ensure the bacterial suspensions were not contaminated. Following 24 h incubation, each bacteria suspension was placed into a 50 mL CELLSTAR® polypropylene tubes (2014-03, Greiner Bio-One, Interpath Services P/L, Heidelberg West, Australia) and centrifuged at 3864 g for 10 min (37 °C) using the Universal 32 R centrifuge (HD Scientific Supplies Pty. Ltd.). The supernatant was removed. The pellets were washed with 1 mL cDMEM-AF and re-centrifuged as outlined previously. The supernatant was removed and each pellet was resuspended in 15 mL of cDMEM-AF. The bacterial suspensions were incubated at 37 °C in the respective conditions (aerobic or anerobic). At 2, 5 and 8h, spread plates were prepared in 6 replicates for each bacterium. A dilution series was conducted for each bacteria suspension in sterile NaCl (0.85%) (S5886, Sigma-Aldrich, Australia) solution at $10^{-2}$, $10^{-4}$, $10^{-6}$ for *L. acidophilus* and *B. lactis*, and $10^{-2}$, $10^{-4}$ and $10^{-5}$ for *S. pyogenes*. For *L. acidophilus* and *B. lactis*, a concentration of $10^{-6}$ was used for the spread plates (MRS agar). A concentration of $10^{-5}$ was used for *S. pyogenes* spread plates (BHI agar). The spread plates were incubated for 24 h at 37 °C prior to colony counting in the Thermoline Scientific Laboratory Incubator. The six replicates were averaged at each time point and plotted to generate a growth curve.
2.3.3 Viability Standard Curve

2.3.3.1 Reagents

The LIVE/DEAD® BacLight™ Bacterial Viability Counting Kit (L7012) was purchased from Invitrogen (Mulgrave, Australia).

2.3.3.2 Staining of Probiotic Bacteria with LIVE/DEAD® BacLight™ Bacterial Viability and Counting Kit (Invitrogen) and Development of a Viability Standard Curve

The bacteria cultures were prepared and stained using the LIVE/DEAD® BacLight™ Bacterial Viability and Counting Kit and the manufacturer’s instructions (Invitrogen) was used as a guide to develop the viability standard curve. The *L. acidophilus* LAFTI® L10, *B. lactis* HN019 (DR10™) and *S. pyogenes* cultures were grown in 15 mL of media for 5 h (until late log phase) as outlined in Section 2.3.1.1. The bacteria cultures were centrifuged at 3864 g for 10 min (37 ºC) using the Universal 32 R centrifuge and the supernatant was removed. The pellets were resuspended in sterile NaCl solution (0.85 %, 2 mL) and allocated into 2 x 1 mL aliquots. To one aliquot for each bacteria culture, NaCl solution (0.85 %, 20 mL) was added and ethanol (100 %, 20 mL) was added to the second aliquots. All bacteria cultures were incubated at room temperature for 1 h and mixed every 20 min. The bacteria cultures were centrifuged as outlined above and the supernatant was removed; the pellets were resuspended in NaCl (0.85 %, 20 mL) and re-centrifuged. The supernatant was removed and the pellets were resuspended in NaCl (0.85 %, 10 mL). Samples were prepared as outlined in Table 2.1 in a Tc black 96
well microplate (655086, Greiner Bio-One; Interpath Services P/L, Heidelberg West, Australia).

Table 2.1. Microplate assay preparation of live and dead cell ratio prior to staining with the LIVE/DEAD® BacLight™ Bacterial Viability and Counting Kit (Invitrogen).

<table>
<thead>
<tr>
<th>Ratio Live/Dead</th>
<th>Live Cell Suspension (µL)</th>
<th>Dead Cell Suspension (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0:100</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>10:90</td>
<td>10</td>
<td>90</td>
</tr>
<tr>
<td>50:50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>90:10</td>
<td>90</td>
<td>10</td>
</tr>
<tr>
<td>100:00</td>
<td>100</td>
<td>0</td>
</tr>
</tbody>
</table>

The SYTO® 9 and PI stains were prepared by adding 8.1 µL of SYTO® 9 to 8.1 µL of PI; the stains were mixed by pipetting. A 2x staining solution was prepared by adding the mixed stains to 2.7 mL of filter sterilized MilliQ water and mixed further. To each well containing the bacteria cultures, 100 µL of the staining solution was added and mixed by pipetting. The solution was incubated at room temperature in the dark for 15 min. The fluorescence intensity was measured by the Perkin Elmer LS50B Luminescence Spectrophotometer using the software FL Winlab (Perkin Elmer, 2001). Excitation for SYTO® 9 and PI was conducted at 485 nm ± 10 nm slit; emission for SYTO® 9 (live cells) was measured at 530 nm ± 15 nm slit and the emission for PI (dead cells) was measured at 630 nm ± 15 nm slit. The fluorescence ratio of Live/Dead cells was calculated using the following equation 2.1 and plotted against the live bacteria (%).

\[
\text{Ratio}_{\text{Live/Dead}} = \frac{\text{Fluorescence SYTO 9}}{\text{Fluorescence PI}}
\]
2.3.4 Microencapsulation of Bacteria and the Development of a Viability Standard Curve

2.3.4.1 Microencapsulation Materials

Alginic acid sodium salt (A2158-500g), chitosan from crab shells (C3646-100g) and calcium chloride (C2661-500g) were purchased from Sigma-Aldrich (Castle Hill, Australia). Glacial acetic acid (UN No. 2789) was purchased from Ajax Finechem (Taren Point, Australia). Alginate solution (1.8 %) was prepared by dissolving 9 g of alginic acid sodium salt in 450 mL of milli Q water using a magnetic stirrer and heated to 50 °C. The volume was adjusted to 500 mL once the alginic acid was completely dissolved. The alginate solution (1.8 %) was filtered using a sterile Sartopure PP2 capsule (0.65 µM, 5591 305P9—SS, Sartorius, Dandenong, Australia) and the Millipore easy-load pump (Masterflex®, North Ryde, Australia) into a sterile 500 mL Schott bottle; the alginate solution was further filtered into a sterile 500 mL Schott bottle using the 0.22 µm Steritop™ Filter Unit (SCGPT10RE, Millipore, Kilsyth, Australia) and stored at room temperature. The cation solution contained chitosan (0.1 % w/v) in calcium chloride (0.1 M). The chitosan was initially dissolved in glacial acetic acid (20 %) and CaCl₂ (0.1 M, pH 6.2) was later added making a volume of 1 L. The cation solution was stored at 4 °C. The Brij solution consisted of zinc chloride (0.2 mM) (1687-500G, Ajax Chemicals, Taren Point, Australia) and Brij solution (150 µL) (430AG-6, Sigma-Aldrich, Sydney, Australia) at a final volume of 100 mL.
2.3.4.2 Microencapsulation of Probiotic Bacteria and Incubation on MRS Agar and in Mammalian Cell Culture Media

MRS Agar and Standard Anaerobic Bacteria Incubation Conditions

The *L. acidophilus* LAFTI® L10 and *B. lactis* HN019 (DR10™) cultures were grown in MRS broth as outlined in Section 2.3.1.1, for 24 h. The bacteria cells were enumerated using the Jenway spectrophotometer at 600 nm and harvested by centrifugation as outlined in Section 2.3.2.2. The supernatant was decanted and the pellet was initially resuspended in alginate solution (1.8 %, 1 mL) and Brij solution (6 % final concentration). Further alginate solution was added making a final bacteria cell concentration of $1.0 \times 10^9$ CFU mL$^{-1}$. Aseptically, the alginate and bacteria slurry was passed through a sterile, non-toxic, non-pyrogenic 10 mL Terumo Syringe (DVR-5174, Terumo Cooperation Australian Branch, Macquarie Park, Australia) and a 25 g x 32 mm needle (NN-2325R, Terumo Cooperation Australian Branch, Macquarie Park, Australia) into chilled chitosan (0.1 %) and CaCl$_2$ (0.1 M) solution at a drop distance of 3 – 5 cm. This solution was stirred using a magnetic stirrer and the capsules were allowed to harden for 15 min. The capsules were removed from the chitosan (0.1 %) and CaCl$_2$ (0.1 M) solution and washed with milliQ water. The capsules were placed on MRS agar and incubated for 24 h at 37 °C in the Thermoline Scientific Laboratory incubator in anaerobic conditions. Control capsules were also prepared as outlined above without the bacteria culture, placed on MRS agar and incubated in the same conditions as the microencapsulated probiotic cultures.
Mammalian Cell Culture Media and Conditions

The probiotic cultures were grown overnight at 37 °C as outlined in Section 2.3.1.1. The cultures were enumerated and centrifuged as outlined above for 10 min, the supernatant was discarded. The pellets were initially resuspended in alginate solution (1.8 %, 1 mL) and Brij solution (6 % final concentration). Further alginate solution was added making a final bacteria cell concentration of $1.0 \times 10^9$ CFU mL$^{-1}$. The capsules were prepared as outlined above. Following the milli Q water wash step, the capsules were further washed with stock DMEM until all traces of milli Q water and chitosan (0.1 %) and CaCl$_2$ (0.1 M) solution were removed (the capsules turned the same colour as the cDMEM-AF). The capsules were placed into 50 mL Falcon tubes and resuspended in cDMEM-AF (same volume that was used for the alginate resuspension) making a final cell concentration of $1.0 \times 10^9$ CFU mL$^{-1}$. The encapsulated bacteria in cDMEM-AF media were incubated at 37 °C for 24 h in the Forma Scientific CO$_2$ water jacketed incubator (Model 3111 S/N 26931-617) in 5 % CO$_2$.

2.3.4.3 Staining of Bacteria with LIVE/DEAD® BacLight™ Bacterial Viability and Counting Kit (Invitrogen) and Microencapsulation of Bacteria

*L. acidophilus* LAFTI® L10, *B. lactis* HN019 (DR10TM) and *S. pyogenes* were grown as outlined in Section 2.3.1.1 for 24 h. The cultures were enumerated by absorbance measurements at 600 nm using the Bio-Rad Benchmark Plus Microplate Spectrophotometer (Model No. Benchmark Plus, Serial No. 10687, Bio-Rad, Australia) and standard curves equations (Appendix A1.1). The bacteria cultures were harvested by centrifugation using the Universal 32 R centrifuge (HD Scientific.
Supplies Pty. Ltd.) at 3864 g for 10 min (37 ºC). The supernatant was decanted and the bacteria cells were each initially resuspended in the respective media (MRS or BHI broth) making a final concentration of $1.0 \times 10^{10} \text{ CFU mL}^{-1}$. From this suspension, 2 x 2.5 mL aliquots were placed into a sterile 15 mL Falcon™ polypropylene conical tube (352096, BD Bioscience, North Ryde, Australia); 20 mL of sterile NaCl (0.85 %) (S5886, Sigma-Aldrich, Australia) solution was added to the first aliquot and ethanol (100 %, 20 mL) was added to the second aliquot. All bacteria cultures were incubated at room temperature for 1 h and mixed every 20 min. The bacteria cultures were centrifuged as outlined above and the supernatant was removed; the pellets were resuspended in NaCl (0.85 %, 20 mL) and re-centrifuged. The supernatant was removed and the pellets were resuspended in NaCl (0.85 %, 2.5 mL). Samples were prepared as outlined in Table 2.2.

**Table 2.2.** Preparation of live and dead cell ratio prior to staining with the LIVE/DEAD® BacLight™ Bacterial Viability and Counting Kit (Invitrogen) and microencapsulating.

<table>
<thead>
<tr>
<th>Ratio Live/Dead</th>
<th>Live Cell Suspension (µL)</th>
<th>Dead Cell Suspension (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0:100</td>
<td>0</td>
<td>1000</td>
</tr>
<tr>
<td>10:90</td>
<td>100</td>
<td>900</td>
</tr>
<tr>
<td>50:50</td>
<td>500</td>
<td>500</td>
</tr>
<tr>
<td>90:10</td>
<td>900</td>
<td>100</td>
</tr>
<tr>
<td>100:00</td>
<td>1000</td>
<td>0</td>
</tr>
</tbody>
</table>

The SYTO® 9 and PI stains were prepared as outlined in Section 2.3.3.2 and the 1 mL bacteria aliquots (prepared as outlined in Table 2.2) were stained with SYTO® 9 and PI mix (1:1 dye ratio); the bacteria and dye suspension was incubated for 10 min at room temperature in the dark. An aliquot of Brij solution (0.2 mL, contains 0.2 mM ZnCl$_2$) was added to the bacteria suspensions, the alginate solution (1.8 %) was added making a final volume of 10 mL (final cell concentration was
approximately $1.0 \times 10^9$ CFU mL$^{-1}$). Microencapsulation of the 10 mL bacteria/alginate slurry was performed using the Inotech Encapsulator (Inotech AG, Dottikon, Switzerland) with a distance of approximately 5 cm from the 300 µm nozzle to the chitosan (0.1 %)/CaCl$_2$ (0.1 M) solution. The pump speed was approximately 253.0, the electrical current was approximately 1.57 kV and the amplitude was approximately 2885 Hz. The capsules were allowed to harden for approximately 15 min in the chitosan/CaCl$_2$ solution and then rinsed with milli Q water.

### 2.3.4.4 Laser Scanning Confocal Microscopy (LSCM) Analysis of Microencapsulated Bacteria

All LSCM images and data were collected using the hardware Leica TCS SP5 inverted LSCM with the DM600 fixed stage and software Leica Application Suite: Advanced Fluorescence. The microencapsulated bacteria were analysed by LSCM to investigate if the viability of microencapsulated bacteria cells can be determined using the LIVE/DEAD® BacLight Bacterial Viability Kit, to identify the distribution of bacteria cells within the capsules and to develop a method to potentially enumerate microencapsulated bacteria cells. The LSCM settings were originally chosen as outlined by the manufacturer’s description of excitation and emission profiles for the SYTO® 9 and PI stains; SYTO® 9 emission was measured at 500 to 550 nm and PI emission at 590 to 625 nm. Excitation was performed using the Argon-488 nm laser for both fluorescent stains; the power was set to 20 % with a 50 % maximum intensity. The 633 nm laser was set to 10 % maximum intensity and the PMT NDD3 detector was used for bright-field images, gain set to 254.5 V. Fluorescence emission for SYTO® 9 was detected using the Photomultiplier Tube
(PMT) 2 and PMT 3 was used for PI. The gain for SYTO® 9 and PI was 965.9 and 923.7 V, respectively. Scanning speed was 800 Hz with pixel resolution at 512 x 512. The HCX PL APO CS 10.0 x 0.4 water immersion UV objective was used for scans. The xyz acquisition mode was selected. The microencapsulated bacteria were placed on the 42 x 0.17 mm circular slides and covered with the 32 x 0.17 mm circular cover slip. The pinhole size was 1 airy unit (95.5 µm). Sequential scanning parameters were defined for SYTO® 9 and PI as outlined above and the “between lines” function was selected. All z-stacks were performed with a z-step size of 5.98 µm using a zoom factor of 2.1. The line average for all scans was set to 1; Z-stack images were performed in triplicate on different microcapsules.

2.3.4.5 Analysis of Microencapsulated Bacteria Viability using the Bitplane Imaris Software

The viability of microencapsulated bacteria cells was analysed using the Bitplane Imaris software version 6.3.1 (Bitplane A.G.). The Bitplane Imaris spots application (parameters outlined below) was used to determine the quantity of cells expressing green fluorescence (live) and red fluorescence (dead).

Image series (z-stacks) collected from the Leica LSCM were converted into a Bitplane Imaris format using the Bitplane Imaris batch converter. The measurement points application was used to measure the size of the fluorescent bacteria cells for further analysis using the spots application. The average length of all bacteria cells was determined to be 5 µm. The ‘segment a region of interest’ option was selected for Channel 1 (live cells) in the algorithm step of the spots applications and the capsular area was chosen by manually adjusting the segment box in the region of
interest step. The spot detection estimated diameter was set to 5 µm and the centre point at a 5 pixel width was selected in the source channel step. The quality filter was manually set to 2 % of the total pixels and the spots were calculated for bacteria cells expressing SYTO® 9. This was repeated for Channel 2 (dead cells expressing PI) using the same settings with the exception for the total pixels in the spots calculation, this was set to 1 %. The clipping pane option was used to obtain a cross-section view of the capsules as a 3D image.

2.3.5 Statistics

The relevant statistical calculations have been outlined in the relevant section throughout this Chapter.

2.4 RESULTS

2.4.1 Bacteria Growth Curves in Broth and Mammalian Cell Culture Conditions

The L. acidophilus, B. lactis and S. pyogenes strains were grown in broth and mammalian cell culture media to compare the growth of the strains in the two different media and to determine if the three strains were able to grow in mammalian cell culture conditions for further experimentation with mammalian immune cell co-cultures. The generation time for L. acidophilus LAFTI® L10 and B. lactis HN019 grown in broth was 1.6 h for both strains (Fig. 2.1 and 2.2) and 1.0 h for S. pyogenes (Fig. 2.3). The absorbance method employed for the broth growth curves was conducted on numerous occasions for the growth curves in mammalian cell culture and conditions. However, the media contains the pH indicator phenol red and the
colour readily changed from pink/red to yellow due to the production of lactic acid by the strains; the absorbance values were found to fluctuate due to the colour changes and bacteria growth producing varied results over time. Many different approaches were conducted to overcome these obstacles with little success including varying the absorbance wavelengths to be more compatible with the media colour. The spread plate method was employed to determine if the three strains were able to grow in mammalian cell culture media and conditions since insufficient results were obtained for the absorbance growth curves. As outlined previously, the ability of the three strains to grow in mammalian cell culture and conditions was required for further co-culture experimentation with mammalian progenitor immune cells (Chapters 4 to 7). As shown in Figures 2.3, 2.4 and 2.5, all three bacteria species exhibited increased growth over 6 h. The *L. acidophilus* LAFTI® L10, *B. lactis* HN019 (DR10™) and *S. pyogenes* growth increased by 44, 50 and 43 %, respectively, from 0 to 6 h. The *S. pyogenes* concentration was 10-fold less than the two probiotic stains. The three strains were allowed to adapt to the cDMEM-AF and mammalian cell culture incubation conditions by growing the bacteria in media for 48 h prior to experimentation. The growth of the bacteria was observed to decrease over the 48 h period (results not shown).
Figure 2.1. Growth curve of *Lactobacillus acidophilus* LAFTI L10 in MRS broth. Generation time is 1.6 h.

Figure 2.2. Growth curve of *Bifidobacteria lactis* HN019 (DR10™) in MRS broth. Generation time is 1.6 h.
Figure 2.3. Growth curve of *Streptococcus pyogenes* in BHI broth. Generation time is 1.0 h.

Figure 2.4. Growth curve of *L. acidophilus* LAFTI® L10 in complete DMEM [antibiotic free supplemented with FBS (10%) and non-essential amino acids (1%)].
The LIVE/DEAD® BacLight™ Bacterial Viability and Counting Kit (L7012, Invitrogen, Mulgrave, Australia) was used to develop a viability standard curve for Figure 2.6. Growth curve of *S. pyogenes* in complete DMEM [antibiotic free supplemented with FBS (10%) and non-essential amino acids (1%)].

**2.4.2 Viability Standard Curve**

The LIVE/DEAD® BacLight™ Bacterial Viability and Counting Kit (L7012, Invitrogen, Mulgrave, Australia) was used to develop a viability standard curve for...
*L. acidophilus* LAFTI L10, *B. lactis* HN019 (DR10™) and *S. pyogenes*. The *L. acidophilus* LAFTI L10, *B. lactis* HN019 (DR10™) and *S. pyogenes* viability standard curves produced $R^2$ values of 0.9932, 0.9931 and 0.98, respectively (Fig. 2.7, 2.8 and 2.9). These values indicate that the ratio of live/dead cells produced by the fluorescence of SYTO 9 and PI, respectively, is a reliable method to measure the viability of free bacteria cells.

**Figure 2.7.** Viability standard curve of *L. acidophilus* LAFTI® L10 using the LIVE/DEAD® BacLight™ Bacterial Viability and Counting Kit (L7012, Invitrogen, Mulgrave, Australia)
Figure 2.8. Viability standard curve of *B. lactis* HN019 (DR10™) using the LIVE/DEAD® BacLight™ Bacterial Viability and Counting Kit (L7012, Invitrogen, Mulgrave, Australia).

Figure 2.9. Viability standard curve of *S. pyogenes* using the LIVE/DEAD® BacLight™ Bacterial Viability and Counting Kit (L7012, Invitrogen, Mulgrave, Australia).
2.4.3 Microencapsulation of Bacteria

2.4.3.1 Laser Scanning Confocal Microscopy Analysis of Microencapsulated Probiotic Bacteria

The probiotic bacteria, \textit{L. acidophilus} LAFTI® L10 and \textit{B. lactis} HN019 (DR10™), were stained with the LIVE/DEAD® BacLight™ Bacterial Viability Kit (Invitrogen, Mulgrave, Australia) to distinguish between live and dead bacteria cells. Analysis using the Leica LSCM was performed on non-encapsulated (Fig. 2.10) and encapsulated bacteria (Fig. 2.12). Both stains in this kit are specific for DNA and RNA. Live cells express SYTO® 9, a stain capable of penetrating the intact cell walls of bacteria. Dead cells express propidium iodide (PI) and require a damaged cell wall to access the cell’s nucleic acid. In the event that the bacteria cell wall has been damaged, the fluorescence of SYTO® 9 is quenched by PI resonance energy transfer allowing non-viable cells to be distinguished from viable cells (Stocks, 2004). As shown in Figure 2.10, live (red) and dead (blue) bacteria cells were easily distinguished for both non-encapsulated probiotic strains.

![Figure 2.10. LIVE/DEAD® BacLight™ Bacterial Viability Kit (Invitrogen) treated non-microencapsulated probiotics a) Lactobacillus acidophilus LAFTI® L10 b) Bifidobacterium lactis HN019 (DR10™). Live cells (expressing SYTO® 9) are shown in red and dead cells (expressing propidium iodide) are shown in blue.](image-url)
Initial analysis was performed on empty 1.8 % alginate capsules to ensure no auto-fluorescence was produced by the capsular ingredients using the 488 nm laser. As shown in Figure 2.11, no auto-fluorescence was produced between 500 to 628 nm.

![Image of empty capsule analysed using the Leica laser-scanning confocal microscope.](image)

**Figure 2.11.** Empty capsule analysed using the Leica laser-scanning confocal microscope.

The encapsulated bacteria were analysed to determine the distribution of the bacteria cells and if viable cells could be distinguished from non-viable cells. As shown in Figure 2.12, the capsule edge, stained bacteria cells and bacterial viability status could be identified at a close zoom level. The non-viable probiotic cells were indicated by the blue colouration and the live cells by the red. The outline of out-of-focus bacteria cells was also visible. Figure 2.13 exhibits the 2D images of microencapsulated *L. acidophilus* LAFTI® L10 and *B. lactis* HN019 (DR10™). The capsules appear as a spherical shape with individual cells visible; however, the live and dead cells are difficult to distinguish at this zoom level. The 3D images of the microencapsulated bacteria are shown in Figure 2.14.
Individual bacteria cells are visible with little differentiation between the live and dead cells; similar to Figure 2.13, the alginate (1.8 %) capsules exhibit a spherical shape.

**Figure 2.12.** LIVE/DEAD® BacLight™ Bacterial Viability Kit (Invitrogen) stained microencapsulated probiotic bacteria a) *L. acidophilus* LAFTI® L10, b) *B. lactis* HN019 (DR10™). Live cells (expressing SYTO® 9) are shown in red and dead cells (expressing propidium iodide) are shown in blue.
Figure 2.13. 2D image of LIVE/DEAD® BacLight™ Bacterial Viability Kit (Invitrogen) stained microencapsulated probiotic bacteria a) *L. acidophilus* LAFTI® L10, b) *B. lactis* HN019 (DR10™).

Figure 2.14. 3D-projection of LIVE/DEAD® BacLight™ Bacterial Viability Kit (Invitrogen) stained microencapsulated probiotic bacteria a) *L. acidophilus* LAFTI® L10, b) *B. lactis* HN019 (DR10™).
2.4.3.2 Growth of Microencapsulated Probiotic Bacteria on MRS Agar and in Mammalian Cell Culture Media

The capsules were grown on MRS agar for 24 h in anaerobic conditions to determine if probiotic bacteria growth occurs in the encapsulated environment. As shown in Figure 2.15, the empty capsules appeared clear with no microbial growth; comparatively, the \( L. \) acidophilus LAFTI® L10 and \( B. \) lactis HN019 (DR10\(^{TM}\)) capsules contained growth by the two strains as exhibited by the turbid colouration as well as colony growth on the MRS agar. The colony growth outside the capsules was potentially due to the presence of bacteria cells on the capsules surfaces that ‘washed off’ with the sterile mill Q water used to wash the capsules following the hardening step (the capsules were not completely dried prior to applying to the agar plates).

![Figure 2.15. Alginate (1.8 %) capsules on MRS agar following 24 h incubation in anaerobic conditions](image)

The microencapsulated probiotic strains were grown in mammalian cell culture media and conditions for 24 h to determine if bacteria growth occurred in
these conditions. As shown in Figure 2.16, the cDMEM-AF colour was pink in the control cells (all the capsules were originally this colour). Within 24 h, the microencapsulated probiotic strains had changed the pH of the media (as indicated by the colour change of phenol red to yellow) due to the production of lactic acid. This colour change was generally observed to occur within 1 h of incubation.

![Image of capsules](image)

**Figure 2.16.** Alginate (1.8 %) capsules in mammalian cell culture media following 24 h incubation in standard cell culture conditions (37 °C, 5 % CO₂) a) empty capsules (control), b) microencapsulated *Lactobacillus acidophilus* LAFTI® L10, c) microencapsulated *Bifidobacterium lactis* HN019 (DR10™).

### 2.4.3.3 Analysis and Development of a Viability Standard Curve for Microencapsulated Bacteria using the Bitplane Imaris Software

Laser scanning confocal microscopy (LSCM) was used to produce z-stack images of microencapsulated bacteria that were stained with the LIVE/DEAD® BacLight™ Bacterial Viability and Counting Kit. The z-stack images were analysed using the Bitplane Imaris software to develop a viability standard curve of the
microencapsulated bacteria by plotting the viability of the bacteria against the green/red fluorescent spot ratio (produced by Bitplane Imaris analysis). The *L. acidophilus* LAFTI L10, *B. lactis* HN019 (DR10™) and *S. pyogenes* viability standard curves produced $R^2$ values of 0.9939, 0.9831 and 0.9962, respectively (Figures 2.17, 2.18 and 2.19). These values indicate that the ratio of live/dead cells produced by the fluorescence of SYTO 9 and PI, respectively, and determined by the Bitplane Imaris spots analysis can be used to measure the viability of microencapsulated bacteria cells.

![Graph](image)

**Figure 2.17.** Viability standard curve of microencapsulated *L. acidophilus* LAFTI L10 using the LIVE/DEAD® BacLight™ Bacterial Viability and Counting Kit (L7012, Invitrogen, Mulgrave, Australia). The Bitplane Imaris spots application software was used to determine the quantity of live and dead cell spots.
**Figure 2.18.** Viability standard curve of microencapsulated *B. lactis* HN019 (DR10™) using the LIVE/DEAD® *BacLight™* Bacterial Viability and Counting Kit (L7012, Invitrogen, Mulgrave, Australia). The Bitplane Imaris spots application software was used to determine the quantity of live and dead cell spots.

\[ y = 0.0687x + 1.4833 \]
\[ R^2 = 0.9831 \]

**Figure 2.19.** Viability standard curve of microencapsulated *Streptococcus pyogenes* using the LIVE/DEAD® *BacLight™* Bacterial Viability and Counting Kit (L7012, Invitrogen, Mulgrave, Australia). The Bitplane Imaris spots application software was used to determine the quantity of live and dead cell spots.

\[ y = 0.0435x + 1.2124 \]
\[ R^2 = 0.9962 \]
The 3D projections of the microencapsulated bacteria used for the development of the viability standard curves are shown in Figures 2.20, 2.21 and 2.22; for these images, the LSCM settings were selected to display live cells as red and dead cells as green colouration. The 100% live bacteria capsules appeared to express more red fluorescence (indicates live cells in Fig. 2.20, 2.21 and 2.22) than green fluorescence (indicates dead cells in Fig. 2.20, 2.21 and 2.22) comparatively to the lower viability concentrations for all three bacterial species (shown in Fig. 2.20e, 2.21e & 2.22e). Green fluorescence was observed for the 0 and 10% viable capsules with yellow fluorescence (indicates fluorescence from both the red and green channels) observed in the lower viable capsules (0, 10 and 50%) particularly for \textit{L. acidophilus} LAFTI® L10 and \textit{B. lactis} HN019 (DR10™) (Fig. 2.20a, b & c, 2.21a, b & c, and 2.22a, b & c).

Similar to the non-treated microencapsulated bacteria in Section 2.4.3.1, the bacteria cells appear distributed throughout the capsule; however, individual bacteria cells are not evident at the 10 x magnification. The Bitplane Imaris spots analysis of the 3D capsules is shown in Figures 2.20, 2.21 and 2.22 (f to k). The capsules have been ‘cut’ in the middle section to give a view of the internal capsular area. Bacteria cells appear distributed throughout the entire capsule; a preference for either internal occupation or residing on the edge of the capsules by the bacteria cells was not observed. The live spots are shown in red and the dead spots are shown in green.
Figure 2.20. Microencapsulated *Lactobacillus acidophilus* LAFTI L10 3D projection images acquired by laser-scanning confocal microscopy: a) 0 % live bacteria cells, b) 10 % live bacteria cells, c) 50 % live bacteria cells, d) 90 % live bacteria cells, e) 100 % live bacteria cells. Microencapsulated *L. acidophilus* LAFTI L10 3D-cross section images acquired by Bitplane Imaris spots analysis: f) 0 % live bacteria cells; g) 10 % live bacteria cells; h) 50 % live bacteria cells; i) 90 % live bacteria cells; and k) 100 % live bacteria cells. In these images, the live cells (green emission spectrum) are represented by the red colouration and the dead cells (red emission spectrum) by the green colouration.
Figure 2.21. Microencapsulated *Bifidobacterium lactis* HN019 (DR10™) 3D projection images acquired by laser-scanning confocal microscopy: a) 0 % live bacteria cells, b) 10 % live bacteria cells, c) 50 % live bacteria cells, d) 90 % live bacteria cells, e) 100 % live bacteria cells. Microencapsulated *B. lactis* HN019 (DR10™) 3D-cross section images acquired by Bitplane Imaris spots analysis: f) 0 % live bacteria cells; g) 10 % live bacteria cells; h) 50 % live bacteria cells; i) 90 % live bacteria cells; and k) 100 % live bacteria cells. In these images, the live cells (green emission spectrum) are represented by the red colouration and the dead cells (red emission spectrum) by the green colouration.
Figure 2.22. Microencapsulated *Streptococcus pyogenes* 3D projection images acquired by laser-scanning confocal microscopy: a) 0 % live bacteria cells, b) 10 % live bacteria cells, c) 50 % live bacteria cells, d) 90 % live bacteria cells, e) 100 % live bacteria cells. Microencapsulated *S. pyogenes* 3d-cross section images acquired by Bitplane Imaris spots analysis: f) 0 % live bacteria cells; g) 10 % live bacteria cells; h) 50 % live bacteria cells; i) 90 % live bacteria cells; and k) 100 % live bacteria cells. In these images, the live cells (green emission spectrum) are represented by the red colouration and the dead cells (red emission spectrum) by the green colouration.
2.5 **DISCUSSION**

2.5.1 *Bacteria Growth Curves in Broth and Mammalian Cell Culture Media and Conditions*

Difficulties were experienced in obtaining bacterial growth curves in mammalian cell culture conditions using absorbance measurements; the readings fluctuated due to changes of the phenol red indicator in the media during the 8 h measurement period. Spread plates were used as an alternative method to enumerate the colonies of bacteria grown in mammalian cell culture media and conditions. Therefore, comparisons between the absorbance growth curves obtained from bacteria growth in broth and spread plate results from bacteria growth in mammalian cell culture and conditions are not possible as the two measurement methods were different. However, within 8 h of incubation, the growth curve of the three bacterial strains in their standard broth (MRS or BHI broth) occurred at an exponential rate, whereas the bacterial growth in mammalian cell culture media and conditions did not appear to reach the exponential growth phase during the 8 h period.

For this thesis, the inability of the three strains to grow exponentially in mammalian cell culture media and conditions is beneficial for the application of the probiotics and pathogenic strains to the progenitor immune cells. In the respective broth, the growth of bacteria occurs at accelerated rates as encouraged by the availability of many nutrients and compounds, high growth rates would not have normally been observed in natural systems e.g. the gastrointestinal tract. The lack of exponential growth by the three stains in mammalian cell culture media and conditions would to some extent resemble the real-life growth characteristics of bacteria in the gastrointestinal environment.
Composition of Mammalian Cell Culture Media & Broth – Effects on Bacterial Growth

The mammalian cell culture media consists of numerous amino acids, vitamins, inorganic salts, glucose, phenol red and sodium pyruvate (Appendix A.2.1); further additives include non-essential amino acids (glycine, L-alanine, L-asparagine, L-aspartic acid, L-glutamic acid, L-proline and L-serine) and FBS. Carbohydrates, such as glucose, are utilised by bacteria to produce energy. Lactobacilli homofermentatively processes glucose via the glycolysis pathway producing lactate; bifidobacteria heterofermentatively processes glucose via the phosphoketotase pathway also producing lactate as a final product (Kandler, 1983). *S. pyogenes* ferments glucose to lactic acid for energy production and shifts to mixed acid fermentation when glucose availability is limited (Thomson, 1979). Sodium pyruvate is formed prior to lactate for the three strains; the presence of sodium pyruvate in DMEM would assist in lactate production. Within the gastrointestinal tract, bacteria ferment amino acids to short-chain fatty acids when carbohydrate content is limited (Russell et al., 1991).

Lactobacilli and bifidobacteria reportedly require arginine, glutamic acid, isoleucine, leucine, tryptophan, tyrosine, cysteine and valine for stimulation or growth in bovine milk (Poch and Bezkorovainy, 1988; Poch and Bezkorovainy, 1991); all these amino acids are present in the mammalian cell culture media. Lactic acid bacteria (LAB) reportedly catabolises all amino acids; the degradation ability differs between species potentially due to variations in the genetic coding of enzymes required to break down the different amino acids (Fernandez´ and Zu´niga, 2006). It was reported by Soska (1966) that different concentrations of amino acids
(together or individually) did not affect the growth rate of *Lactobacillus acidophilus* R-26; however, higher concentrations of these amino acids encouraged bacterial growth (Soska, 1966). The amino acids investigated in the Soska (1966) study were present in the mammalian cell culture media with the exception of glutamine and aspartic acid (not tested by Soska). Steiner and Malke (2000) evaluated the adaptive genetic changes of *Streptococcus pyogenes* strains SF370 and NZ131 to amino acid starvation, in particular, the genes required to produce enzymes that break down oligopeptides. Group A streptococci are multiple auxotrophs requiring certain amino acids to be provided by the local environment for growth and stimulation (Steiner and Malke, 2000).

The MRS and BHI broths both contain ingredients (peptone, protease peptone, lab lemco powder and yeast extract) that consist of amino acids and small peptides; these mediums provide the essential nutrients and compounds required for the growth of *L. acidophilus* and *B. lactis* (MRS broth) and *S. pyogenes* (BHI broth). Therefore, the presence of the amino acids in the mammalian cell culture media should not have an adverse effect on the growth of the bacteria strains, in some instances, bacterial growth would be encouraged by the availability of the amino acids.

The inorganic salts, sodium chloride and magnesium sulphate, are included in both MRS and BHI broth, as well as DMEM. The concentration of magnesium salt is reported to affect the growth and morphology of *L. acidophilus*; slow growth occurs in lower salt concentrations with the cells appearing swollen and transparent, whereas the cells clump and sediment in higher salt concentrations (Soska, 1966).
The other inorganic salts (i.e. calcium chloride, ferric nitrate, potassium chloride and sodium phosphate monobasic) found in DMEM are not included in MRS or BHI broth. However, considering the fastidious growth requirements of mammalian cells, these compounds would not be expected to have adverse effects on the growth of *L. acidophilus, B. lactis* or *S. pyogenes*. Calcium chloride has buffering effects in fermented foods containing lactic acid bacteria and reportedly encourages bacterial growth (Seo et al., 2009). Calcium chloride reportedly protects *L. acidophilus* NCFM cells from damage during freezing (Wright and Klaenhammer, 1981) and is used to form capsules when mixed with alginate by acting as a hardening agent for probiotic bacterial microencapsulation (Kailasapathy, 2002). Potassium chloride has also been used as a hardening agent for κ-carrageenan microencapsulation of *L. bulgaricus* ATCC 11842 (Buyukgungor, 1992) and bifidobacteria (Kebary et al., 1998). Further, sodium chloride, potassium chloride and calcium chloride are used as food processing and preserving compounds at much higher concentrations (Ravishankar and Juneja, 2000). Ferric nitrate has been reported to induce the growth of *E. coli* as a source of nitrogen (Roberts et al., 2005). The other inorganic salt present in DMEM, sodium phosphate monobasic, has buffering capacities and is an ingredient in phosphate buffered saline (PBS). The presence of these compounds should not have an adverse effect on the growth of *L. acidophilus, B. lactis* and *S. pyogenes*. The concentrations of these inorganic salts are low in the DMEM comparatively to MRS and BHI broth with milligram quantities in DMEM as opposed to gram quantities in the two broths.

The slow growth of the three strains in the DMEM (comparative to the two broths) could be from the low salt concentrations. As outlined previously, Soska
(1966) described slower bacteria growth by *L. acidophilus* in media containing low concentrations of magnesium salt. Further, the pH of the DMEM could also affect bacterial growth, in particular for *L. acidophilus* and *B. lactis*; the MRS broth pH is recommended at 6.2 ± 0.2 and the pH of DMEM is 7.2 (Appendix Table A.1.1). Milk is commonly used as a growth medium for *L. acidophilus* and *B. lactis* with a pH range of 6.4 to 6.8. The pH of BHI broth is recommended at 7.4 ± 0.2; the growth of *S. pyogenes* should not be affected by the pH of DMEM.

The mammalian cell culture media additive, FBS, provides growth factors and a rich-variety of proteins necessary for the growth of the mammalian cells. The three strains would not be expected to be affected by the FBS content; bacterial growth could potentially be encouraged by the availability of the proteins and growth factors. Further, DMEM contains the vitamins choline chloride, calcium pantothenate, folic acid, niacinamide, pyridoxine, riboflavin, thiamine and inositol. Vitamins act as coenzymes and functional groups of certain enzymes in bacterial metabolic activities; some bacteria are capable of synthesizing the necessary vitamins whereas others require certain vitamins to be added to the culture media (Todah, 2009). For instance, riboflavine, calcium pantothenate and pyridoxine were evaluated to determine if *L. acidophilus* could grow in their absence and what the effects would be with varying concentrations. The cell size of *L. acidophilus* reportedly decreased in lower vitamin concentrations and could not grow without riboflavine and calcium pantothenate; however the cells could grow without pyridoxine (Soska, 1966). Lactic acid bacteria isolated from brewery materials reportedly required pantothenic acid, nicotinic acid, riboflavin and thiamine; the requirement of individual vitamins was strain-dependent (Russell et al., 1954).
Therefore, the presence of the vitamins in DMEM (as listed previously) should not have an undesirable effect on the growth of the three stains. In some cases, the presence of certain vitamins would assist with the metabolic activities of the strains.

The components of the mammalian cell culture media should support the growth of the three bacteria strains without adverse affects. To allow the bacteria to adapt to the available of amino acids, growth factors, etc. in cDMEM-AF, the three bacteria strains were grown for 24 to 48 h in cDMEM-AF prior to conducting the growth curves (results not included). The adaptation step was performed to allow short-term genetic changes by the bacteria to the new environment, for instance, to produce some enzymes required to breakdown the amino acids and other compounds of DMEM. It was found that the bacteria growth decreased over this time with the longer exposure to the DMEM; the lower concentrations of glucose, inorganic salts, amino acids and vitamins could have potentially slowed the bacterial growth. The higher pH could have affected the growth of *L. acidophilus* and *B. lactis*. As outlined previously, the slow growth of the three strains would somewhat resemble the growth of bacteria in the gastrointestinal environment and therefore would be applicable to the co-culture experimentation with the immune cells.

### 2.5.2 Microencapsulation of Probiotic Bacteria

Distinguishing Viable and Non-viable Cells using Fluorescent Stains

The LIVE/DEAD® *BacLight™* Bacterial Viability Kit has been used to investigate the viability states of bacteria (Boulos et al., 1999), Achaea (Leuko et al., 2004) and eukaryotic cells (Zhang and Fang, 2004). The two stains enter cells based on the integrity of the cells walls; therefore, the kit differentiates between bacteria
with intact and damaged cytoplasmic membranes. The manufacturer states that cells expressing PI are considered non-viable (http://tools.invitrogen.com/content/sfs/manuals/mp07007.pdf). Gram-negative bacteria intermediate cell states (e.g. livings cells with a sustained injury) have been identified by flow cytometry analysis using the BacLight™ kit; however, intermediate states of Gram-positive bacteria were not observed (Berney et al., 2007). It was proposed that the ability to distinguish an intermediate cellular state of stained Gram-negative bacteria and not Gram-positive bacteria was due to the presence of an outer cell membrane in Gram-negative bacteria; upon damage of the outer cell membrane, PI can access and fully quench SYTO® 9 fluorescence (Berney et al., 2007). Gram-positive bacteria lack this outer cell membrane and the PI stain gains access to the cellular nucleic acid regardless of the cellular state (Berney et al., 2007). For this thesis, only Gram-positive bacteria were used and all cells expressing PI will be regarded as non-viable. As shown in Figure 2.10, live and dead cellular fluorescence of free bacteria can be distinguished as well as those that have been microencapsulated and viewed under a high magnification (Fig. 2.12). However, fluorescence from live and dead cells cannot be distinguished when the entire capsule is viewed under a low magnification (x 10); nonetheless, the overall microencapsulated bacteria population is visible (Fig. 2.13, 2.14, 2.20, 2.21 and 2.22). The Bitplane Imaris software allowed for the viable cells to be differentiated from non-viable cells based on the fluorescence from the individual channels i.e. fluorescence from SYTO® 9 as detected by PMT 2 (Channel 1) and from PI detected by PMT 3 (Channel 2) (Fig. 2.20, 2.21 and 2.22).
Viability Standard Curve for Microencapsulated Bacteria

Developing a viability standard curve of the microencapsulated bacteria was possible using the Bitplane Imaris software spots application statistics for the two channels (viable and non-viable); the same formula used for the viability standard curve of free bacteria was applied for the microencapsulated bacteria with the exception of using a spots count instead of fluorescence intensity for the live/dead ratio. To date, the most common method for enumerating microencapsulated bacteria is to release the bacteria from the capsules (usually by homogenisation) and to grow the bacteria on media (Annan et al., 2007; Ding and Shah, 2008; Zou et al., 2011). This method offers the ability to determine the concentration of viable bacteria cells; however, cellular viability is potentially lost during the homogenising steps and does not provide real-time live and dead cell information. At present, fluorescent stains, microscopy instruments and analysis software provide the tools to evaluate the real-time viable nature of bacteria cells in a capsular environment.

Microencapsulation Ingredients – Effects on the Diffusion of Soluble Factors and Mammalian Cell Culture Media

Probiotic bacteria viability is reportedly affected during food processing and storage (Kosin and Rakshit, 2006). As outlined previously, the viability of probiotic strains is considered important for the health-conveying properties to hosts (Fuller, 1992). Microencapsulation has been proposed as a tool to protect probiotics from stresses experienced during food processing procedures, storage and transit through the GI tract (Kailasapathy, 2002 and 2008). Alginate is commonly used as a microencapsulation ingredient as it is a natural occurring biopolymer with the advantage of requiring a mild environment to develop into a polymer (Blandino et
the microencapsulation processing environment is important to ensure probiotic bacterial viability is not affected. Similar to alginate, chitosan forms a gel by ionotropic gelation (Guerin et al., 2003), is a biodegradable, non-toxic biological compound (Anal and Singh, 2007) originating from the chitin of crustacean shells (Guerin et al., 2003). However, chitosan as a gelling agent alone can have adverse effects on cellular viability of LAB and is often used to coat alginate capsules to reduce these effects (Anal and Singh, 2007). Calcium chloride is used as a hardening agent of alginate. Interfacial polymerization takes place upon mixing sodium alginate and calcium chloride; the calcium alginate precipitates and slow gelation of the core occurs from the infusion of calcium ions through the alginate structure (Anal and Singh, 2007); therefore longer exposure of the alginate to the calcium chloride solution results in increased hardening of capsules.

Further, the surface of calcium alginate capsules is porous allowing for metabolite diffusion (Anal and Singh, 2007; Kailasapathy, 2008). This was demonstrated in Figure 2.16a, the capsule surface allowed cDMEM-AF to diffuse into the internal cavity (shown in pink); therefore oxygen and liquid material are able to diffuse into the capsule core gaining access to the bacteria. In addition, the microencapsulated probiotic bacteria were capable of metabolising the mammalian cell cultured media and readily produced lactic acid within 24 h as shown by the colour change of the pH indicator (Fig. 2.16b and c). Therefore, microencapsulated bacteria can be used to condition mammalian cell culture media for further experimentation with the mammalian progenitor immune cells. Due to the high production of lactic acid that would affect the viability of the mammalian progenitor immune cells, the incubation duration was shortened to 1 h (as opposed to 24 h) for
the conditioning of mammalian cell culture media by the probiotic bacteria (Chapters 4 and 5).

**Viability and Distribution of Microencapsulated Bacteria**

Microencapsulated probiotic bacteria have been applied to a number of food products including yoghurts, cheese, ice cream and milk powders (Anal and Singh, 2007). The viability of microencapsulated *L. acidophilus* (DD910) and *B. lactis* (DD920) was reportedly higher than free bacteria cells in yoghurt (Kailasapathy, 2006). Similarly, *L. acidophilus* CSCC2401 and *B. infantis* CSCC1912 were reported to have higher survival rates for the encapsulated cells comparative to free cells (Kailasapathy and Godward, 2001; Kailasapathy, 2003). However, the behaviour of the probiotic cells in capsules requires further research as this may lead to increased knowledge of how to protect cells and maintain cellular viability; such information could potentially be applied to increase the efficiency of probiotic incorporation in food products. As shown in Section 2.4.3, the distribution of the bacteria cells was observed to be throughout the capsule with the highest amount of cells concentrated in the middle section of the capsule (due to the larger diameter). Considering the anaerobic preference by the two probiotic strains, the bacteria near the capsular surface areas did not appear to have many non-viable cells where the highest oxygen content would be located. Oxygen has reportedly decreased the viability of free probiotic bacteria in food products during storage (Kailasapathy and Chin, 2000). Laser scanning confocal microscopy and Bitplane Imaris analysis of the 100 % viable capsules (Fig. 2.20, 2.21 and 2.22e and j) showed a mixture of viable (majority) and non-viable cells lining the internal capsular edge.
Release of Microencapsulated Bacteria

The ability of the bacteria cells to escape the capsules requires further investigation. Figure 2.15 shows the growth of probiotic bacterial colonies inside and outside of the capsules following 24 h incubation in anaerobic conditions; the possibility exists that the bacteria colonies outside the capsules were due to the growth of bacteria cells present on the capsule surface that ‘washed off’ with the sterile milli Q water used in the washing step (the capsules were not completely dried after the washing step when applied to the MRS agar plates). Chitosan alginate has been reported to decrease the rate of *L. lactis* ssp. *lactis* cellular release in fermented milk (Klinkenberg et al., 2001). It is possible that the pores of the calcium alginate and chitosan capsules are not large enough to facilitate the escape of bacteria cells. Generally the release of probiotic cells is controlled based on the composition of the capsule polymer and environment that results in the release of the cells. For instance, alginate capsules are commonly used for targeted delivery of probiotics in the GI tract (Zou et al., 2011).

At this point, LSCM analysis of capsules will not provide high resolved images required to determine the pore size on the surface of the calcium alginate capsules. Scanning electron microscopy (SEM) has been used to investigate the pore size of alginate-polyethersulfone capsules prepared at difference concentrations of polyethersulfone and porophore providing high detailed images (Kupikowska et al., 2009). Transmission electron microscopy (TEM) and SEM have been used to investigate the microstructure of microencapsulated probiotics (Allan-Wojtas et al., 2008). However, the distribution and behaviour of cells is altered during the sample preparation procedure that involves dehydrating fixing the capsules for SEM and
TEM, respectively. The SEM analysis revealed pockets of bacteria cells in the capsules and the capsule surface; the pore composition of the surface was not clear (Allan-Wojtas et al., 2008). Further, the SEM analysis of alginate capsules containing the fungi *Piromyces* sp. KSX1 showed rhizomycelial growth that covered the capsule surface (McCabe, 1998). The combination of analysis from LSCM and other microscopy methods, such as SEM, can provide detailed information regarding the structure of the capsules, distribution of bacteria cells and real-time viability.

Future investigations into stress-related changes of microencapsulated bacteria are possible by using plasmids with a gene of interested (e.g. stress-related proteins, etc.) tagged to a fluorescent protein such as GFP. The long term stress responses potentially could be observed using fluorescent microscopy if the proteins are expressed. For instance, the pGLO plasmid contains the reporter genes GFP and ampicillin resistance; an arabinose metabolising gene shares a bidirectional promoter with GFP and in the presence of arabinose, GFP is expressed when excited by UV light.

The real-time viability of microencapsulated probiotics in food products for long durations, such as yoghurt, could be investigated using the LIVE/DEAD® BacLight™ Bacterial Viability Kit. Microencapsulated microorganisms were successfully stained with this kit following microencapsulation (Pereira et al., 2005). Using a viability standard curve, such as the one developed in Section 2.4.3.3, the real-time viability of the microencapsulated probiotics can be determined without disrupting the capsule and internal contents that could result in the loss of viable cells.
2.6 CONCLUSION

The bacteria strains, *L. acidophilus* LAFTI® L10, *B. lactis* HN019 (DR10™) and *S. pyogenes*, were able to metabolise the mammalian cell culture media. This was demonstrated by the generation of a growth curve. The growth rate of the three strains in the mammalian cell culture media was shown to be slower than growth of these three strains in conventional broth (MRS or BHI broth). This was attributed to the lower concentrations of inorganic salts, vitamins, amino acids, growth factors and glucose in the mammalian cell culture media comparative to the two broths. However, the lower growth rate is considered beneficial for the conditioning of mammalian cell culture media by the three strains in this thesis as it potentially resembles the slow growth rate of the strains experienced in real-life systems.

Microencapsulated probiotic bacteria were shown to grow on MRS agar after 24 h incubation in anaerobic conditions at 37 °C. The presence of colonies from free bacteria was also observed, however, the possibility these colonies developed from cells escaping the capsules requires further research. The microencapsulated bacteria were shown to metabolise mammalian cell culture media. The production of lactic acid by the microencapsulated probiotic bacteria was determined from the colour change of the pH indicator (phenol red) in the mammalian cell culture media; a 1 h conditioning of mammalian cell culture media by the three strains was determined to be the maximum duration prior to high lactic acid production. Therefore, the bacteria-conditioned mammalian cell culture media can be applied for further experimentation with the mammalian progenitor immune cells.
A viability standard curve was developed for the three microencapsulated strains using the LIVE/DEAD® BacLight™ Bacterial Viability Kit. This standard curve could be applied to investigate the real-time viability of microencapsulated microorganisms, particularly those used in food products.
3.CHAPTER THREE

Development of a Fluorescent Immune Cell Model

3.1 Introduction

Fluorescent Proteins as Reporter Molecules of Gene & Protein Expression

Fluorescence from intracellular-expressed fluorescent proteins can be used as an indicator of gene and protein expression providing the ability to tag specific intracellular genes and protein products (Low and Gong, 2005). The green fluorescent protein (GFP) was initially used as a reporter gene for β-tubulin in Caenorhabditis elegans (Chalfie et al., 1994). Cellular protein interactions, activities and expression are commonly determined by tracking fusion GFP and other fluorescent proteins (Low and Gong, 2005).

For this study, the effects of probiotic interactions with immune cells were determined by the fluorescence expression of two fluorescent proteins (phMGFP and pCIneo-DsRed2) transfected into the porcine progenitor immune cell lines L45 (T-cell) and L23 (B-cell); changes in cellular fluorescence expression are able to be analysed using confocal microscopy and fluorescence imaging analysis software. However, the hMGFP and pCIneo-DsRed2 fluorescent proteins have not been tagged to any particular gene and protein product. Therefore, the transfected plasmid potentially integrates randomly into the progenitor immune cell’s DNA, undergoes transcription and translation (including modifying procedures during these stages) and produces a fluorescent protein. These stages can be influenced by a number of cellular and environmental factors rendering either a functional or non-functional protein product. Identification of gene expression timing and protein dynamics can provide a way to understand the physiological activities of a protein and what
influences its expression and activities in the presence of other cellular molecules. Therefore, the resulting fluorescence expression is being used as a downstream bioindicator of cellular responses to probiotic bacterial treatments and hence, as an indirect indicator of the effects of probiotic-produced soluble factors on the activities of the progenitor immune cell’s gene expression. However, reports have indicated that intracellular fluorescent proteins accumulate in lysosomes (Katayama et al., 2008) and induce apoptosis (Liu et al., 1999).

Fluorescent Dyes to Confirm Localization of Fluorescent Protein Expression

This Chapter presents an investigation into the localization of fluorescence expression by analysing the co-localization of the fluorescent protein expression with DNA, RNA and lysosomal fluorescent dyes and probes. Unlike fluorescent proteins that tag cellular components via genetic encoding, these dyes/probes ‘tag’ components via chemical interactions. For instance, the dyes Hoechst 33342, SYTO 59® and propidium iodide (PI) are DNA-specific probes that fluorescence (at different wavelengths) upon binding to nucleic acid. Hoechst 33342 binds the minor-grooves of double-stranded (ds) DNA via bisbenzimidazole derivatives with AT selectivity (fluorescence is enhanced 2-fold compared to GC binding) and can be used to detect nuclear damage due to the sensitivity to DNA conformation and chromatin (Portugal and Waring, 1988). The SYTO 59® stain is less specific than Hoechst 33342 binding to DNA and RNA, cytoplasmic regions and mitochondria; information regarding the mechanisms of SYTO 59® binding is limited as this dye is a commercial product (Molecular Probes, 2001 http://tools.invitrogen.com/content/sfs/manuals/mp11340.pdf). Propidium iodide
(PI) intercalates between DNA bases with minimal specificity (Waring, 1965) and also binds RNA (Suzuki et al., 1997).

The L45 and L23 Progenitor Immune Cell Lines

The L45 (T-cell) and L23 (B-cell) cell lines are two of five porcine lymphoid cell lines derived from peripheral blood mononuclear cells (PMBC) of a histocompatible miniature boar (Sus scrofa domestica) infected with the Tsukuba-1 retrovirus. Shimozuma cells (Kodama et al., 1981) producing the porcine Tsukuba-1 retrovirus were taken from a pig infected with a malignant lymphoma (Suzuka et al., 1985) and injected into the miniature boar. A parent lymphoblastoid cell line (B1) was isolated and the derivative cell lines produced by Kaeffer et al. (1990). Following mitogenic stimulation using phytohaemagglutinin (PHA) and sub-cloning, two T cell and three B cell lines were established and remained unchanged at 100 passages (Kaeffer et al., 1990).

The L23 cell lines are lymphoblastoid in morphology with a larger size than most circulating lymphocytes. In suspensions, these cells grow in clumps mediated through cellular junctions and suggestive of active cellular cooperation (Kaeffer et al., 1990). The L23 cells were characterised by Kaeffer et al. (1990) who described the expression of the major histocompatibility complex (MHC) class I (7 % expressed MHC class II), membrane immunoglobulins (IgG and IgM) of the μ isotype and Fc receptors on the cell surface. The L23 cells possess normal chromosome numbers as determined by karyotype analysis. In addition, these cells did not express reverse transcriptase (characteristic of retroviruses) but did secrete retrovirus-like particles as established by electron microscopy. Similar to L23 cells,
the L45 cells exhibit lymphoblastoid morphology (with a high nucleocytoplasmic ratio), form cellular clumps and contain inactive retrovirus particles (Kaeffer et al., 1990). However, karyotype analysis revealed an average of 36.5; this differs from the porcine diploid chromosome number of 38 therefore demonstrating usual gene expression. The L45 cells display MHC class I molecules (3 % contain class II) and 72 % express membrane antibody receptors (Kaeffer et al., 1990). The presence of CD2a (marker of differentiation) on cell surfaces indicates that these cells are T lymphocytes, however the lack of CD4 and CD8 surface molecules suggests L45 cells are at the commencement of T cell commitment and have not matured (Kaeffer, 1994). These cell lines are unipotent (develop into one cell type) progenitor cells (immature cells committed to B (L23) and T (L45) cell development) capable of self-renewing.

3.2 AIMS AND OBJECTIVES

The aims of this Chapter are:

- To develop a fluorescent immune cell model by transfecting populations of the porcine progenitor immune cell lines, L45 (T-cell) and L23 (B-cell), with the fluorescent protein plasmids humanized Monster Green® Fluorescent Protein (phMGFP) and pCIneo-DsRed2 resulting in four new cell lines i.e. L45hMGFP, L45pCIneo-DsRed2, L23hMGFP and L23pCIneo-DsRed2, with fluorescent protein production capabilities.

- To identify the location of fluorescence expression by the fluorescent proteins within the L45 and L23 cells by investigating the nucleic and lysosomal regions.
- To investigate the relationship between high fluorescence expression and apoptosis in immune cells.

The objectives of this Chapter are:

- To produce porcine immune cell lines (B & T cells) expressing the phMGFP and pCIneo-DsRed2 plasmids and to confirm fluorescence production by laser scanning confocal microscopy (LSCM).

- To stain and locate the DNA, RNA and lysosomal regions of the cells by using the DNA and RNA-specific fluorescent probe SYTO 59® (Invitrogen, Mulgrave, Australia) and the lysosomal fluorescent probe, Lysosensor™ Blue DND-167 (Invitrogen, Mulgrave, Australia).

- To determine if any co-localization exists between the fluorescence expression of nucleic acids, lysosomes and the intracellular expressed fluorescent proteins.

- To induce apoptosis in the transfected immune cells lines and identify if any correlation exists between intracellular fluorescence expression and the apoptotic state of the cells.

- To perform LSCM analysis on the apoptosis-induced cells using the dead cell fluorescence probe, propidium iodide (PI), to confirm the apoptotic state of the cells and to further identify if a correlation exists between intracellular fluorescence expression and the apoptotic state of the cells as indicated by PI staining.
3.3 MATERI ALS AND METHODS

3.3.1 Transfection of the Mammalian Immune Cells, L23 and L45, with the Fluorescent Protein Plasmids, phMGFP and pCIneo-DsRed2

3.3.1.1 Transfection reagents

Transfections were performed using the LipoTAXI Mammalian Transfection Kit (204110, Strategene) purchased from Integrated Sciences (Melbourne, Australia).

3.3.1.2 Fluorescent Protein Plasmids

Two fluorescent vectors were utilised. The monster green®, fluorescent protein phMGFP vector (Appendix Fig. A.2.1) was purchased from the Promega Corporation. The pCIneo-DsRed2 plasmid was a gift from B. Sainsbury (Sainsbury, 2008) and was constructed using the pDsRed2 vector (632404, Clontech, USA) (Appendix Fig. A.2.2) and pCI-neo mammalian expression vector (E1841, Promega, Australia) (Appendix Fig. A.2.3). The phMGFP and pCIneo-DsRed2 plasmids were transformed into the mutated strain of Escherichia coli called One shot® BL21Star™ (DE3, Invitrogen, Mulgrave, Australia) – this procedure was performed by S. Bowman (Bowman, 2008) for the phMGFP and B. Sainsbury for the pCIneo-DsRed2 plasmids. Extraction and purification of the plasmids were performed using the QuickLyse Miniprep Kit (27405, Qiagen, Australia) – the phMGFP extraction and purification was performed by S. Bowman (Bowman, 2008). The One shot® BL21Star™ E coli cells transformed with pCIneo-DsRed2 plasmids were stored at -80 °C by B. Sainsbury. An aliquot of cells (10 µL) was placed into sterile Luria-Bertani (LB) (Maqueda et al., 2008) broth containing tryptone (1 % w/v), yeast
extract (0.5 % w/v) and NaCl (0.5 % w/v) supplemented with ampicillin (1 mg mL\(^{-1}\)). The cells were grown for 24 h at 37 °C in the Thermoline Scientific Australia Incubator (Model I60G-290-D); a streak plate was prepared using LB agar containing ampicillin (1 mg mL\(^{-1}\)) to isolate colonies and incubated at 37 °C for 24 h. A colony was selected and grown in two 50 mL CELLSTAR® polypropylene tubes LB broth containing ampicillin (1 mg mL\(^{-1}\)) for a further 24 h. Plasmid extractions were conducted according to the manufacturer’s instructions of the QuickLyse Miniprep Kit (27405, Qiagen, Australia). Both plasmid/bacteria 50 mL suspensions were centrifuged at 1391 g for 10 min with the Universal 32 R centrifuge (HD Scientific Supplies Pty. Ltd.); the supernatant was decanted and the pellet was resuspended in LB broth (1.5 mL). The suspension was placed into a QuickLyse Lysis tube and centrifuged at 15, 682.2 g with the Eppendorf Bench Centrifuge (5415D) for 1 min at room temperature. The supernatant was removed by decanting; the pellet was resuspended in ice cold complete lysis solution (400 µL) and vortexed for 30 s. The suspension was incubated at room temperature for 3 min. The suspension was placed into a QuickLyse spin column and centrifuged at 15, 682.2 g for 30 s by the Eppendorf Bench Centrifuge (5415D). The QuickLyse spin column was washed by diluted buffer QLW (400 µL) and re-centrifuged for 60 s at 15, 682.2 g. The flow through was discarded and the QuickLyse spin column was dried by centrifuging at 15, 682.2 g for 1 min. The plasmid DNA was eluted by buffer QLE (50 µL) into a sterile collection tube and centrifuged at 13, 000 rpm for 1 min. The plasmids were stored at -20 °C. Both plasmids were quantified using the Thermo Scientific NanoDrop (2000) Spectrophotometer. The concentration of phMGFP and pCIneo-DsRed2 were 341.6 ng µL\(^{-1}\) and 206.1 ng µL\(^{-1}\), respectively.
3.3.1.3 Porcine Progenitor Immune Cell Lines

The mammalian immune cell lines, L23 (ECACC No. 91012318) and L45 (ECACC No. 91012320), were sourced from the University of Western Sydney’s cell culture collection. Both cell lines are non-adherent, immortalised, porcine peripheral blood progenitor B (L23) and T (L45) cells (European Collection of Animal Cell Cultures catalogue number 91012318 and 90102320, respectively). The cell cultures were maintained in CELLSTAR® 24 Well Cell Culture Multiwell Plates (662102, Greiner Bio-One; Interpath Services P/L, Australia) and Corning® 25cm$^2$ Rectangular Canted Neck Cell Culture Flask with Vent Cap (430639, Corning, Australia). Cells from the T25 flasks were passaged 24 to 48 h prior to experimentation (unless otherwise stated) and grown in complete Dulbecco’s Modified Eagle’s Medium (DMEM) (Invitrogen; Mulgrave, Australia) (Appendix A.2.1.) supplemented with foetal bovine serum (FBS) (Invitrogen; Mulgrave, Australia) at a final concentration of 10%. Cell suspensions grown in the 24 Well Cell Culture Plates were passaged approximately every 7 d, depending on cell growth.

Passaging was performed by adding cell suspension (0.5 mL) to fresh complete DMEM (1.0 mL) and serially diluting the cell suspension for 5 wells; the final well volume was 1 mL with the exception of the last well with a total of 1.5 mL. The cell suspensions were incubated at 37 °C in 5 % CO$_2$ in the Forma Scientific CO$_2$ water jacketed incubator (Model 3111 S/N 26931-617) and cell culturing was performed in the Clyde-Apac BH2000 series laminar flow hood (class 2 safety cabinet) and cell culture inspections (contamination and general overall culture wellbeing) were conducted with the Olympus CK2 inverted light microscope.
3.3.1.4 Transfection Procedure

The progenitor immune cells (L45 and L23) were passaged as detailed in Section 3.3.1.3, 18 to 24 h prior to transfection. Cell suspensions were enumerated using the Neubauer - Improved hematocytometer (Marienfeld, Germany) and trypan blue stain (0.4 %; T8154, Sigma, Sydney, Australia). A mixture of cell suspension (10 μL) and trypan blue (10 μL) was prepared in a clear, polystyrene, flat bottom 96-well plate (655101, Greiner bio-one; Interpath Services P/L, West Heidelberg, Australia), mixed by pipetting and placed on the hematocytometer. Cell enumeration was performed with the Olympus BH2 light microscope (Model No. 0M0180). Cells excluding the trypan blue stain were identified as viable and those coloured blue as non-viable. It should be noted that trypan blue does not distinguish between necrotic and apoptotic cells. The cell suspensions were placed into a sterile 15 mL Falcon™ polypropylene conical tube (352096, BD Bioscience, Australia) and centrifuged with the Universal 32 R centrifuge (HD Scientific Supplies Pty. Ltd.) for 5 min at 320 g; the supernatant was removed. The pellets were washed with stock DMEM and resuspended in stock DMEM for a final concentration of 1.0 x 10^6 cells mL^-1. The transfections were performed using the LipoTAXI Mammalian Transfection Kit (204110, Strategene; Integrated Sciences, Australia) as per the manufacturer’s instructions with modifications for a 24-well plate. Two wells were prepared for each fluorescent protein plasmid treatment (phMGFP and pCIneo-DsRed2) for the L45 and L23 cells in a 24-well plate. To each well, cell suspension (500 μL) was added with a cell concentration of 5.0 x 10^5 cells mL^-1. Two transfection complex formation solutions were prepared, one each for the phMGFP and pCIneo-DsRed2 plasmids, in a sterile 1.5 mL microcentrifuge tube (121000, Greiner Bio-One; Interpath Services P/L, Heidelberg West, Australia); LipoTAXI (30 μL) was added
to stock DMEM (90 μL) making a final volume of 120 μL, the solution was mixed by gently tapping the tube. Two microlitres of phMGFP (341.6 ng μL\(^{-1}\)) and pCIneo-DsRed2 (206.1 ng μL\(^{-1}\)) were added to the complex formation solution and incubated at room temperature for 20 min. The activated transfection solution was prepared by adding complete DMEM (200 μL) to the complex formation solution making a final volume of 320 μL. The activated solution (80 μL) was added to each treatment well and incubated at 37 °C, in 5% CO\(_2\) for 4 h. Following the 4 h incubation, complete DMEM (420 μL) was added to each well making a final volume of 1 mL, and the cells were further incubated at 37 °C in 5% CO\(_2\).

3.3.1.5 Laser Scanning Confocal Microscopy (LSCM) Analysis of Transfected Progenitor Immune Cells

All LSCM images and data were collected using the Leica TCS SP5 inverted LSCM with the DM600 fixed stage and software Leica Application Suite: Advanced Fluorescence (Leica Microsystems, Heidelberg, Germany). At 24 h and 14 days post-transfection, the transfected L45 cells (L45hMGFP and L45pCIneo-DsRed2) and L23 cells (L23hMGFP and L23pCIneo-DsRed2) were analysed for fluorescence expression. The LSCM settings were originally chosen as outlined by the manufacturer’s description of excitation and emission profiles for the two fluorescent protein plasmids. Excitation was performed using the Argon-488 nm laser for both fluorescent proteins. The excitation maximum for the pDsRed2 is recommended at 558 nm (Clontech), however excitation with the Argon-561 nm laser produced little to no fluorescence emission using this system. Further experimentation was conducted using the Argon-488 nm laser to excite the pCIneo-DsRed2 fluorescent protein. The 633 nm laser was set to 10 % maximum intensity and the
Photomultiplier Tube (PMT) NDD3 detector was used for bright-field images, gain set to 254.5 V. Fluorescence emission for both fluorescent proteins was detected using the PMT 2. The gain was randomly selected but kept lower than 1000 V to ensure the autofluorescence of cells did not interfere with the detected fluorescence emission of the proteins. Scanning speed was 400 Hz with pixel resolution at 512 x 512. The HCX PL APO CS 63.0 x 1.4 (numerical aperture) oil UV objective was used for all imaging and scans. Cell suspensions (4 samples at 10 µL each) were placed on the 42 x 0.17 mm circular slides and covered with the 32 x 0.17 mm circular cover slip. The pinhole size was 1 airy unit (95.5 µm). Further observations were performed after 14 days post-transfection with the Leica LSCM to ensure the fluorescence expression of cells.

3.3.1.6 Comparison of the Growth-Rates of Immune Cells Expressing Fluorescent Proteins and not Expressing Fluorescent Proteins

Cell

Changes in the growth-rate of transfected cells, in response to the fluorescent protein plasmids, were determined by comparing the growth rates of transfected and non-transfected (not expressing fluorescent proteins) cells. All cells were grown as outlined in Section 3.3.1.3. The cells were enumerated as outlined in Section 3.3.1.4, and centrifuged as described in Section 3.3.1.3. The supernatants were decanted and the pellets resuspended in complete DMEM making a final cell concentration of 5.0 x 10^5 cells mL^-1. Individual 24-well plates were used for each cell line with a 1 mL final volume of cell suspension placed into every well. The cell lines were enumerated at random time intervals up to 47 h. Three aliquots of cells (10 µL) were taken from the same well for enumeration; aliquots from each well were only
performed once to ensure cell-cell interactions were not disturbed prior to enumeration.

3.3.2 Identification of Transfected Cellular Fluorescence Expression by Comparative Analysis with Nucleic Acid and Lysosomal-specific Fluorescent Probes

3.3.2.1 Fluorescent Probes

The fluorescent probes, SYTO 59® (S11341) and LysoSensor™ Blue DND-167 (L-7533), were purchased from Invitrogen (Mulgrave, Australia). SYTO 59® is a cell-permeate nucleic acid stain with a fluorescence emission in the red spectrum. This stain can be used for both live and dead cells. The SYTO range of stains are not exclusive to nuclear staining in live cells and can also stain cytoplasmic regions and mitochondria (Molecular Probes Product Information, 2001). LysoSensor™ Blue DND-167 is used as a lysosomal stain that measures the pH of acidic organelles. Fluorescence is increased in acidic environments with LysoSensor™ Blue DND-167 only exhibiting fluorescence in acidic cellular regions due to possessing the pKₐ of 5.1. The LysoSensor™ probes are commonly used to study lysosomal acidification, changes in lysosomal activities and trafficking. These two stains are being used to study the localization of fluorescence expression by the pCIneo-DsRed2 and phMGFP fluorescent plasmids within the L23 and L45 cells. SYTO 59® was chosen as the nucleic acid stain as the fluorescence emission is within the far-red region and does not overlap the fluorescence emission of the two fluorescent protein plasmids. Similarly, LysoSensor™ Blue DND-167 was chosen as the fluorescence emission is in the blue range and to identify lysosomes without overlapping the fluorescence emission of the two fluorescent proteins.
A working concentration of 20 µM was prepared for both stains by adding SYTO 59® (5 mM, 1 µL) to stock DMEM (249 µL) and LysoSensor™ Blue DND-167 (1 mM, 1 µL) to stock DMEM (99 µL).

### 3.3.2.2 Cell Suspension Preparation and Staining with SYTO 59® and LysoSensor™ Blue DND-167

The L45, L23, L45hMGFP, L45pCIneo-DsRed2, L23hMGFP and L23pCIneo-DsRed2 cells were grown as outlined in Section 3.3.1.3. The cell suspensions were enumerated as outlined in Section 3.3.1.4. Experimentation was conducted on cell suspensions with at least 80 % viability. The cell suspensions were centrifuged as outlined in Section 3.3.1.4, the supernatants were decanted and the cell pellets resuspended in stock DMEM making a final concentration of 1.0 x 10^6 cells mL⁻¹. The L45 and L23 cell suspensions were treated with LysoSensor™ Blue DND-167 in a 1.5 mL microcentrifuge tube at a 100 µL final volume for 30 min at 37 ºC in the Thermoline Australia water bath (Model No. TSB1, Serial No. 8012). SYTO 59® treated L45 and L23 cell suspensions were incubated at 37 ºC for 10 min in the Thermoline Australia water bath (Model No. TSB1, Serial No. 8012) at a final volume of 100 µL in 1.5 mL microcentrifuge tubes. The final concentration for both stains was 2 µM. All cell suspensions were centrifuged briefly for 5 sec at room temperature using the Eppendorf Bench Centrifuge (5415D).

The autofluorescence intensity of unstained L45 and L23 cells were determined by wavelength scans using the Leica LSCM. Wavelength scans were
also conducted for the L45 and L23 cells stained with SYTO 59® and LysoSensor™ Blue DND-167 to identify the wavelength emission profile of these stains. Emission wavelength scans were also performed on the transfected L45 and L23 cells (L45hMGFP, L45pClneo-DsRed2, L23hMGFP and L23pClneo-DsRed2). The autofluorescence of L45 and L23 were compared to the fluorescence emission profile of L45 and L23 cells stained with SYTO 59® and LysoSensor™ Blue DND-167. Comparisons were also made between the autofluorescence of L45 and L23 cells with the fluorescence of L45hMGFP, L45pClneo-DsRed2, L23hMGFP and L23pClneo-DsRed2 fluorescence emission.

### 3.3.2.3 Laser-Scanning Confocal Microscopy (LSCM) Settings and Wavelength Scans

All LSCM images and data were collected using the hardware Leica TCS SP5 inverted LSCM with the DM600 fixed stage and software Leica Application Suite: Advanced Fluorescence. For wavelength scans, the software was set to the xyλ mode. Excitation was performed by the 405-Diode (405 nm) for LysoSensor™ Blue DND-167, Argon-488 nm for the intracellular fluorescent proteins (hMGFP and pClneo-DsRed2) and HeNe-633 nm lasers for SYTO 59®; a fluorescence emission profile scan was produced for stain by these three lasers. Wavelength scans were performed between 410 to 600 nm for the 405-Diode laser, 500 to 600 nm for the Argon-488 nm laser excitation and 640 to 700 nm for the HeNe-633 nm laser using the PMT 2. Bandwidth measurements were 10 nm with 20 steps for all lasers; the lambda step size was 10.00 nm, 5.26 nm and 3.16 nm for the 405-Diode, Argon-488 nm and HeNe-633 nm lasers, respectively. The 405-Diode laser was set to 30 % maximum intensity, the Argon-488 nm laser to 20 % power with a 50 % maximum
intensity and HeNe-633 nm laser to 10 % maximum intensity. The gain was set to 821.8 V and scans were performed at the best focus setting as determined by the Leica LAS software. Scanning speed was 400 Hz with pixel resolution at 512 x 512. The pinhole size was 1 airy unit (95.5 µm) and the zoom factor was 2.5. Line averages were set to 3 for all scans and to 16 for image collection. Imaging of cells was performed prior to each wavelength scan; bright-field (not used in wavelength scans) illumination was used for imaging. The HeNe-633 nm laser and the PMT NDD3 detector were used for bright-field images, gain set to 254.5 V. The HCX PL APO CS 63.0 x 1.4 (numerical aperture) oil UV objective was used for all imaging and scans. Cell suspensions (4 samples at 10 µL each) were placed on the 42 x 0.17 mm circular slides and covered with the 32 x 0.17 mm circular cover slip. The settings described above were used for all scans of non-transfected L45 and L23 cells.

3.3.2.4 Laser-Scanning Confocal Microscopy (LSCM) Analysis of DNA, RNA and Lysosomal Regions in Transfected Cells using SYTO 59® and LysoSensor™ Blue DND-167

To determine the intracellular location of fluorescence expression in the transfected progenitor immune cell lines (L45hMGFP, L45pCIneo-DsRed2, L23hMGFP and L23pCIneo-DsRed2), co-localization analysis between the intracellular fluorescent protein, SYTO 59® and LysoSensor™ Blue DND-167 staining was conducted. Staining of the transfected cell suspensions were prepared as outlined in Section 3.3.2.2. LSCM analysis was conducted using the system and settings outlined in Section 3.3.2.3 with the following modifications. The 405-Diode laser was set to 50 % maximum intensity, the gain set to 916.1 V and PMT 1 was
used to detect LysoSenor™ Blue DND-167 fluorescence expression between 450 to 470 nm. For L45hMGFP and L23hMGFP cells, PMT 2 was set to 520 to 550 nm detection range at a gain of 944.5 V using the Argon-488 nm laser for excitation. SYTO 59® fluorescence emission was detected using PMT 3 set to a gain of 536.9 V and excited by the HeNe-633 nm laser. The power intensities remained the same for the Argon-488 nm and HeNe-633 nm lasers as outlined in Section 3.3.2.3. The Leica LSCM software was set to the xyz mode to obtain z-stacks (3-dimensional) images of the cells. Sequential scanning parameters were defined for SYTO 59®, LysoSenor™ Blue DND-167 and intracellular fluorescence emission using the emission scan profiles as outlined and the “between lines” function. All z-stacks were performed at a 0.99 µM z-step size using a scanning speed of 700 Hz and a zoom factor of 2.5. The line average for all scans was set to 3. Z-stack images were performed in triplicate on different sample groups consisting of 20 - 25 cells (due to cellular aggregation).

3.3.2.5 Co-localization Analysis using Bitplane Imaris Software

The co-localization of SYTO 59® and intracellular fluorescent protein expression, and LysoSenor™ Blue DND-167 and the intracellular fluorescent protein expression were analysed using Bitplane Imaris software version 6.3.1. The Bitplane Imaris co-localization program was used to determine the level of spatial overlap of both channels by analysing each pixel of the raster confocal image to produce a frequency histogram of the channel’s intensities and associated statistical information.

Image series (z-stacks) collected from the Leica LSCM were prepared for co-localization analysis by employing 3D deconvolution, median smoothing and
background subtraction to gain a more accurate co-localization result (Landmann, 2002). The Leica TCS SP5 software application, 3D deconvolution, was performed using 10 total iterations, a refractive index of 1.52 (from the immersion medium - oil), with the blind method (automatically calculates the best image resolution and PSF using the microscopic parameters of the system), rescale intensity, remove background and autogenerate the PSF selected. The resulting 3D series were converted into a Bitplane Imaris format using the Bitplane Imaris batch converter. The series images were smoothed using the median filter option offered by Bitplane Imaris and a background subtraction of the three channels (Channel 1 - LysoSenor™ Blue DND-167; Channel 2 - intracellular fluorescent protein; and Channel 3 – SYTO 59®) was performed on all series with a filter width of 24.65 µM (as determined by the software). The ‘single points’ option was selected in the histogram mode Section and the ‘build co-localization channel’ option was applied to all channel combinations. The automatic threshold option was used for each channel combination (Channel 1 and Channel 2, Channel 1 and Channel 3, Channel 2 and Channel 3) that employs an algorithm developed by Costes et al. (2004); the PSF was 1.025. This algorithm excludes intensity pairs lacking any correlation by performing separate analysis of each channel and calculates the probability of having non-random co-localization (Costes et al., 2004). The Pearson’s correlation coefficient and Mander’s coefficient were determined. The Pearson’s correlation coefficient indicates a positive (1), negative (-1) or no correlation (0) between the overlap of an image’s intensity voxels in a localized region or volume; the average intensity distributions of the respective channels is measured indicating similarities in shapes as opposed to signal intensities (Zinchuk et al., 2007). The Mander’s coefficient provides a statistical value of co-localization by measuring the overlap of signals...
(Zinchuk et al., 2007). The average intensities are not considered in the calculation of the Mander’s coefficient (absolute intensities of signal are normalised against total pixel intensity); a value of 0 indicates no overlapping of signals whereas 1 indicates 100% co-localization (Bitplane, 2011). The average of the Pearson’s correlation coefficient and Mander’s coefficient were determined from the triplicate series to identify any correlation between the intracellular fluorescent protein, DNA, RNA and lysosomal stains.

3.3.3 Anisomycin Treatment of Transfected Cells to Evaluate the Relationship between Apoptosis and Fluorescence Expression

3.3.3.1 Anisomycin Preparation

Apoptosis of L45hMGFP, L45pClneo-DsRed2, L23hMGFP and L23pClneo-DsRed2 cells were induced by anisomycin (flagecidin), a protein inhibitor produced by *Streptomyces griseolus*. Anisomycin inhibits the peptidyl transferase activity in eukaryote ribosomes and reversibly inhibits DNA synthesis in the mammalian cell lines, HeLa and rabbit reticulocytes; no activity was reported against the prokaryote *Escherichia coli* (Grollman, 1967). Anisomycin was purchased from Sigma-Aldrich (A9789, Sydney, Australia) and prepared by adding of dimethyl sulfoxide (DMSO, 1 mL) (D2650, Sigma-Aldrich; Sydney, Australia) to anisomycin (5 mg) making a final stock concentration of 19 mM. A working solution of 9.22 mM was prepared by adding DMSO (500 µL) to the 19 mM (500 µL) stock solution.
3.3.3.2 Anisomycin Treatment of Transfected Cells

Apoptosis of L45hMGFP, L45pCIneo-DsRed, L23hMGFP and L23pCIneo-DsRed2 cells were induced with anisomycin to determine the relationship between cellular fluorescence expression and cell death. Two concentrations of anisomycin (10 µM and 25 µM) were used to treat the transfected cell lines. The cells were passaged 24 to 48 h prior to treatment as described in Section 3.3.1.3. Cells were enumerated as described in Section 3.3.1.4 and the suspensions were centrifuged at 320 g for 5 min. The supernatant was decanted and the pellets were resuspended in fresh complete DMEM making a final cell concentration of $1.0 \times 10^6$ cells mL$^{-1}$. The control, 10 µM and 25 µM treatments were prepared in 1.5 mL microcentrifuge tubes as outlined in Table 3.1.

**Table 3.1** Preparation of control and anisomycin treatments in a 96-well microplate.

<table>
<thead>
<tr>
<th></th>
<th>Cell suspension volume (µL)</th>
<th>DMSO volume (µL)</th>
<th>Anisomycin* volume (µL)</th>
<th>Total volume (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>593.4</td>
<td>6.60</td>
<td>0.00</td>
<td>600.00</td>
</tr>
<tr>
<td>10 µM treatment</td>
<td>593.4</td>
<td>5.95</td>
<td>0.65</td>
<td>600.00</td>
</tr>
<tr>
<td>25 µM treatment</td>
<td>593.4</td>
<td>4.97</td>
<td>1.63</td>
<td>600.00</td>
</tr>
</tbody>
</table>

* From the anisomycin 9.22 mM working solution

3.3.3.3 Fluorescent Probes

The fluorescent probe, Hoechst 33342 (H1399, Invitrogen, Mulgrave, Australia), binds the minor groove of DNA with AT specificity (Portugal and Waring, 1988) and is capable of permeating intact cell membranes, therefore indicating live cells. Propidium iodide (PI) intercalates between DNA bases with minimal specificity (Waring, 1965) and also binds RNA (Suzuki et al., 1997);
however, PI gains access to cellular nucleic acid via compromised cell membranes and is commonly used as a dead cell indicator (Moore et al., 1998). A stock solution of Hoechst 33342 was prepared by adding Hoechst 33342, trihydrochloride, trihydrate (10 µg) to distilled water (1000 µL) making a stock solution of 0.17 mM. A working solution of 100 µM was prepared and all cells were stained with a final Hoechst 33342 concentration of 5 µM. The stain, PI, was purchased as a component in the LIVE/DEAD BacLight Bacterial Viability Kit (L-7012, Invitrogen, Mulgrave, Australia) at a concentration of 20 mM. A 1.5 mM working solution was prepared and cells were stained at a final concentration of 3 µM.

Aliquots of non-transfected L45 and L23 cells were individually stained with Hoechst 33342 and PI in a final volume of 100 µL. Hoechst 33342 stained cells were incubated for 10 min at 37 °C in the Thermoline Australia waterbath (Model No. TSB1, Serial No. 8012) and PI stained cells were incubated for 5 min at room temperature. All cell suspensions were pulse-centrifuged for 5 sec using the Eppendorf Bench Centrifuge prior to the wavelength scans. Wavelength scans were performed for Hoechst 33342 and PI stained L45 and L23 cells as described in Section 3.3.2.3. The gain was set to 856.6 V for all channels.

3.3.3.4 Laser Scanning Confocal Microscopy Analysis of Apoptosis-Induced Immune Cells

Analysis was conducted with the Leica LSCM to determine if a correlation and co-localization exists between the intracellular fluorescence expression of apoptotic transfected cells, PI stained (i.e. DNA and RNA) and Hoechst 33342 stained (DNA) cellular areas.
The transfected cells were treated with anisomycin for 24 h (as described in Section 3.3.3.2) in Tc black, 96 well microplates in standard cell culture conditions. A control and treatment well were prepared for each cell line at a final volume of 200 µL with a starting cell concentration of 1.0 x 10⁶ cells mL⁻¹. The L45hMGFP and L23hMGFP cells were treated with anisomycin (10 µM) and L45pCIneo-DsRed2 and L23pCIneo-DsRed2 cells were treated with anisomycin (25 µM) as determined from the apoptosis assays. The viability (%) of the cells were determined using trypan blue as described in Section 3.3.1.4 and stained with Hoechst 33342 (5 µM) for 10 min at 37 ºC (as outlined above). The cells were subsequently stained with PI (3 µM) at room temperature for 5 min. The final volume was 100 µL.

The Leica LSCM software was set to the xyz acquisition mode to obtain z-stacks (3-dimensional) images of the cells. The 405-diode laser was set to 20 % maximum intensity and used to excite Hoechst 33342; wavelength emission of Hoechst 33342 was measured from 450 to 470 nm using PMT 1 and a gain of 935.0 V. The argon (488 nm) laser was set to 50 % power with 50 % maximum intensity and used to excite the intracellular fluorescent proteins (pMGFP and pCIneo-DsRed2) and PI. The L45hMGFP and L23hMGFP cellular emission was measured from 520 to 540 nm using PMT 2 with a gain of 940 V. The L45pCIneo-DsRed2 and L23pCIneo-DsRed2 cellular emission was measured from 560 to 580 nm using  PMT 2 with a gain of 933 V; PI fluorescence emission was measured from 640 to 660 nm using PMT 3 with a gain of 626 V. Sequential scanning parameters were defined for Hoechst 33342, PI and intracellular fluorescence emission using the emission scan profiles as outlined and the “between lines” function. All z-stacks were performed at a 0.99 µM z-step size using a scanning speed of 1000 Hz and a
3.3.3.5 Co-localization Analysis using Bitplane Imaris Software

The co-localization of Hoechst 33342 and intracellular fluorescent protein expression, Hoechst 33342 and PI, and PI and the intracellular fluorescent protein expression were analysed using Bitplane Imaris software version 6.3.1 as described in Section 3.3.2.5. A filter width of 20.54 µM (as determined by the software) was used to compare the co-localization between Hoechst 33342 (Channel 1) and the intracellular fluorescent protein (Channel 2), the intracellular fluorescent protein (Channel 2) and PI (Channel 3), and Hoechst 33342 (Channel 1) and PI (Channel 3).

3.3.4 Mitogen Treatment of Transfected Cells to Evaluate the Relationship between Increased Cellular Activity and Fluorescence Expression

3.3.4.1 Concanavalin A (Con A) and Lipopolysaccharide (LPS) Preparation

The transfected cells lines were treated with the mitogens, Concanavalin A (Con A) and lipopolysaccharide (LPS), to determine if the intracellular fluorescent protein expression is influenced by increased cellular activity; the co-localization results were calculated for comparison with the apoptosis-induced co-localization results. Con A is a carbohydrate-binding protein (lectin) extracted from *Canavalia*
ensiformis (a jack-bean) that binds specific structures on sugars, glycoproteins, glycolipids, and internal and non-reducing terminal α-D-mannosyl and α-D-glucosyl groups (Sumner et al., 1938). This lectin acts as a T cell mitogen (encourages cells to commence division and mitosis) (Dwyer and Johnson, 1981). Lipopolysaccharide (LPS) is a cell wall component of Gram-negative bacteria that acts as a mitogen of B cells encouraging immunoglobulin M (IgM) production (Moller et al., 1973). Con A (C5275, Sigma-Aldrich, Sydney, Australia) was dissolved in PBS at a concentration of 1 mg mL\(^{-1}\). A solution of LPS (0.5 mg mL\(^{-1}\)) (from \textit{E. coli} 026:B6; L2654, Sigma-Aldrich, Sydney, Australia) was prepared in RPMI medium 1640. Both the Con A and LPS solutions were prepared by other researchers.

### 3.3.4.2 Mitogen Treatment of Transfected Cells

The transfected cells were passaged and enumerated as described in Sections 3.3.1.3 and 3.3.1.4. The suspensions were centrifuged at 320 g for 5 min and resuspended in complete DMEM making a final cell concentration of 1.0 x 10\(^6\) cells mL\(^{-1}\). A final volume of 500 μL was prepared for all cell suspension (including controls) in a sterile 24-well plate and incubated under standard cell culture conditions. The L45hMGFP and L45pCIneo-DsRed2 were treated with Con A (1 mg mL\(^{-1}\)) by adding Con A (0.5 μL) to cell suspension (499.5 μL); the respective controls were prepared by adding PBS (0.5 μL) to the cell suspensions. The L23hMGFP and L23pCIneo-DsRed2 cells were treated with LPS (1 mg ml\(^{-1}\)) by adding LPS (1.0 μL) to cell suspension (499.0 μL); the control was prepared by adding RPMI (1.0 μL) media to the cell suspensions.
3.3.4.3 Fluorescent Probes

The fluorescent probes, Hoechst 33342 and PI, were used to stain the mitogen-stimulated transfected cells. The staining procedure was conducted as described in Section 3.3.3.3.

3.3.4.4 Laser-Scanning Confocal Microscopy Analysis of Mitogen-Stimulated Immune Cells

Analysis of mitogen-stimulated transfected immune cells by LSCM was conducted as described in Section 3.3.3.4.

3.3.4.5 Co-localization Analysis using Bitplane Imaris Software

The co-localization of Hoechst 33342 and intracellular fluorescent protein expression, Hoechst 33342 and PI, and PI and the intracellular fluorescent protein expression were analysed using Bitplane Imaris software version 6.3.1 as described in Section 3.3.2.5. The LSCM and Bitplane Imaris settings were applied as described in Section 3.3.3.5. Comparative analysis between the apoptosis-induced transfected cells and mitogen-stimulated transfected cells were performed using the Pearson’s correlation and Mander’s co-localization coefficients obtained for the three channel combinations (Hoechst 33342 vs. PI, Hoechst 33342 vs. fluorescent protein, fluorescent protein vs. PI).

3.3.5 Statistical Analysis

All statistical analysis was performed using Microsoft Excel (2007) unless stated otherwise.
3.4 RESULTS

3.4.1 Fluorescence Expression of Immune Cells following Transfection

Transfected L45 progenitor immune cells (with phMGFP and pCIneo-DsRed2) and transfected L23 progenitor immune cells (with phMGFP and pCIneo-DsRed2) were monitored by the Leica LSCM to determine the fluorescence expression of the plasmid within the cells (Fig. 3.1, 3.2, 3.3 and 3.4). Fluorescence imaging with the LSCM was conducted at 24 h and 14 day post-transfection to assess for continued fluorescence expression. Further observations were conducted following 14 days post-transfection.

All cell lines exhibited fluorescence within 24 h of transfection with the majority of cells appearing to contain fluorescent intracellular vesicles. The transfected L45 cells appeared to exhibit a high amount of intracellular vesicles in comparison to the transfected L23 cells. At 14 days post transfection, all cells were observed to express fluorescence, including healthy cells; however, fluorescence was observed to be contained within cellular vesicles or expressed in outer regions of the cells. Low fluorescence was observed in cells of a healthy appearance at 20 days post-transfection. Cells expressing high quantities of intracellular vesicles appeared to be apoptotic and observed to express higher levels of fluorescence compared to cells without/less intracellular vesicles. The transfected cell lines were stained with the DNA-specific fluorescent stain SYTO 59® and LysoSensor™ Blue DND-167 to determine the intracellular areas expressing fluorescence by the transfected fluorescent plasmids (Section 3.4.2). In addition, the relationship between
fluorescence expression and the apoptotic state of cells was investigated (Section 3.4.3) and compared to mitogen-stimulated cells (Section 3.4.4).

Changes in cellular morphology and growth characteristics were also observed in the cell suspension. Non-transfected cells usually formed circular cellular aggregates in cell culture media that were visible without microscopic imaging, in particular the L45 cells. Transfected cells were still observed to form cellular aggregates however not of a consistent circular type but that of a flake-shape. Microscopically, transfected cells seem to aggregate of a less frequency than non-transfected cells.

![Figure 3.1](image1.png)

Figure 3.1. LSCM analysis of L45 immune cells transfected with the pCIneo-DsRed2 (L45pCIneo-DsRed2) a) 24 h post-transfection; and b) 14 days post-transfection.

![Figure 3.2](image2.png)

Figure 3.2. LSCM analysis of L45 immune cells transfected with the phMGFP (L45hMGFP) a) 24 h post-transfection; and b) 14 days post-transfection.
3.4.1.1 Growth-Rate of Transfected and Non-Transfected Cells

The cellular proliferation of transfected cells were compared to non-transfected cells to determine if the fluorescent plasmids potentially influence the growth-rate of the L45 and L23 cells.
Growth Rate of L45, L45hMGFP and L45pCIneo-DsRed2

The L45hMGFP cells exhibited a slower growth than L45 (non-transfected cells) for approximately 24 h with an increase in growth from 24 to 30 h that exceeded the L45 cell proliferation (Fig. 3.5). However, at approximately 47 h, both L45 and L45hMGFP cells exhibited no differences. Significant differences between the concentration of L45 cells and L45hMGFP cells were observed at 4, 7 and 23 h. The L4hMGFP cells exhibited a diauxy-type of growth behaviour between 25 and 30 h. The L45pCIneo-DsRed2 cells exhibited a high proliferation rate than L45 cells at all time points except at 7 h where the cell concentration was approximately the same. At 47 h, the L45pCIneo-DsRed2 cell concentration significantly exceeded the L45 cell concentration. Significant differences between the concentration of L45 cells and L45pCIneo-DsRed2 cells were observed at 4, 28 and 47 h.

Growth Rate of L23, L23hMGFP and L23pCIneo-DsRed2

The L23hMGFP cells exhibited a higher cellular proliferation than L23 cells at all time points with significant differences observed at 23, 28 and 47 h (Fig. 3.6). Similar to L45pCIneo-DsRed2 cells, the L23pCIneo-DsRed2 cells also showed a higher proliferation but at all time points (all significantly different than the L23 cell growth).
Figure 3.5. Mean ± SEM (n = 6) for the cell proliferation of L45, L45pCIneo-DsRed2 and L45hMGFP cells for approximately 47 h. The significant differences between the non-transfected and transfected cells were calculated using two-sample T-tests with either equal or unequal variance as determined from the F-test two sample for variances (* p < 0.05; ** p < 0.01; *** p < 0.001).

Figure 3.6. Mean ± SEM (n = 6) for the cell proliferation of L23, L23pCIneo-DsRed2 and L23hMGFP cells for approximately 47 h. The significant differences between the non-transfected and transfected cells were calculated using two-sample T-tests with either equal or unequal variance as determined from the F-test two sample for variances (* p < 0.05; ** p < 0.01; *** p < 0.001).
3.4.2 Identification of Transfected Cellular Fluorescence Expression by Comparative Analysis with Nucleic Acid and Lysosomal-specific Fluorescent Probes

To determine the location of the intracellular fluorescent protein expression, the transfected cells were stained with the nucleic acid stain SYTO 59® and the lysosomal stain LysoSensor™ Blue DND-167. As described in Section 3.3.2.1, SYTO 59® stains DNA and RNA, as well as cytoplasmic regions and mitochondria. LysoSensor™ Blue DND-167 stains the acidic organelles, lysosomes. All transfected cells exhibited areas of DNA, RNA and cytoplasmic staining by SYTO 59® and fluorescent protein expression. However, the LysoSensor™ Blue DND-167 fluorescent probe did not produce distinct lysosomal staining but exhibited faint fluorescence throughout the cells, as shown in Figures 3.7a, 3.8a, 3.9a and 3.10a. The L45hMGFP and L23hMGFP cells exhibited faint fluorescence throughout the cell from the hMGFP expression (Fig. 3.7b and 3.9b). The Pearson’s correlation coefficient obtained from Bitplane Imaris (Table 3.2) indicated that no correlation (overlap of pixels) exists between LysoSensor™ and SYTO 59® staining, LysoSensor™ and the fluorescent proteins, nor the fluorescent proteins and SYTO 59® staining with the exception of L23pClneo-DsRed2 and SYTO 59® with a value of 0.5396 (although this does not indicate a strong correlation between DsRed2 and SYTO 59®). The Mander’s coefficient A indicates the amount of pixels from the first channel that colocalize with the second channel; Mander’s Coefficient B is the opposite i.e. second channel co-localization with the first channel. The values for Mander’s Coefficient A indicated very little to no co-localization for the LysoSensor™ with SYTO 9, LysoSensor™ with the fluorescent proteins, and the fluorescent proteins with SYTO 9 combinations (Table 3.2). Similarly, the Mander’s
Coefficient B indicated very little to no co-localization for SYTO 59® with LysoSensor™, and the fluorescent proteins with LysoSensor™. However, the SYTO 59® with L45hMGFP and L23hMGFP indicated some co-localization with a Mander’s Coefficient B of 0.7395 and 0.6686, respectively (Table 3.2). The Mander’s Coefficient B values of SYTO 59® with L45pCIneo-DsRed2 and L23pCIneo-DsRed2 (0.5594 and 0.3300, respectively) indicated very little co-localization existed. The Bitplane Imaris 2D frequency histograms provide a visual interpretation of the pixel frequency from the two channels i.e. hMGFP with LysoSensor™, etc. All 2D frequency histograms indicated that little to no co-localization between the two channels existed as shown by the distribution of pixels to the edges of the 2D frequency histogram. The exception to this observation is L23hMGFP with LysoSensor™ (Fig. 3.9f) and L23pCIneo-DsRed2 with SYTO 59® (Fig. 3.10g) as exhibited by the positive correlation between the pixels from the two channels (near diagonally straight line).
Figure 3.7. Laser Scanning Confocal Microscopy project maximum images of L45hMGFP cells a) LysoSensor™-stained DNA; b) hMGFP fluorescence; c) SYTO 59 stained DNA and RNA; d) overlay of a, b and c; e) 2D frequency histogram of fluorescence correlation between LysoSensor™ (red) and hMGFP (green); f) 2D frequency histogram of fluorescence correlation between LysoSensor™ (red) and SYTO 59 (purple); g) 2D frequency histogram of fluorescence correlation between hMGFP (green) and SYTO 59 (purple).

Figure 3.8. Laser Scanning Confocal Microscopy project maximum images of L45pClneo-DsRed2 cells a) LysoSensor™-stained DNA; b) pClneo-DsRed2 fluorescence; c) SYTO 59® stained DNA and RNA; d) overlay of a, b and c; e) 2D frequency histogram of fluorescence correlation between LysoSensor™ (yellow) and pClneo-DsRed2 (red); f) 2D frequency histogram of fluorescence correlation between LysoSensor™ (yellow) and SYTO 59® (purple); g) 2D frequency histogram of fluorescence correlation between pClneo-DsRed2 (red) and SYTO 59® (purple).
Figure 3.9. Laser Scanning Confocal Microscopy project maximum images of L23hMGFP cells a) LysoSensor™-stained DNA; b) hMGFP fluorescence; c) SYTO 59® stained DNA and RNA; d) overlay of a, b and c; e) 2D frequency histogram of fluorescence correlation between LysoSensor™ (yellow) and hMGFP (green); f) 2D frequency histogram of fluorescence correlation between LysoSensor™ (yellow) and SYTO 59® (purple); g) 2D frequency histogram of fluorescence correlation between hMGFP (green) and SYTO 59® (purple).

Figure 3.10. Laser Scanning Confocal Microscopy project maximum images of L23pClneo-DsRed2 cells a) LysoSensor™-stained DNA; b) pClneo-DsRed2 fluorescence; c) SYTO 59® stained DNA and RNA; d) overlay of a, b and c; e) 2D frequency histogram of fluorescence correlation between LysoSensor™ (yellow) and pClneo-DsRed2 (red); f) 2D frequency histogram of fluorescence correlation between LysoSensor™ (yellow) and SYTO 59® (purple); g) 2D frequency histogram of fluorescence correlation between pClneo-DsRed2 (red) and SYTO 59® (purple).
Table 3.2: Co-localization between lysosomal, nucleic acid stains and intracellular fluorescent proteins (n = 3) of transfected cells as determined by Pearson’s Coefficient and Manders Coefficient.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Pearson’s correlation</th>
<th>Thresholded Mander’s Coefficient</th>
<th>Thresholded Manders Coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean ± SD</td>
<td>Mean ± SD</td>
</tr>
<tr>
<td></td>
<td>A*</td>
<td>B*</td>
<td></td>
</tr>
<tr>
<td>LysoSensor™ vs SYTO 59®</td>
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<td>0.1451 ± 0.0011</td>
<td>0.3551 ± 0.1823</td>
</tr>
<tr>
<td>L45hMGFP</td>
<td>LysoSensor™ vs MGFP</td>
<td>0</td>
<td>0.0002 ± 0.0004</td>
</tr>
<tr>
<td>MGFP vs SYTO 59®</td>
<td>0.1897 ± 0.0555</td>
<td>0.3544 ± 0.0514</td>
<td>0.7395 ± 0.1611</td>
</tr>
<tr>
<td>LysoSensor™ vs SYTO 59®</td>
<td>0.0878 ± 0.0286</td>
<td>0.0612 ± 0.0874</td>
<td>0.2661 ± 0.0512</td>
</tr>
<tr>
<td>L45pCIneo-DsRed2</td>
<td>LysoSensor™ vs DsRed2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>DsRed2 vs SYTO 59®</td>
<td>0.2891 ± 0.0813</td>
<td>0.1885 ± 0.1366</td>
<td>0.5594 ± 0.3479</td>
</tr>
<tr>
<td>LysoSensor™ vs SYTO 59®</td>
<td>-0.1500 ± 0.1018</td>
<td>0.2865 ± 0.0923</td>
<td>0.1533 ± 0.0638</td>
</tr>
<tr>
<td>L23hMGFP</td>
<td>LysoSensor™ vs MGFP</td>
<td>0</td>
<td>0.0006 ± 0.0005</td>
</tr>
<tr>
<td>MGFP vs SYTO 59®</td>
<td>0.0874 ± 0.0938</td>
<td>0.4893 ± 0.0957</td>
<td>0.6686 ± 0.2046</td>
</tr>
<tr>
<td>LysoSensor™ vs SYTO 59®</td>
<td>0.3212 ± 0.0544</td>
<td>0.3311 ± 0.0864</td>
<td>0.3258 ± 0.0558</td>
</tr>
<tr>
<td>L23pCIneo-DsRed2</td>
<td>LysoSensor™ vs DsRed2</td>
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<td>0.0003 ± 0.0001</td>
</tr>
<tr>
<td>DsRed2 vs SYTO 59®</td>
<td>0.5396 ± 0.0248</td>
<td>0.3730 ± 0.0812</td>
<td>0.3300 ± 0.0939</td>
</tr>
</tbody>
</table>

3.4.3 Co-localization Analysis of Apoptotic-induced Intracellular Fluorescence, Hoechst 33342 and PI Expression using Bitplane Imaris Software

Non-treated (control) L45hMGFP cells exhibited distinct areas of Hoechst 33342 and PI staining indicating DNA (for both stains) and some RNA (PI staining) as shown in Figures 3.11a, c and d. Cells exhibiting PI staining imply that these cells are either necrotic or apoptotic due to the impenetrable nature of PI across intact cell membranes (Moore et al., 1998). The fluorescent protein expression (hMGFP) (Fig. 3.11b), however, was very faint in all live cells and showed little expression in the necrotic/apoptotic cells expressing both PI and Hoechst 33342 staining (Fig. 3.11b and d). The 2D frequency histogram (Fig. 3.11e, f and g and Table 3.2) indicated no co-localization between Hoechst 33342 and hMGFP (Fig. 3.11e), Hoechst 33342 and PI (Fig. 3.11f), and PI and hMGFP (Fig. 3.11g) existed. Table 3.3 consists of the mean value for Pearson’s correlation, and thresholded Mander’s coefficients A and B – the calculated values indicate very little to no co-localization from the Hoechst 33342 with PI and Hoechst 33342 with L45hMGFP combinations. However, the thresholded Manders Coefficient B for PI with L45hMGFP indicated a co-localization relationship with a result of 0.753; the Pearson’s correlation for this combination indicated little co-localization with no co-localization indicated by the thresholded Manders Coefficient A.
Similar to the control L45hMGFP cells, the anisomycin treated cells exhibited areas of Hoechst 33342 and PI staining indicating DNA, and some RNA (PI staining) as shown in Figures 3.12a, b and d. All cells exhibited PI staining indicating that these cells were either necrotic or apoptotic. The hMGFP fluorescent protein expression (Fig. 3.12b) appeared faintly distributed throughout the entire cell. Co-localization analysis using Bitplane Imaris (Fig. 3.12e, f and g and Table 3.3) indicated some co-localization of Hoechst 33342 with PI (Fig. 3.12f) and very little between Hoechst 33342 and hMGFP (Fig. 3.12e) and hMGFP with PI (Fig. 3.12f). Table 3.3 results indicate that the Mander’s coefficient A of Hoechst 33342 with PI expression for the anisomycin treated L45hMGFP produced a significant colocalized result compared to the control. The thresholded Mander’s coefficient B of PI with...
Hoechst 33342 did not significantly differ from the control cells but did indicate that co-localization occurred between these two stains with a value of 0.737. The Pearson’s correlation and both Mander’s coefficients (A and B) of PI with the control L45hMGFP indicated a higher co-localization correlation than the anisomycin-treated L45hMGFP, however not significantly.

![Image](image1.png)

**Figure 3.12.** Laser Scanning Confocal Microscopy project maximum images of anisomycin treated L45hMGFP cells a) Hoechst 33342-stained DNA; b) pClneo-DsRed2 fluorescence; c) propidium iodide (PI) stained DNA and RNA; d) overlay of a, b and c; e) 2D frequency histogram of fluorescence correlation between Hoechst 33342 (blue) and pClneo-DsRed2 (red); f) 2D frequency histogram of fluorescence correlation between Hoechst 33342 (blue) and PI (purple); g) 2D frequency histogram of fluorescence correlation between pClneo-DsRed2 (red) and PI (purple).

The control L45pClneo-DsRed2 cells also exhibited areas of Hoechst 33342 and PI staining as well as DsRed2 expression as shown in Figures 3.13a, b, c and d. The DsRed2 expression appeared more pronounced in the cells expressing PI (Fig. 3.13b). Co-localization analysis using Bitplane Imaris (Fig. 3.13e, f and g and Table 3.3) indicated no co-localization between Hoechst 33342 and DsRed2 (Fig. 3.13e), and Hoechst 33342 and PI (Fig. 3.13f) existed. However, co-localization between
DsRed2 and PI appeared to exist based on Figure 3.13g. The co-localization statistics in Table 3.3 indicated that little to no co-localization existed for the Hoechst 33342 with PI and Hoechst 33342 with DsRed2 combinations (Table 3.3). However the Pearson’s correlation value of 0.738 from DsRed2 with PI denoted that a co-localization correlation existed.

Figure 3.13. Laser Scanning Confocal Microscopy project maximum images of non-treated (control) L45pCIneo-DsRed2 cells a) Hoechst 33342-stained DNA; b) pCIneo-DsRed2 fluorescence; c) propidium iodide (PI) stained DNA and RNA; d) overlay of a, b and c; e) 2D frequency histogram of fluorescence correlation between Hoechst 33342 (blue) and pCIneo-DsRed2 (red); f) 2D frequency histogram of fluorescence correlation between Hoechst 33342 (blue) and PI (purple); g) 2D frequency histogram of fluorescence correlation between pCIneo-DsRed2 (red) and PI (purple).

The anisomycin treated L45pCIneo-DsRed2 cells expressed areas of Hoechst 33342, PI and DsRed2 expression as shown in Figures 3.14a, b, c and d. Similar to the control L45pCIneo-DsRed2 cells, the pCIneo-DsRed2 expression appeared pronounced in the cells expressing PI (Fig. 3.14b). Co-localization analysis using Bitplane Imaris (Fig. 3.14e, f and g and Table 3.3) indicated no co-localization between Hoechst 33342 and pCIneo-DsRed2 (Fig. 3.14e), and Hoechst 33342 with
PI (Fig. 3.14f) existed. However, co-localization between DsRed2 and PI appeared to exist based on Figures 3.14g. Table 3.3 results indicate that the Mander’s coefficient A of Hoechst 33342 with PI colocalization by anisomycin treated L45pCIneo-DsRed2 cells was significantly different than the control, although the value of 0.670 indicates little co-localization existed. However, the thresholded Mander’s coefficient A indicated that Hoechst 33342 with pCIneo-DsRed2 and the thresholded Mander’s coefficient B for PI with pCIneo-DsRed2 combinations were highly colocalized producing a result of 0.939 and 0.899, respectively (Table 3.3). The former was significantly different from the control whereas the latter was not.

**Figure 3.14.** Laser Scanning Confocal Microscopy project maximum images of anisomycin treated L45pCIneo-DsRed2 cells a) Hoechst 33342-stained DNA; b) pCIneo-DsRed2 fluorescence; c) propidium iodide (PI) stained DNA and RNA; d) overlay of a, b and c; e) 2D frequency histogram of fluorescence correlation between Hoechst 33342 (blue) and pCIneo-DsRed2 (red); f) 2D frequency histogram of fluorescence correlation between Hoechst 33342 (blue) and PI (purple); g) 2D frequency histogram of fluorescence correlation between pCIneo-DsRed2 (red) and PI (purple).
Table 3.3 Pearson’s correlation, and Mander’s coefficients A and B to determine co-localization between DNA & RNA stains and intracellular fluorescent proteins by anisomycin treated transfected-L45 cells (n = 3).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Pearson’s Correlation</th>
<th>Thresholded Manders Coefficient A&lt;sup&gt;*&lt;/sup&gt;</th>
<th>Thresholded Manders Coefficient B&lt;sup&gt;+&lt;/sup&gt;</th>
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<tr>
<td></td>
<td>Mean ± SD</td>
<td>Mean ± SD</td>
<td>Mean ± SD</td>
</tr>
<tr>
<td>L45hMGFP Control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hoechst 33342 vs PI&lt;sup&gt;1&lt;/sup&gt;</td>
<td>0.308 ± 0.192</td>
<td>0.122 ± 0.064</td>
<td>0.677 ± 0.207</td>
</tr>
<tr>
<td>Hoechst 33342 vs MGFP&lt;sup&gt;2&lt;/sup&gt;</td>
<td>0.272 ± 0.047</td>
<td>0.388 ± 0.256</td>
<td>0.098 ± 0.062</td>
</tr>
<tr>
<td>MGFP vs PI&lt;sup&gt;3&lt;/sup&gt;</td>
<td>0.502 ± 0.222</td>
<td>0.091 ± 0.058</td>
<td>0.753 ± 0.183</td>
</tr>
<tr>
<td>L45hMGFP Treatment</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hoechst 33342 vs PI&lt;sup&gt;1&lt;/sup&gt;</td>
<td>0.623 ± 0.084</td>
<td>0.608 ± 0.098</td>
<td>0.737 ± 0.045</td>
</tr>
<tr>
<td>Hoechst 33342 vs MGFP&lt;sup&gt;2&lt;/sup&gt;</td>
<td>0.286 ± 0.155</td>
<td>0.631 ± 0.145</td>
<td>0.087 ± 0.048</td>
</tr>
<tr>
<td>MGFP vs PI&lt;sup&gt;3&lt;/sup&gt;</td>
<td>0.297 ± 0.135</td>
<td>0.093 ± 0.044</td>
<td>0.581 ± 0.265</td>
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<tr>
<td>L45pCIneo-DsRed2 Control</td>
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<tr>
<td>Hoechst 33342 vs PI&lt;sup&gt;1&lt;/sup&gt;</td>
<td>0.216 ± 0.309</td>
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<td>0.390 ± 0.279</td>
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<tr>
<td>Hoechst 33342 vs DsRed2&lt;sup&gt;4&lt;/sup&gt;</td>
<td>0.081 ± 0.188</td>
<td>0.188 ± 0.195</td>
<td>0.160 ± 0.038</td>
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<tr>
<td>DsRed2 vs PI&lt;sup&gt;5&lt;/sup&gt;</td>
<td>0.738 ± 0.061</td>
<td>0.382 ± 0.015</td>
<td>0.576 ± 0.342</td>
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<tr>
<td>L45pCIneo-DsRed2 Treatment</td>
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<td></td>
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<tr>
<td>Hoechst 33342 vs PI&lt;sup&gt;1&lt;/sup&gt;</td>
<td>0.293 ± 0.052</td>
<td>0.670 ± 0.144</td>
<td>0.592 ± 0.120</td>
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<tr>
<td>Hoechst 33342 vs DsRed2&lt;sup&gt;4&lt;/sup&gt;</td>
<td>0.301 ± 0.088</td>
<td>0.939 ± 0.023</td>
<td>0.303 ± 0.093</td>
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<tr>
<td>DsRed2 vs PI&lt;sup&gt;5&lt;/sup&gt;</td>
<td>0.606 ± 0.089</td>
<td>0.362 ± 0.120</td>
<td>0.899 ± 0.015</td>
</tr>
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</table>

Bolded figures indicate significance difference (p < 0.05) between control & treatments as determined by the Students T-test. A<sup>*</sup> Denotes first listed stain co-localization with the second listed stain. B<sup>+</sup> Denotes second listed stain co-localization with the second listed stain. 1 DNA stain (Hoechst 33342) coloc. with DNA/RNA stain (Propidium iodide). 2 DNA stain (Hoechst 33342) coloc. with monster green® fluorescent protein (MGFP). 3 MGFP coloc. with DNA/RNA stain (PI). 4 DNA stain (Hoechst 33342 coloc. with pCIneo-DsRed2 fluorescent protein (FP). 5 pCIneo-DsRed2 FP coloc. with DNA/RNA stain (PI)
The control L23hMGFP cells displayed areas of Hoechst 33342 (DNA), PI (DNA and RNA of apoptotic or necrotic cells) staining and hMGFP expression (Fig. 3.15a, b, and c), the overlay of the three channels indicated very little overlap from the three channels (Fig. 3.15d). The Bitplane Imaris 2D frequency histograms (Fig. 3.15e, f and g) indicated that no co-localization existed for Hoechst 33342 with hMGFP, Hoechst 33342 with PI and hMGFP with PI combinations. This was further supported by the Pearson’s correlation and thresholded Mander’s coefficient A and B values as shown in Table 3.4.

The anisomycin treated L23hMGFP cells also exhibited areas of Hoechst 33342 and PI staining. However comparatively to the control, the anisomycin treated cells displayed almost entire cellular hMGFP expression and brightly
fluorescent vesicular areas of Hoechst 33342 and PI staining (Fig. 3.16a, b and c), the majority of these areas exhibited fluorescence overlap from all three channels (Fig. 3.16d). The Bitplane Imaris 2D frequency histogram for Hoechst 33342 and PI exhibits some co-localization between the two stains (Fig. 3.16f). This was also indicated by the Pearson’s correlation, thresholded Mander’s coefficient A and thresholded Mander’s coefficient B with values of 0.577, 0.370 and 0.580 respectively (Table 3.4); although these values are not indicative of high co-localization between Hoechst 33342 and PI (vice versa for the thresholded Manders coefficient B), all values were significantly different from the respective control values (p < 0.05). The thresholded Manders coefficient A value for Hoechst 33342 with hMGFP was also significantly different to the respective control (p < 0.05) but did not indicate a co-localization result with a value of 0.296 (Table 3.4).

**Figure 3.16.** Laser Scanning Confocal Microscopy project maximum images of anisomycin treated L23hMGFP cells a) Hoechst 33342-stained DNA; b) hMGFP fluorescence; c) propidium iodide (PI) stained DNA and RNA; d) overlay of a, b and c; e) 2D frequency histogram of fluorescence correlation between Hoechst 33342 (blue) and hMGFP (green); f) 2D frequency histogram of fluorescence correlation between Hoechst 33342 (blue) and PI (purple); g) 2D frequency histogram of fluorescence correlation between hMGFP (green) and PI (purple).
The control L23pCIneo-DsRed2 cells displayed areas of Hoechst 33342, PI and pCIneo-DsRed2 expression (Fig. 3.17a, b and c), that are distinctly expressed with little overlap (Fig. 3.17d). The Bitplane Imaris 2D frequency histograms exhibit some degree of co-localization between the two channels for all combinations (Fig. 3.17e, f and g). As shown in Table 3.4, the Pearson’s correlation and thresholded Mander’s coefficients A and B indicate little co-localization for all channel combinations of the control L23pCIneo-DsRed2 with the exception of pCIneo-DsRed2 with PI that produced a Pearson’s correlation of 0.669 and a thresholded Mander’s coefficient B of 0.692 (indicates PI pixel overlap with pCIneo-DsRed2 pixels); although these values do not indicate high co-localization it is notable as other control values were usually close to 0. The anisomycin treated L23pCIneo-DsRed2 cells exhibited high co-localization between all three channels (than the control) as shown in Figures 3.18a, b, c and d and the Bitplane Imaris 2D frequency histogram (Fig. 3.18e, f and g).

The Pearson’s correlation and thresholded Mander’s coefficient A for Hoechst 33342 with PI were significantly different (p < 0.05) from the respective controls with values of 0.611 and 0.512, respectively (Table 3.4); however these values do not indicate high co-localization between the two channels. Similarly the Pearson’s correlation for Hoechst 33342 with pCIneo-DsRed2 was also significant from the respective control but did not indicate high co-localization between the two channels with a result of 0.517 (Table 3.4). Although not significantly different from the respective control, the thresholded Mander’s coefficient B for PI with pCIneo-DsRed2 indicates high co-localization between the two channels with a value of 0.971 (Table 3.4).
Figure 3.17. Laser Scanning Confocal Microscopy project maximum images of non-treated (control) L23pCIneo-DsRed2 cells a) Hoechst 33342-stained DNA; b) pCIneo-DsRed2 fluorescence; c) propidium iodide (PI) stained DNA and RNA; d) overlay of a, b and c; e) 2D frequency histogram of fluorescence correlation between Hoechst 33342 (blue) and pCIneo-DsRed2 (red); f) 2D frequency histogram of fluorescence correlation between Hoechst 33342 (blue) and PI (purple); g) 2D frequency histogram of fluorescence correlation between pCIneo-DsRed2 (red) and PI (purple).

Figure 3.18. Laser Scanning Confocal Microscopy project maximum images of anisomycin treated L23pCIneo-DsRed2 cells a) Hoechst 33342-stained DNA; b) pCIneo-DsRed2 fluorescence; c) propidium iodide (PI) stained DNA and RNA; d) overlay of a, b and c; e) 2D frequency histogram of fluorescence correlation between Hoechst 33342 (blue) and pCIneo-DsRed2 (red); f) 2D frequency histogram of fluorescence correlation between Hoechst 33342 (blue) and PI (purple); g) 2D frequency histogram of fluorescence correlation between pCIneo-DsRed2 (red) and PI (purple).
Table 3.4 Pearson’s correlation, and Mander’s coefficients A and B to determine co-localization between DNA & RNA stains and intracellular fluorescent proteins by anisomycin treated transfected-L23 cells (n = 3).

<table>
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<th>Sample</th>
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<th>Thresholded Manders Coefficient A*</th>
<th>Thresholded Manders Coefficient B*</th>
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<td></td>
<td>Mean ± SD</td>
<td>Mean ± SD</td>
<td>Mean ± SD</td>
</tr>
<tr>
<td>L23hMGFP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hoechst 33342 vs PI1</td>
<td>0.115 ± 0.139</td>
<td>0.003 ± 0.002</td>
<td>0.221 ± 0.176</td>
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<td>Hoechst 33342 vs MGFP</td>
<td>0.011 ± 0.074</td>
<td>0.014 ± 0.006</td>
<td>0.111 ± 0.063</td>
</tr>
<tr>
<td>MGFP vs PI</td>
<td>0.112 ± 0.143</td>
<td>0.026 ± 0.034</td>
<td>0.126 ± 0.108</td>
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<tr>
<td>L23hMGFP</td>
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<td></td>
</tr>
<tr>
<td>Treatment</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Hoechst 33342 vs PI</td>
<td>0.577 ± 0.137</td>
<td>0.370 ± 0.147</td>
<td>0.580 ± 0.119</td>
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<td>Hoechst 33342 vs MGFP</td>
<td>0.015 ± 0.184</td>
<td>0.296 ± 0.035</td>
<td>0.127 ± 0.045</td>
</tr>
<tr>
<td>MGFP vs PI</td>
<td>0.111 ± 0.165</td>
<td>0.180 ± 0.103</td>
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<td>L23pClneo-DsRed2</td>
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<tr>
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<td></td>
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<tr>
<td>Hoechst 33342 vs PI</td>
<td>0.077 ± 0.211</td>
<td>0.045 ± 0.044</td>
<td>0.366 ± 0.241</td>
</tr>
<tr>
<td>Hoechst 33342 vs DsRed2</td>
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<td>0.395 ± 0.338</td>
<td>0.187 ± 0.161</td>
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<td>DsRed2 vs PI</td>
<td>0.669 ± 0.184</td>
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</tr>
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<td>L23pClneo-DsRed2</td>
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<td>Treatment</td>
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<tr>
<td>Hoechst 33342 vs PI</td>
<td>0.611 ± 0.042</td>
<td>0.512 ± 0.154</td>
<td>0.709 ± 0.087</td>
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<tr>
<td>Hoechst 33342 vs DsRed2</td>
<td>0.517 ± 0.043</td>
<td>0.674 ± 0.193</td>
<td>0.402 ± 0.201</td>
</tr>
</tbody>
</table>

Bolded figures indicate significance difference (p < 0.05) between control & treatments as determined by the Students T-test. A* Denotes first listed stain co-localization with the second listed stain. B* Denotes second listed stain co-localization with the second listed stain. ¹ DNA stain (Hoechst 33342) coloc. with DNA/RNA stain (Propidium iodide). ² DNA stain (Hoechst 33342) coloc. with monster green® fluorescent protein (MGFP). ³ MGFP coloc. with DNA/RNA stain (PI). ⁴ DNA stain (Hoechst 33342 coloc. with pClneo-DsRed2 fluorescent protein (FP). ⁵ pClneo-DsRed2 FP coloc. with DNA/RNA stain (PI)
3.4.4 Co-localization Analysis of Mitogen-Stimulated Intracellular Fluorescence, Hoechst 33342 and PI Expression

Similar to the anisomycin treated cells, the mitogen-stimulated cells were stained with Hoechst 33342 and PI to determine if these two stains co-localization with the intracellular fluorescent proteins.

The control L45hMGFP cells exhibited distinct areas of Hoechst 33342 staining (indicating live cellular DNA). Other cells expressed areas of PI (indicates dead cell DNA and RNA) and faint hMGFP expression (Fig. 3.19a, b and c). This supports previous results (Section 3.4.3) that indicate co-localization between PI and the intracellular fluorescent protein in apoptotic cells. It appears the PI has quenched the expression of Hoechst 33342 (Fig. 3.19d) in non-viable cells. According to the Bitplane Imaris 2D frequency histogram (Fig. 3.19e, f and g), no co-localization has occurred between the three channel combinations. This was further supported by the calculated Pearson’s correlation, and thresholded Mander’s coefficients A and B values shown in Table 3.5. Similar fluorescence expression was observed for the three channels by Con A treated L45hMGFP cells (Fig. 3.19a, b, c and d), as well as the Bitplane Imaris 2D frequency histogram (Fig. 3.19e, f and g) and the co-localization statistics (Table 3.5). All treatment co-localization statistics were not significantly different from the respective control statistics.

The control L45pCIneo-DsRed2 cells exhibited distinct areas of Hoechst 33342 staining (indicating the DNA of live cells), however the PI and pCIneo-DsRed2 expression appeared to be colocalized in most areas; all three fluorescence expressing agents produced areas that exhibited higher fluorescence expression than
other parts of the cells (Fig. 3.19a, b, c and d). The Bitplane IMARIS 2D frequency
histograms indicated that only the pCIneo-DsRed2 with PI colocalized (Fig. 3.19e, f
and g). However, the Pearson’s correlation, and thresholded Mander’s coefficients A
and B values indicated that little to no co-localization occurred for all channel
combinations (Table 3.5). Similar effects were observed for the Con A treated
L45pCIneo-DsRed2 cells (Fig. 3.20) with the exception that the Pearson’s
correlation for pCIneo-DsRed2 with PI indicated high co-localization with a value of
0.829 (Table 3.5), however this was not significantly different from the respective
control as were all treatment co-localization statistics.
Figure 3.19. Laser Scanning Confocal Microscopy project maximum images of non-treated L45hMGFP cells a) Hoechst 33342-stained DNA; b) hMGFP fluorescence; c) propidium iodide (PI) stained DNA and RNA; d) overlay of a, b and c; e) 2D frequency histogram of fluorescence correlation between Hoechst 33342 (blue) and hMGFP (green); f) 2D frequency histogram of fluorescence correlation between Hoechst 33342 (blue) and PI (purple); g) 2D frequency histogram of fluorescence correlation between hMGFP (green) and PI (purple).

Figure 3.20. Laser Scanning Confocal Microscopy project maximum images of concanavalin A treated L45hMGFP cells a) Hoechst 33342-stained DNA; b) hMGFP fluorescence; c) propidium iodide (PI) stained DNA and RNA; d) overlay of a, b and c; e) 2D frequency histogram of fluorescence correlation between Hoechst 33342 (blue) and hMGFP (green); f) 2D frequency histogram of fluorescence correlation between Hoechst 33342 (blue) and PI (purple); g) 2D frequency histogram of fluorescence correlation between hMGFP (green) and PI (purple).
**Figure 3.21.** Laser Scanning Confocal Microscopy project maximum images of non-treated L45pClneo-DsRed2 cells a) Hoechst 33342-stained DNA; b) pClneo-DsRed2 fluorescence; c) propidium iodide (PI) stained DNA and RNA; d) overlay of a, b and c; e) 2D frequency histogram of fluorescence correlation between Hoechst 33342 (blue) and pClneo-DsRed2 (red); f) 2D frequency histogram of fluorescence correlation between Hoechst 33342 (blue) and PI (purple); g) 2D frequency histogram of fluorescence correlation between pClneo-DsRed2 (red) and PI (purple).

**Figure 3.22.** Laser Scanning Confocal Microscopy project maximum images of concanavalin A treated L45pClneo-DsRed2 cells a) Hoechst 33342-stained DNA; b) pClneo-DsRed2 fluorescence; c) propidium iodide (PI) stained DNA and RNA; d) overlay of a, b and c; e) 2D frequency histogram of fluorescence correlation between Hoechst 33342 (blue) and pClneo-DsRed2 (red); f) 2D frequency histogram of fluorescence correlation between Hoechst 33342 (blue) and PI (purple); g) 2D frequency histogram of fluorescence correlation between pClneo-DsRed2 (red) and PI (purple).
Table 3.5. Pearson’s correlation, and Mander’s coefficients A & B to determine co-localization between DNA & RNA stains and intracellular fluorescent proteins (n = 3) by Concanavalin A treated transfected-cells.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Pearson’s Correlation</th>
<th>Thresholded Manders Coefficient A*</th>
<th>Thresholded Manders Coefficient B*</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Mean ± SD</td>
<td>Mean ± SD</td>
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</tr>
<tr>
<td>L45hMGFP Control</td>
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<tr>
<td>Hoechst 33342 vs PI</td>
<td>-0.033 ± 0.076</td>
<td>0.014 ± 0.005</td>
<td>0.024 ± 0.025</td>
</tr>
<tr>
<td>Hoechst 33342 vs MGFP</td>
<td>0</td>
<td>0</td>
<td>0.001 ± 0.0005</td>
</tr>
<tr>
<td>MGFP vs PI</td>
<td>0.089 ± 0.174</td>
<td>0.115 ± 0.017</td>
<td>0.270 ± 0.166</td>
</tr>
<tr>
<td>L45hMGFP Treatment</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Hoechst 33342 vs PI</td>
<td>-0.105 ± 0.023</td>
<td>0.163 ± 0.157</td>
<td>0.020 ± 0.013</td>
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<tr>
<td>Hoechst 33342 vs MGFP</td>
<td>0</td>
<td>0</td>
<td>0.0002 ± 0.0001</td>
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<tr>
<td>MGFP vs PI</td>
<td>0.114 ± 0.099</td>
<td>0.129 ± 0.118</td>
<td>0.088 ± 0.083</td>
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<td>L45pCIneo-DsRed2 Control</td>
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<tr>
<td>Hoechst 33342 vs PI</td>
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<td>0.002 ± 0.003</td>
<td>0.014 ± 0.025</td>
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<tr>
<td>Hoechst 33342 vs DsRed2</td>
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<td>0.0003 ± 0.0006</td>
<td>0.00007 ± 0.00006</td>
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<tr>
<td>DsRed2 vs PI</td>
<td>0.294 ± 0.510</td>
<td>0.161 ± 0.279</td>
<td>0.232 ± 0.402</td>
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<tr>
<td>Hoechst 33342 vs PI</td>
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<td>0.070 ± 0.120</td>
<td>0.108 ± 0.093</td>
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<tr>
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<td>0.001 ± 0.001</td>
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<tr>
<td>DsRed2 vs PI</td>
<td>0.829 ± 0.010</td>
<td>0.531 ± 0.135</td>
<td>0.474 ± 0.299</td>
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</tbody>
</table>

Bolded figures indicate significance difference (p < 0.05) between control & treatments as determined by the Students T-test. A* Denotes first listed stain co-localization with the second listed stain, B* Denotes second listed stain co-localization with the second listed stain. ¹ DNA stain (Hoechst 33342) coloc. with DNA/RNA stain (Propidium iodide), ² DNA stain (Hoechst 33342) coloc. with monster green® fluorescent protein (MGFP), ³ MGFP coloc. with DNA/RNA stain (PI), ⁴ DNA stain (Hoechst 33342 coloc. with pCIneo-DsRed2 fluorescent protein (FP), ⁵ pCIneo-DsRed2 FP coloc. with DNA/RNA stain (PI).
The control L23hMGFP cells exhibited distinct areas of Hoechst 33342 staining (Fig. 3.23a). Other cells (not expressing Hoechst 33342) express PI and faint hMGFP fluorescence (Fig. 3.23b, c and d). These observations are similar to the mitogen-stimulated transfected-L45 cells and controls. Further, the Bitplane Imaris 2D frequency histogram shows little co-localization of hMGFP with PI, but no co-localization for the other channel combinations (Fig. 3.23e, f and g). This was also supported by the co-localization statistics shown in Table 3.6; low values were produced for all channel combinations except the thresholded Mander’s coefficient B value for PI with hMGFP with a value of 0.525, however this indicates very little co-localization. Similar to the control L23hMGFP cells, the LPS treated L23hMGFP cells exhibited distinct areas of Hoechst 33342 staining, whereas other cells expressed both hMGFP and PI fluorescence (Fig. 3.24a, b, c, and d). The Bitplane Imaris 2D frequency histograms indicated no co-localization between the channel combinations with the exception for hMGFP with PI (Fig. 3.24e, f and g). The co-localization statistics shown in Table 3.6 also supports this with the exception for the thresholded Mander’s coefficient B for PI with hMGFP with a value of 0.697.
Figure 3.23. Laser Scanning Confocal Microscopy project maximum images of non-treated L23hMGFP cells a) Hoechst 33342-stained DNA; b) hMGFP fluorescence; c) propidium iodide (PI) stained DNA and RNA; d) overlay of a, b and c; e) 2D frequency histogram of fluorescence correlation between Hoechst 33342 (blue) and hMGFP (green); f) 2D frequency histogram of fluorescence correlation between Hoechst 33342 (blue) and PI (purple); g) 2D frequency histogram of fluorescence correlation between hMGFP (green) and PI (purple).

Figure 3.24. Laser Scanning Confocal Microscopy project maximum images of lipopolysaccharide treated L23hMGFP cells a) Hoechst 33342-stained DNA; b) hMGFP fluorescence; c) propidium iodide (PI) stained DNA and RNA; d) overlay of a, b and c; e) 2D frequency histogram of fluorescence correlation between Hoechst 33342 (blue) and hMGFP (green); f) 2D frequency histogram of fluorescence correlation between Hoechst 33342 (blue) and PI (purple); g) 2D frequency histogram of fluorescence correlation between hMGFP (green) and PI (purple).
Both the control and LPS treated L23pClneo-DsRed2 cells exhibited distinct areas of Hoechst 33342 staining, indicating live cellular DNA (Fig. 3.25a and Fig. 3.26a). Other cells, not expressing Hoechst 33342, exhibited PI and pClneo-DsRed2 often in the same cellular areas (Fig. 3.25b, c and d, and Fig 3.26b, c and d). These cells potentially indicate non-viable cells as PI is expressed in apoptotic or necrotic cells; this also supports the results in Section 3.4.3. The Bitplane Imaris 2D frequency histograms indicate little to no co-localization for all channel combinations (Fig. 3.25e and f, and Fig 3.26e and f) with the exception for pClneo-DsRed2 with PI (Fig. 3.25g and Fig. 3.26g). This observation was also supported by the co-localization statistics shown in Table 3.6. The co-localization values for all channel combinations indicate little to no co-localization with the exception of the control and LPS treated PI with pClneo-DsRed2 expression. The thresholded Manders coefficient B value for the control and treatment was 0.926 and 0.916, respectively, indicating high co-localization between these two channels (Table 3.6).
Figure 3.25. Laser Scanning Confocal Microscopy project maximum images of non-treated L23pClneo-DsRed2 cells a) Hoechst 33342-stained DNA; b) pClneo-DsRed2 fluorescence; c) propidium iodide (PI) stained DNA and RNA; d) overlay of a, b and c; e) 2D frequency histogram of fluorescence correlation between Hoechst 33342 (blue) and pClneo-DsRed2 (red); f) 2D frequency histogram of fluorescence correlation between Hoechst 33342 (blue) and PI (purple); g) 2D frequency histogram of fluorescence correlation between pClneo-DsRed2 (red) and PI (purple).

Figure 3.26. Laser Scanning Confocal Microscopy project maximum images of lipopolysaccharide treated L23pClneo-DsRed2 cells a) Hoechst 33342-stained DNA; b) pClneo-DsRed2 fluorescence; c) propidium iodide (PI) stained DNA and RNA; d) overlay of a, b and c; e) 2D frequency histogram of fluorescence correlation between Hoechst 33342 (blue) and pClneo-DsRed2 (red); f) 2D frequency histogram of fluorescence correlation between Hoechst 33342 (blue) and PI (purple); g) 2D frequency histogram of fluorescence correlation between pClneo-DsRed2 (red) and PI (purple).
Table 3.6 Pearson’s correlation, and Mander’s coefficients A & B to determine co-localization between DNA & RNA stains and intracellular fluorescent proteins (n = 3) by lipopolysaccharide treated transfected-cells.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Pearson’s Correlation</th>
<th>Thresholded Manders Coefficient A</th>
<th>Thresholded Manders Coefficient B</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD</td>
<td>Mean ± SD</td>
<td>Mean ± SD</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control L23hMGFP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hoechst 33342 vs PI</td>
<td>0.049 ± 0.380</td>
<td>0.013 ± 0.005</td>
<td>0.105 ± 0.037</td>
</tr>
<tr>
<td>Hoechst 33342 vs MGFP</td>
<td>0</td>
<td>0.0003 ± 0.0006</td>
<td>0.0005 ± 0.0006</td>
</tr>
<tr>
<td>MGFP vs PI</td>
<td>0.277 ± 0.281</td>
<td>0.341 ± 0.224</td>
<td>0.525 ± 0.05</td>
</tr>
<tr>
<td>Control L23hMGFP</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Hoechst 33342 vs PI</td>
<td>0.039 ± 0.322</td>
<td>0.052 ± 0.40</td>
<td>0.171 ± 0.201</td>
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<tr>
<td>Treatment L23hMGFP</td>
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<tr>
<td>Hoechst 33342 vs MGFP</td>
<td>0</td>
<td>0</td>
<td>0.001 ± 0.002</td>
</tr>
<tr>
<td>MGFP vs PI</td>
<td>0.381 ± 0.168</td>
<td>0.346 ± 0.190</td>
<td>0.697 ± 0.245</td>
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<tr>
<td>Control L23pClneo-DsRed2</td>
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<td></td>
</tr>
<tr>
<td>Hoechst 33342 vs PI</td>
<td>0.089 ± 0.032</td>
<td>0.008 ± 0.004</td>
<td>0.252 ± 0.125</td>
</tr>
<tr>
<td>Hoechst 33342 vs DsRed2</td>
<td>0</td>
<td>0.0001 ± 0.000</td>
<td>0</td>
</tr>
<tr>
<td>DsRed2 vs PI</td>
<td>0.580 ± 0.220</td>
<td>0.390 ± 0.048</td>
<td>0.926 ± 0.034</td>
</tr>
<tr>
<td>Treatment L23pClneo-DsRed2</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Hoechst 33342 vs PI</td>
<td>-0.031 ± 0.120</td>
<td>0.007 ± 0.004</td>
<td>0.189 ± 0.155</td>
</tr>
<tr>
<td>Hoechst 33342 vs DsRed2</td>
<td>0</td>
<td>0.00003 ± 0.00006</td>
<td>0.0001 ± 0.0002</td>
</tr>
<tr>
<td>DsRed2 vs PI</td>
<td>0.601 ± 0.211</td>
<td>0.377 ± 0.088</td>
<td>0.916 ± 0.047</td>
</tr>
</tbody>
</table>

Bolded figures indicate significance difference (p < 0.05) between control & treatments as determined by the Students T-test. A* Denotes first listed stain co-localization with the second listed stain, B* Denotes second listed stain co-localization with the second listed stain. * DNA stain (Hoechst 33342) coloc. with DNA/RNA stain (Propidium iodide), † DNA stain (Hoechst 33342) coloc. with monster green® fluorescent protein (MGFP), ‡ MGFP colo. with DNA/RNA stain (PI), § DNA stain (Hoechst 33342 coloc. with pClneo-DsRed2 fluorescent protein (FP), ¶ pClneo-DsRed2 FP coloc. with DNA/RNA stain (PI).
3.5 **DISCUSSION**

3.5.1 **Fluorescence Expression of Progenitor Immune Cells following Transfection with Fluorescent Protein Plasmids**

**Fluorescent Protein Vectors**

Fluorescent proteins are commonly used as a reporter of gene and protein expression in a number of cell systems (i.e. bacterial, mammalian cells, etc.) (Low and Gong, 2005). Fluorescence expression by these proteins are independent of external substrates and cofactors, reportedly stable and applicable to a number of systems due to the production of mutant variants optimized for particular hosts (Chalfie et al., 1994; Low and Gong, 2005). For this thesis, the fluorescent protein plasmids, phMGFP and pCIneo-DsRed2, were transfected into the porcine progenitor immune cell lines, L23 and L45, to be used as a bioindicator of gene and protein expression in response to probiotic bacterial treatments (Chapters 4 to 7).

The phMGFP vector contains mammalian codon genes and optimised-Kozak sequences to improve expression levels and translation efficiency (Almond, 2003). The DsRed2 gene has six amino acid substitutions and a series of silent base-pair changes to increase chromophore maturation, decreases protein aggregation, produce higher expression and lower cytotoxic effects in mammalian cells (Yanushevicha et al., 2002). Further, the DsRed2 gene was inserted into the mammalian expression vector pCIneo (Promega, Australia) by B. Sainsbury (Sainsbury, 2008). Both the phMGFP and pCIneo vectors are produced by the same manufacturer (Promega) and contain the human cytomegalovirus (CMV) immediate-early enhancer/promoter region (encourages cloned DNA expression in mammalian cells), chimeric intron and SV40 late polyadenylation signal (terminates RNA polymerase II transcription and
signals for 200-250 adenosine residue additions to the 3’ end of the RNA transcript to enhance RNA stability and translation efficiency). The pCIneo vector has additional features including the mammalian selectable marker neomycin phosphotransferase and f1 origin of replication and plasmid replicon for bacterial transformation and production of the vector (Promega, 2008). Therefore, the vectors consisting of the hMGFP and DsRed2 genes are almost similar. Any differences in fluorescence expression are possibly due to post-transcriptional modifications of the fluorescent protein’s genetic RNA molecules, post-translational modification of the resulting protein as well as the folding of the fluorescent protein. In addition, the fluorescent protein gene is not tagged to any particular cellular product; the resulting fluorescence expression occurs randomly within the mammalian progenitor immune cells. The manufacturer of DsRed2 (Clontech) indicated that the fluorescence expression of this protein was cytoplasmic and nuclear-region localized (this is discussed in Section 3.5.2). The localization of fluorescence expression by pHMGFP has not been reported and information regarding this plasmid is limited. Stable expression of the fluorescent proteins was observed post 14-days (in both cell lines by both pHMGFP and pCIneo-DsRed2).

**Fluorescence Protein Expression by the Transfected L45 and L23 Cells**

The fluorescence expression of both hMGFP and DsRed2 appeared to be expressed differently in the T- (L45) and B-cells (L23). The cell types, i.e. immune, skin, liver, muscle, etc. reportedly influences the expression of fluorescent proteins (Bell et al., 2007). *In vivo* experimentation revealed hMGFP fluorescence was low in a number of cell types including lung, muscle and liver cells, whereas DsRed2 fluorescence was weak in liver cells, stronger in muscle cells but undetectable in
However, it appears that there have been no reports comparing the fluorescence expression of hMGFP and DsRed2 in different immune cell lines. Numerous types of analyses were performed to identify what conditions influenced the fluorescence expression of the transfected cells as well as the cellular location of the fluorescence expression. Sub-cloning (to obtain a homogenous population of fluorescence-expressing cells) and antibiotic treatments (relative to the selective marker on the plasmid) were performed to isolate a homogenous population of fluorescing cells (results not shown). It should be noted that the antibiotic selection marker on the phMGFP was a bacteria-selective marker (ampicillin) and not a mammalian selective marker; although different concentrations were applied, no apparent changes to the fluorescence expression outcome of cells was observed.

**Effects on Fluorescence Expression of the Transfected Immune Cells following Mitogen Treatments**

Mitogen treatments (concanavalin A and lipopolysaccharide for L45 and L23 cells, respectively) were used to stimulate the growth of cells to evaluate the effects on fluorescence expression (discussed in Section 3.5.4). The fluorescence expression of sub-cloned, antibiotic and mitogen-treated cells appeared to be similar to the untreated cells. A small quantity of cells exhibited some fluorescence within intracellular vesicles and other cells expressed no fluorescence at all. However, analysis with the LSCM revealed that cells with almost entire cellular fluorescence appeared to be undergoing apoptosis (programmed cell death) as outlined in Section 3.4.3. This observation supports previous research describing similar characteristics (Liu et al., 1999). Four different cell types (mouse fibroblast, baby hamster kidney cells and two different hepatoma cancer cell lines) expressing one of three mutant
GFPs, reportedly underwent apoptosis regardless of the fluorescence expression level and photobleaching rate (Liu et al., 1999). The authors (Liu et al., 1999) suggested that further investigations into GFP cytotoxicity should be conducted to understand the underlying mechanisms.

The aim of this Chapter is not to address the underlying mechanisms of potential cytotoxic effects from hMGFP or DsRed2 fluorescent proteins, however, cellular apoptosis was observed including cellular size reduction, apoptotic body (cytoplastic and nucleic membrane bounded vesicles containing ribosomes, mitochondria and nuclear material) formation and nucleic acid condensation. Apoptotic cells exhibited high fluorescence expression often localized in intracellular vesicles. Therefore, apoptosis assays were conducted to investigate the link between apoptotic cell death and fluorescence expression of the cells as such expression was used as an indicator of cellular responses to probiotic treatments. Further reports have indicated that GFP-like protein aggregates, often thought to be cytosolic aggregates, were accumulated GFP in lysosomes. The continued fluorescence expression was attributed to the fluorescent protein’s resistance to acidic lysosomal conditions and proteases (Katayama et al., 2008). The accumulation of hMGFP and DsRed2 proteins in the lysosomes of L23 and L45 were investigated using the lysosomal-specific fluorescent dye LysoSensor® DND-167 (Molecular Probes, Invitrogen, Mulgrave, Australia) as outlined in Section 3.5.2.

**Growth Rates of Transfected Immune Cells**

Comparisons between the growth rates of the non-transfected and transfected cells indicated that the fluorescent protein influenced the proliferation and growth of
the cells potentially due to plasmid genetic composition. For both cell types, L45 and L23, the pCIneo-DsRed2 transfected cells exhibited higher proliferation than the non-transfected and phMGFP-transfected cells (Fig. 3.5 and 3.6). This could be attributed to the six amino acid substitutions and series of silent base-pair changes of the DsRed2 fluorescent protein gene as outlined by Yanushevicha et al. (2002). These modifications are reported to improve mammalian cellular expression of the DsRed2 protein including increased chromophore maturation, decreased protein aggregation, higher expression and lower cytotoxic effects in mammalian cells (Yanushevicha et al., 2002). Differences between L45hMGFP and L23hMGFP growth curves were observed; the L23hMGFP cells exhibited higher proliferation than the respective control, whereas the L45hMGFP cellular proliferation was relatively similar to the respective control (Fig. 3.5 and 3.6). Such differences could be attributed to the cell type itself; the karyotype of L45 cells is 36.5 on average (porcine diploid chromosome number is 38) and is attributed to unusual gene expression (Kaeffer et al., 1990). However, the connection between the fluorescent proteins physiochemical characteristics and subsequent effects on mammalian cellular growth require further investigations beyond the scope of this thesis.

3.5.2 Identification of Transfected Cellular Fluorescence Expression by Comparative Analysis with Nucleic Acid and Lysosomal-specific Fluorescent Probes

As described previously, the fluorescent proteins were not linked to any specific gene/protein product and are randomly expressed within the cells. Previous studies have indicated that fluorescent proteins aggregate and oligomerize in the cytosol of eukaryotic cells (Miyawaki et al., 2003; Müller-Taubenberger, 2006).
Conversely, it was reported that fluorescent proteins accumulate in cellular lysosomes and do not aggregate in the cytosol (Katayama et al., 2008). The transfected cells used in this thesis were stained with SYTO 59® and LysoSensor™ Blue DND-167 to investigate the location of the intracellular fluorescent protein expression.

**Lysosomal and Nucleic Acid Staining of Transfected Immune Cells**

The results in Section 3.4.2 identified very little correlation and colocalization between the intracellular fluorescent proteins with LysoSensor™ Blue DND-167. The LysoSensor™ Blue DND-167 stain is used to indicate the pH of acidic organelles by producing fluorescence in acidic conditions and providing a tool to study cellular lysosomes. Previous research has identified the accumulation of chemotherapy drugs in lysosomes of drug-resistant cells using the LysoSensor™ Blue DND-167 stain (Altan et al., 1998).

The reported excitation maxima for LysoSensor™ Blue DND-167 is 373 nm with an excitation tail that extends to about 415 – 420 nm. The excitation wavelength used in this thesis was at 405 nm (the 405-diode laser, only laser available on the Leica LSCM system close to 373 nm) and since it was towards the end of the excitation tail, it is possible that the emission intensity of the LysoSensor™ stain was lowered. Given that the expression of the LysoSensor™ Blue DND-167 stain appeared faintly distributed throughout the transfected cells, it is difficult to account for the staining of lysosomes and is therefore not a reliable indication that the intracellular fluorescent proteins accumulate in cellular lysosomes as previously reported by Katayama et al. (2008). However, the accumulation of the
fluorescent protein in lysosomes cannot be discounted based on these results. Katayama et al., (2008) identified colocalization between the fluorescent protein expression in HeLa cells with the anti-lysosomal-associated membrane protein-1 (conjugated to a fluorescent probe) and texas red dextran (a hydrophilic polysaccharide linked to the fluorescent probe texas red used to track endocytosis) concluding that fluorescent proteins enter lysosomes via an autophagy-pathway.

Co-localization of DsRed2 Fluorescent Protein with Lysosomal and Nucleic Acid Stains

The fluorescence expression of DsRed2 is influenced by pH as reported by the manufacturer (Clontech, USA); the optimal range is between pH 5.5 and 9.5 with fluorescence expression reduced outside this range due to inefficient protein folding. Since the pH of lysosomes is about 5 and much lower than the optimal range for DsRed2, very little fluorescence (if any) would be produced by any accumulated DsRed2 protein in the cellular lysosomes and this could have contributed to the low correlation and colocalization of LysoSensor™ Blue DND-167 with the DsRed2 fluorescent proteins. This was supported by the correlation and colocalization statistics shown in Table 3.2 with all values very close to 0. This does not discount the possibility that the pCIneo-DsRed2 protein is accumulating in cellular endosomes and still expressing fluorescence as the pH of early endosomes is approximately 5.9 - 6.0. Nonetheless, pCIneo-DsRed2 did produce fluorescence within the transfected cells and it is possible that this protein was accumulating in the cellular cytosol. The DsRed1 fluorescent protein was described by the manufacturer (Clontech) to produce fluorescence in the cytosol and nuclear regions of cells. The SYTO 59® fluorescent probe was used to investigate if DsRed2 fluorescence
expression occurs in similar areas as DsRed1; SYTO 59® stains nucleic acid, cytoplasmic regions and mitochondria. Some correlation was produced by L23pCIneo-DsRed2 and SYTO 59® as indicated by Pearson’s Correlation of 0.5396 and some colocalization was produced by SYTO 59® with L45pCIneo-DsRed2 as calculated by the thresholded Mander’s coefficient B (Table 3.2). This could be due to expression of fluorescence by the pCIneo-DsRed2 protein in the cellular cytosol and to a lesser extent, in the cellular nucleus (Fig. 3.8 and 3.10). To date, no reports have indicated that non-targeted DsRed2 protein accumulates in cellular mitochondria.

Co-localization of hMGFP Fluorescent Protein with Lysosomal and Nucleic Acid Stains

The fluorescence expression of variant GFPs (not MGFP) is reportedly stable over a wide pH range (Campbell and Choy, 2001) and both modified and native GFP have been described as resistant to proteases (Bokman and Ward, 1981). However, no research has described the stability and resistance of hMGFP to pH and proteases, respectively. Further, no information is available regarding the cellular areas the non-tagged hMGFP fluorescence expression occurs. Based on the results in Section 3.4.2, no correlation was produced for LysoSensor™ Blue DND-167 with hMGFP fluorescence (Pearson’s correlation shown in Table 3.2) indicating that hMGFP may not accumulate in the lysosomal regions of cells. However, as described previously in this discussion section, the probability of hMGFP accumulation in cellular lysosomes cannot be discounted without further research using other methods such as those described by Katayama et al. (2008). The fluorescence produced by hMGFP (for both cells lines) appeared distributed throughout the cells. Based on the
thesholded Mander’s coefficient B for SYTO 59® with hMGFP fluorescence (for both cell lines), some colocalization occurred with values of 0.7395 and 0.6686 for L45hMGFP and L23hMGFP, respectively (Table 3.2). From these results, the hMGFP fluorescence expression potentially occurs in the cytosol and some nuclear regions; however this would require further investigating to confirm.

3.5.3 Anisomycin Treatment of Transfected Mammalian Immunological Cells to Evaluate the Relationship between Fluorescence Expression and Apoptosis

3.5.3.1 Anisomycin Treatment of Transfected Cells

The expression of the fluorescent proteins in the anisomycin treated cells appeared to increase as the cells progressed through the apoptotic stages. This supports previous research that describes cell lines expressing GFP becoming apoptotic regardless of the fluorescent protein expression levels (Liu et al., 1999). Anisomycin is a protein synthesis inhibitor and is known as a ribotoxin; the formation of peptide bonds between amino acids of proteins is inhibited by the binding of anisomycin to the 28S subunit of an actively translating ribosome (Iordanov et al., 1997). The mitogen activated protein kinases (MAPK), stress-activated protein kinases (SAPKs) and cJun NH2-terminal kinases (JNKs), are mammalian cellular signal transduction pathways activated by anisomycin treatment that subsequently initiates one of the apoptosis cascades upon the modification of cellular gene expression (Iordanov et al., 1997; Stadheim and Kucera, 2002). Inducement of the SAPKs/JNKs by anisomycin reportedly occurs prior to protein synthesis inhibition; a dose of anisomycin that causes 50 % of SAPKs/JNKs maximum activation only results in a maximum of 10 % of translation inhibition
(Iordanov et al., 1997). Rudy et al. (2006) postulated that messengers involved in apoptosis are activated prior to the inhibition of protein synthesis (Rudy et al., 2006). Although anisomycin targets ribosome’s and inhibits protein translation, it is possible that the SAPKs/JNKs pathways induced by anisomycin have initiated apoptosis of the transfected cells initially with little effect on the translation of the fluorescent protein and subsequent expression; therefore the fluorescence expression of cells would have been minimally affected.

As outlined previously in the introduction of this Chapter, the phMGFP and pClneo-DsRed2 vectors are not genetically linked to any specific cellular protein/processes, therefore fluorescence expression occurred randomly. It is possible that the cells are ‘disposing’ of the mature fluorescent protein in viable/healthy cells keeping the fluorescence expression low; as the fluorescent protein content increases, the cell is no longer able to maintain this content and eventually enters apoptosis with the associated high fluorescence expression. The aggregation of the fluorescent proteins potentially could have induced apoptosis in these mammalian immune cell lines. Cellular toxicity reportedly occurs from fluorescent protein aggregation (Miyawaki et al., 2003). Improper folding of the fluorescent protein can cause protein aggregation (Müller-Taubenberger, 2006) as well as electrostatic or hydrophobic interactions between fluorescent proteins and the formation of large cross-linked masses from fluorescent protein oligomerization (Miyawaki et al., 2003). To overcome aggregation-associated cellular toxicity, the removal of hydrophobic side chains on oligomeric complex surfaces was postulated to generate non-aggregated fluorescent protein mutants (Miyawaki et al., 2003). The DsRed2 fluorescent protein reportedly has a reduced ability to aggregate due to the
point mutations of the DsRed gene and removal of basic residues near the amino termini that would normally interact via electrostatic exchanges (Yanushevicha et al., 2002). The description of the hMGFP gene does not indicate any gene-related changes to reduce potential aggregation of the resulting fluorescent protein. Nonetheless, the results presented in Section 3.4.1 indicates that the fluorescent proteins accumulate in what appears to be intracellular vesicles; potential associations between the fluorescent protein and cellular elements e.g. other proteins, lipids, etc. via hydrophobic or other interactions could be the mechanisms underlying the production of these fluorescent protein vesicles.

It should be noted that many investigations have used gene linked/tagged fluorescent protein products to identify the location and expression of proteins or organelles without cellular apoptosis being reported (Yokeo and Meyer, 1996). For instance, GFP cDNA with a mitochondria targeting sequence allowed researchers to visualize mitochondria movement within living cells and morphological changes induced by drugs (Rizzuto et al., 1995); cellular apoptosis was not described by Rizzuto et al. (1995). Further, the location of secretory proteins tagged by enhanced GFP (EGFP) and DsRed2 were identified in Mardin-Darby canine kidney epithelial cells (Maruyama et al., 2004); similar to the previous study, no apoptotic events were reported. However, the tagged protein itself reportedly influences the folding of the fluorescent protein i.e. well-folded host proteins assist with the folding of the associated fluorescent protein (Sacchetti and Alberti, 1999); this could potentially reduce fluorescent protein aggregation and therefore lessen the probability of cellular apoptosis induced by the fluorescent protein. Since the hMGFP and pCIneo-DsRed2 fluorescent proteins are not tagged to any cellular proteins, it is possible that the
random fluorescent protein expression is not ‘controlled’ resulting in fluorescent proteins accumulation in different cellular regions that are more likely to form aggregates and induce cellular apoptosis.

3.5.3.2 Mitogen-stimulation of Transfected Cells

The L45- and L23-transfected cells were stimulated with the mitogens, Con A and LPS (respectively), to determine if mitogen-stimulation (as opposed to apoptosis inducement) influences the co-localization between the intracellular fluorescent proteins and fluorescent stains (similar to the anisomycin-treated cells) and to compare with the results from the anisomycin treated cells. Unlike the anisomycin-treatment correlation and co-localization results, the mitogen-stimulated results for all transfected cell lines were not significantly different from the respective control results (Section 3.4.4). The L45-transfected cells produced low correlation and co-localization results for all control and treatment combinations with the exception of the Pearsons correlation for L45pCIneo-DsRed2 with PI; a high correlation was produced as indicated by the value of 0.829 (Table 3.5). It should be noted that the anisomycin treated L45pCIneo-DsRed2 Pearson’s correlation value was 0.606 and comparatively lower than the Con-A treated L45pCIneo-DsRed2 cells; however, the thresholded Mander’s coefficient B for the anisomycin treated cells was much higher than the mitogen-stimulated cells with a value of 0.899 and 0.474, respectively. Since the PI stain is used to indicate apoptotic/necrotic cells, this result supports the theory that high fluorescence production is produced by the intracellular fluorescent protein pCIneo-DsRed2. Similarly, the L23-transfected cells treated with a mitogen (LPS) produced low correlation and co-localization results with the exception of the thresholded Mander’s coefficient B for PI with both
fluorescent proteins for all control and treatments (Table 3.6). The L23hMGFP cellular results indicate that moderate co-localization occurred for PI with hMGFP with the values of 0.525 and 0.697 for the control and treatment, respectively. The L23pClneo-DsRed2 control and treated cells indicated high co-localization of PI with pClneo-DsRed2 producing a value of 0.926 and 0.916, respectively. The LPS treated cells produced a higher thresholded Manders coefficient B compared to the anisomycin treated cells with the exception of the anisomycin treated L23pClneo-DsRed2 cells. As outlined above, the PI stain is used to indicate non-viable cells and these results also support the theory that high intracellular fluorescence is expressed in such cells.

Further, the correlation and co-localization values of Hoechst 33342 with both intracellular fluorescent proteins from the anisomycin-treated cells were higher than all respective mitogen-stimulated cells. The thresholded Mander’s coefficient A for Hoechst 33342 with the intracellular fluorescent protein indicated colocalization for all treated transfected cells except the treated L23hMGFP cells although it was significantly different to the respective control (Tables 3.3 and 3.4). As the cells undergo apoptosis, the nuclear regions of the cells fragmentize. It was established in Section 3.5.2 that both intracellular fluorescent proteins potentially localise in cellular nuclear regions and could potentially contribute to the colocalization between Hoechst 33342 and the intracellular fluorescent protein.

3.6 CONCLUSION

The porcine progenitor immune cell lines, L45 and L23, were successfully transfected with the fluorescent protein plasmids humanized Monster Green®
Fluorescent protein (hMGFP) and pCIneo-DsRed2. The resulting cell lines, L45hMGFP, L45pCIneo-DsRed2, L23hMGFP and L23pCIneo-DsRed2, all produced fluorescence within 24 h of transfection and exhibited stable fluorescence 14 days post-transfection. The co-localisation results presented in this Chapter indicate that the expression of the intracellular fluorescent proteins is localized to the cytosol and nuclear regions of the cells. Further research is required to investigate the accumulation of fluorescent proteins in cellular lysosomes. Increased fluorescence from both intracellular fluorescent proteins in both cell lines were produced during induced cellular apoptosis. Correlation between intracellular fluorescent proteins with Hoechst 33342 and PI in apoptotic cells was identified using LSCM and the Bitplane Image Analysis software. These results indicate that cells undergoing apoptosis produced high intracellular fluorescence; a potential cause of cellular apoptosis is the inability of the cells to maintain the fluorescent protein levels and the accumulation and aggregation of the intracellular fluorescent protein content eventually induces cellular apoptosis. Conversely, mitogen-stimulated transfected cells did not exhibit correlation and co-localization between the intracellular fluorescent protein and Hoechst 33342, however co-localization was produced for the intracellular fluorescent proteins with PI (indicates apoptotic/necrotic cells) supporting the results from the apoptosis induced cells. It was determined that these cell lines will be utilised as a negative control in subsequent experiments with probiotic bacteria to monitor potential apoptotic immunological cell responses to these microorganisms.
4. CHAPTER FOUR

Free and Microencapsulated Probiotic Bacteria Effects on Immune Cell Proliferation

4.1 INTRODUCTION

Immune Cell Interactions with Probiotic Bacteria

A number of studies have described the enhancement of innate immune cell activities from probiotic bacterial exposure including dendritic cells (Mileti et al., 2009), monocytes (Parra et al., 2004; Ghadimi et al., 2008) and natural killer cells (Parra et al., 2004). Adaptive immunological responses to probiotic bacteria have also been reported (Gill et al., 2000; Paturi et al., 2007; Schareka et al., 2007; Paturi et al., 2008). However, the response of T and B cells to probiotic bacteria-produced soluble factors is rarely described. Further, the majority of studies have used either in vivo murine models or human immunological cells ex vivo to investigate the effects of probiotics on immunological cells; porcine adaptive immune cell responses to probiotics are infrequently researched.

Immunomodulation of innate and adaptive immune cells have been reported in mice that were fed L. acidophilus LAFTI® L10 (Paturi et al., 2008) and B. lactis HN019 (DR10™) (Gill et al., 2000). Within the gastrointestinal system, dendritic cells are believed to be one of the first immunological cells to be in contact with the microflora of the gastrointestinal tract. A number of studies have treated dendritic cells with probiotic conditioned media and described the effects from co-culturing the treated dendritic cells with adaptive immune cells (von der Weid et al., 2001; Braat et al., 2004; Smits et al., 2005; Zeuthen et al., 2007). Direct interactions of
probiotic bacterial-produced soluble factors on T- and B-cells have rarely been described.

**Immune Response to Microencapsulated Probiotics**

As described in Chapter 1, the harsh gastrointestinal environment has been suggested to affect probiotic bacterial viability that is considered important for health-conveying benefits to hosts. Microencapsulation of probiotics has been proposed to protect the cells from food processing and the gastrointestinal environment. The effect of microencapsulated probiotics on the immunological responses of innate and adaptive immune cells has not been addressed at this point. To determine the effects of microencapsulation on probiotics, the proliferation responses of immunological cells may be used as a downstream indicator.

The effects on the transfected porcine progenitor immune cells developed in Chapter 3 (L45hMGFP, L45pClneo-DsRed2, L23hMGFP and L23pClneo-DsRed2) by microencapsulated bacteria-produced soluble factors are addressed in this Chapter. The proliferation of the immune cells in response to the microencapsulated bacteria-conditioned media was compared to the responses of the immune cells treated with free bacteria-conditioned media. In addition, the proliferation of the immune cells to the probiotic bacteria-conditioned media was compared to the immune cell proliferation responses to the pathogenic bacteria-conditioned media.
4.2 AIMS & OBJECTIVES

The aim of this Chapter is:

- To investigate the effects of free- and microencapsulated bacteria (*L. acidophilus* LAFTI® L10, *B. lactis* HN019 (DR10™) and *S. pyogenes*) conditioned mammalian cell culture media (CM) on the proliferation of the porcine progenitor immune cell lines that have been transfected with the fluorescent proteins phMGFP and pClneo-DsRed2 (i.e. L45hMGFP, L45pClneo-DsRed2, L23hMGFP and L23pClneo-DsRed2 cell lines).

The objectives of this Chapter are:

- To produce mammalian cell culture media (without antibiotics known as cDMEM-AF) that has been conditioned with free and microencapsulated bacteria (*L. acidophilus* LAFTI® L10, *B. lactis* HN019 (DR10™) and *S. pyogenes*). This medium will be applied to the L45hMGFP, L45pClneo-DsRed2, L23hMGFP and L23pClneo-DsRed2 cell lines. The control is the cDMEM-AF media without bacteria.

- To compare the total cell concentration of the respective controls with the total cell concentration of free and microencapsulated *L. acidophilus* LAFTI® L10, *B. lactis* HN019 (DR10™) and *S. pyogenes* conditioned mammalian cell culture media treated L45hMGFP, L45pClneo-DsRed2, L23hMGFP and L23pClneo-DsRed2 cells.
4.3 MATERIALS AND METHODS

4.3.1 Treatment of Porcine Progenitor Immune Cells with Free and Microencapsulated Bacteria Conditioned Mammalian Cell Culture Media

4.3.1.1 Bacteria-Conditioning of Mammalian Cell Culture Media

The bacteria cultures were grown in the respective broth as outlined in Chapter 2, Section 2.3.1.1, for 24 h. A 1 mL aliquot of *L. acidophilus* LAFTI® L10, *B. lactis* HN019 (DR10™) and *S. pyogenes* was added to 14 mL of the respective broth. The cultures were grown until mid-log phase (4 h) at 37 °C in the Thermoline Scientific Laboratory incubator in the appropriate conditions (anaerobic and aerobic); the cultures were harvested at mid-log phase (4 h) due to the active growth of the culture as previously determined from growth curves conducted in the respective media (Chapter 2). The Jenway spectrophotometer was used to enumerate the cultures at 600 nm. The bacteria cells were harvested by centrifugation at 3864 g for 10 min (37 °C) using the Universal 32 R centrifuge (HD Scientific Supplies Pty. Ltd.). The supernatant was decanted and the pellet washed with 1 mL of cDMEM-AF and re-centrifuged. The supernatant was removed by a 2 mL serological pipette (710 160, Greiner Bio-one; Interpath Services P/L, West Heidelberg, Australia) and the pellets were resuspended in cDMEM-AF making a final bacteria cell concentration of $1.0 \times 10^9$ CFU mL$^{-1}$.

Conditioning of the mammalian cell culture media (cDMEM-AF) by the bacteria cultures was conducted by incubating the bacteria/cDMEM-AF in the Forma Scientific CO$_2$ water jacketed incubator (Model 3111 S/N 26931-617) for 1 h at 37 °C in 5 % CO$_2$; high quantities of lactic acid were produced for incubations longer
than 1 h (outlined in Chapter 2). Following the conditioning of cDMEM-AF, the cultures were harvested by centrifugation (as outlined above). The supernatant (conditioned media – cDMEM-AF) was removed by a 5 mL serological pipette (606 160, Greiner Bio-One; Interpath Services P/L, West Heidelberg, Australia) and placed into a sterile 10 mL Terumo® syringe (DVR-5174, Terumo Cooperation Australian Branch, Macquarie Park, Australia). The conditioned media was passed through a 0.20 µm sterile, non-pyrogenic filter (194-2520, Nalgene; Thermo Fisher Scientific Inc, Scoresby, Australia) and placed into a 50 mL polypropylene tube (227 261, Greiner Bio-One; Interpath Services P/L, West Heidelberg, Australia). The conditioned media was re-centrifuged to ensure the absence of bacteria cells. In the event of a pellet present following centrifugation, the supernatant was again filtered and re-centrifuged. The probiotic- and bacterial-conditioned cDMEM-AF was stored at -20 ºC.

### 4.3.1.2 Treatment of Porcine Progenitor Immune Cells with Bacteria-Conditioned Media

The porcine progenitor immune cell lines, L45hMGFP, L45pCIneo-DsRed2, L23hMGFP and L23pCIneo-DsRed2 (developed as described in Chapter 3), were used to evaluate proliferation in response to the bacterial-conditioned cDMEM-AF. The progenitor immune cell suspensions were passaged 24 to 48 h prior to experimentation in complete DMEM as outlined in Section 3.3.1.3. The cells were placed into a sterile 15 mL Falcon™ polypropylene conical tube (352096; BD Bioscience; North Ryde, Australia) and 10 µL of suspension was removed for counting as described previously (Chapter 3, Section 3.3.1.4). The remaining cell suspensions were centrifuged at 98.92 g for 5 min using the Universal 32 R
The supernatant was decanted and the pellet resuspended in fresh cDMEM-AF making a final cell concentration of $6.0 \times 10^6$ cells mL$^{-1}$. Four aliquots of approximately $1.5 \times 10^6$ cells mL$^{-1}$ were placed into 15 mL Falcon tubes by removing 1 mL of the cell suspension and adding 0.25 mL to each tube. The cell suspension were washed in 1 mL of cDMEM-AF and re-centrifuged. The supernatant was removed by a 2 mL sterile serological pipette and the pellet resuspended in 3 mL of either cDMEM-AF (control), *L. acidophilus*-conditioned cDMEM-AF, *B. lactis*-conditioned cDMEM-AF or *S. pyogenes*-conditioned cDMEM-AF. The 3 mL suspensions were allocated into 1 mL aliquots in a sterile 24-well plate containing approximately $5.0 \times 10^5$ cells mL$^{-1}$ and incubated in the Forma Scientific CO$_2$ water jacketed incubator (Model 3111 S/N 26931-617) at 37 $^\circ$C in 5 % CO$_2$. Cell counts were conducted at 4, 8 and 24 h by removing 30 µL of cell suspension. The 30 µL aliquots were divided into three 10 µL aliquots and cells were counted by trypan blue live cell exclusion staining using a haematocytometer (Chapter 3, Section 3.3.1.4). Each well was disturbed only once to maintain cellular junctions contributing to growth dynamics. Treatments of each progenitor immune cell lines with bacteria-conditioned cDMEM-AF were repeated in triplicate.

### 4.3.1.3 Conditioning of Mammalian Cell Culture Media by Microencapsulated Bacteria

The bacteria cultures (*L. acidophilus* LAFTI® L10, *B. lactis* HN019 (DR10™) and *S. pyogenes*) was grown overnight at 37 $^\circ$C in the respective culture conditions (refer to Chapter 2, Section 2.3.1.1). The Jenway spectrophotometer was used to enumerate the cultures at 600 nm. The cultures were centrifuged as outlined in Section 2.3.1.1 for 10 min and the supernatant was discarded. The pellets were
initially resuspended in alginate solution (1.8 %, 1 mL). Further alginate solution (1.8 %) and Brij solution (6 % final concentration) was added making a final bacteria cell concentration of $1.0 \times 10^9$ CFU mL$^{-1}$. The alginate and bacteria slurry was passed through a 10 mL Terumo Syringe and a 23 g x 32 mm needle (NN-2325R, Terumo Cooperation Australian Branch, Macquarie Park, Australia) into the chilled chitosan (0.1 %) and CaCl$_2$ (0.1 M) solution at a drop distance of 3 to 5 cm. This solution was stirred using a magnetic stirrer and the capsules were allowed to harden for 15 min. The capsules were removed from the chitosan (0.1 %) and CaCl$_2$ (0.1 M) solution and washed with milliQ water. The capsules were further washed with stock DMEM until all traces of milliQ water and chitosan (0.1 %) and CaCl$_2$ (0.1 M) solution were removed (the capsules appeared the same colour as the cDMEM-AF).

The capsules were placed into 50 mL Falcon tubes and resuspended in cDMEM-AF making a final cell concentration of $1.0 \times 10^9$ CFU mL$^{-1}$ (same volume of alginate used to resuspend the bacteria pellet). The encapsulated bacteria in cDMEM-AF media was incubated at 37 °C for 1 h in the Forma Scientific CO$_2$ water jacketed incubator for 1 h at 37 °C in 5 % CO$_2$. The encapsulated bacterial-conditioned cDMEM-AF was removed using a 10 mL Terumo syringe and passed through a 0.20 µm sterile, non-pyrogenic filter into a sterile 50 mL polypropylene tube. The conditioned media was centrifuged using the Universal 32 R centrifuge at 3864 g for 10 min (37 °C). Conditioned media that contained a bacteria pellet were re-filtered and re-centrifuged. The conditioned media was stored at 4 °C to minimise precipitation of the capsular compounds.
4.3.1.4 Treatment of Porcine Progenitor Immune Cells with Microencapsulated Bacteria-Conditioned Media

The progenitor immune cell lines were prepared as outlined in Section 4.3.1.2 using the microencapsulated bacteria-conditioned media instead of the non-microencapsulated bacteria-conditioned media. The progenitor immune cells were enumerated at 4, 8 and 24 h as outlined in Section 4.3.1.2 to determine the effects of the microencapsulated bacteria-conditioned media on the progenitor immune cell viability and proliferation. The treatments were repeated in triplicate.

4.3.2 Statistical Analysis

The mean and standard error of the mean (SEM) of the total cell counts from the triple experiments were calculated using Microsoft Excel 2003. Comparisons between the control and treatment means were conducted using the paired-samples T test with the SPSS Statistics 18 Program (2010).

4.4 Results

4.4.1 Free- and Microencapsulated Bacteria-Conditioned Mammalian Cell Culture Media Treatment of Porcine Progenitor Immune Cells

4.4.1.1 Treated L45hMGFP Progenitor Immune Cells

Free Bacteria Soluble Factors Effects on Immune Cells

The total cell concentration of L45hMGFP cells treated with probiotic-conditioned mammalian cell culture media (CM) is shown in Figure 4.1a. The *B. lactis* HN019 (DR10™) treated cells exhibited a lower total cell concentration than
the control, *L. acidophilus* LAFTI® L10 and *S. pyogenes* CM treated cells, at all time points. The *L. acidophilus* LAFTI® L10 treated cells exhibited higher cell concentrations than the control and *B. lactis* HN019 (DR10™) treated cells at 4 and 24 h (exhibiting opposite effects to the *B. lactis* HN019 (DR10™) treatments). The *S. pyogenes* CM treated cells exhibited a higher total cell count at 24 h. Significant differences were observed for the total cell concentration of *B. lactis* HN019 (DR10™) treated cells at 4 h and 8 h, and for *L. acidophilus* LAFTI® L10 treated cells at 24 h (compared to the control).

**Microencapsulated Bacteria Soluble Factors Effects on Immune Cells**

The total cell concentration of L45hMGFP cells treated with microencapsulated bacteria CM are shown in Figure 4.1b. The total cell concentration for microencapsulated *L. acidophilus* LAFTI® L10 and *S. pyogenes* CM treated cells was higher than the control at 4 (significantly different to control) and 8 h. The total cell count for *B. lactis* HN019 (DR10™) CM treatment was lower than the control at 4 and 24 h (significantly different to control). The control total cell count was higher than the three microencapsulated bacteria-CM treated cells at 24 h.

**Comparison between Free and Microencapsulated Bacteria Treatments**

Similarities between the free and microencapsulated *L. acidophilus* LAFTI® L10 CM treated cells were observed at 4 h with a higher cell concentration than the control; opposite results for the free and microencapsulated *L. acidophilus* LAFTI® L10 were observed at 8 and 24 h. The free and microencapsulated *B. lactis* HN019 (DR10™) CM treated cells exhibited similar results with a lower cell concentration
than the control at 4 and 24 h. The free *S. pyogenes* CM treated total cell count (Fig. 4.1a) differed to the microencapsulated *S. pyogenes* CM treated cells (Fig. 4.1b) at 4 and 24 h.
Figure 4.1. Effects on L45hMGFP cell proliferation at 4, 8 and 24 h from treatments with mammalian cell culture media conditioned with a) free *L. acidophilus* LAFTI® L10 (L10 treatment), *B. lactis* HN019 (DR10™ treatment) and *S. pyogenes* b) microencapsulated *L. acidophilus* LAFTI® L10 (L10 treatment), *B. lactis* HN019 (DR10™ treatment) and *S. pyogenes*. Data represents the mean ± SEM from triplicate experiments where n = 9. Significant difference was determined by the paired sample T-test (SPSS Statistics 18) comparing the means of the controls and treatments (*p < 0.05; **p < 0.01).
4.4.1.2 Treated L45pCI neo-DsRed2 progenitor immune cells

Free Bacteria Soluble Factors Effects on Immune Cells

The \textit{L. acidophilus} LAFTI® L10 CM treated L45pCI neo-DsRed2 cells exhibited a higher total cell concentration than the control at all time points (Fig. 4.2a). The \textit{B. lactis} HN019 (DR10™) CM and \textit{S. pyogenes} CM treated cells were lower than the control at all times points. Significant differences were observed for all bacteria CM treated cells at 8 and 24 h compared to the control.

Microencapsulated Bacteria Soluble Factors Effects on Immune Cells

The \textit{L. acidophilus} LAFTI® L10 CM treated L45pCI neo-DsRed2 cells exhibited higher total cell counts than the control at all time points (Fig. 4.2b); significant differences were calculated at 4 and 8 h. The microencapsulated \textit{B. lactis} HN019 (DR10™) CM treated total cell concentration were similar to the control at 8 and 24 h. The cell concentration of microencapsulated \textit{S. pyogenes} treated cells was higher than the control at 4 and 8 h.

Comparison between Free and Microencapsulated Bacteria Treatments

Similar to the free bacteria CM treated L45pCI neo-DsRed2 cells (Fig. 4.3a), the microencapsulated \textit{L. acidophilus} LAFTI® L10 CM treated cells exhibited higher total cell counts than the controls at all time points. For the \textit{B. lactis} HN019 (DR10™) treatments, the only similarity for the free and microencapsulated CM treated L45pCI neo-DsRed2 cells was at 4 h. The microencapsulated \textit{S. pyogenes} exhibited opposite effects at 4 and 8 h with a lower total cell concentration than the control.
Figure 4.2. Effects on L45pClneo-DsRed2 cell proliferation at 4, 8 and 24 h from treatments with mammalian cell culture media conditioned with a) free L. acidophilus LAFTI® L10 (L10 treatment), B. lactis HN019 (DR10™ treatment) and S. pyogenes b) microencapsulated L. acidophilus LAFTI® L10 (L10 treatment), B. lactis HN019 (DR10™ treatment) and S. pyogenes. Data represents the mean ± SEM from triplicate experiments where n = 9. Significant difference was determined by the paired sample T-test (SPSS Statistics 18) comparing the means of the controls and treatments (*p < 0.05; **p < 0.01; *** p < 0.001).
4.4.1.3 Treated L23hMGFP progenitor immune cells

Free Bacteria Soluble Factors Effects on Immune Cells

The *L. acidophilus* LAFTI® L10 CM treated L23hMGFP cells exhibited significantly higher total cell concentrations than the control at all time points (Fig. 4.3a). The *B. lactis* HN019 (DR10™) CM treated cells were similar to the control at 4 and 24 h, but significantly lower at 8 h. The *S. pyogenes* CM treated cells were observed with the lowest total cell concentration at 4 and 8 h (significantly different to the control) with a similar cell concentration as the control at 24 h.

Microencapsulated Bacteria Soluble Factors Effects on Immune Cells

The total cell concentration of microencapsulated *L. acidophilus* LAFTI® L10 CM treated L23hMGFP cells was higher than the control at 8 and 24 h (Fig. 4.3b). Conversely, the microencapsulated *B. lactis* HN019 (DR10™) treated cells exhibited a lower total cell concentration than control at all time points. The microencapsulated *S. pyogenes* treated cells exhibited similar total cell counts to the control at all time points.

Comparison between Free and Microencapsulated Bacteria Treatments

The microencapsulated *L. acidophilus* LAFTI® L10 and *B. lactis* HN019 (DR10™) CM treated L23hMGFP cells exhibited similar results to the respective free-bacteria CM treatments. The free and microencapsulated *S. pyogenes* CM treated L23hMGFP cells exhibited similar results with a lower cell concentration than the control at 4 and 8 h.
Figure 4.3. Effects on L23hMGFP cell proliferation at 4, 8 and 24 h from treatments with mammalian cell culture media conditioned with a) free *L. acidophilus* LAFTI® L10 (L10 treatment), *B. lactis* HN019 (DR10™ treatment) and *S. pyogenes* b) microencapsulated *L. acidophilus* LAFTI® L10 (L10 treatment), *B. lactis* HN019 (DR10™ treatment) and *S. pyogenes*. Data represents the mean ± SEM from triplicate experiments where n = 9. Significant difference was determined by the paired sample T-test (SPSS Statistics 18) comparing the means of the controls and treatments (*p < 0.05; **p < 0.01; *** p < 0.001).
4.4.1.4 Treated L23pClneo-DsRed2 progenitor immune cells

Free Bacteria Soluble Factors Effects on Immune Cells

As shown in Figure 4.4a, the *L. acidophilus* LAFTI® L10 CM treated L23pClneo-DsRed2 total cell concentration was the lowest at 8 and 24 h (both significantly different to the control). The *B. lactis* HN019 (DR10™) CM treated L23pClneo-DsRed2 total cell concentration were lower than the control at 4 h and 8 h (both significantly different to the control). The *S. pyogenes* total cell counts for the CM treated cells were lower than the control at 8 and 24 h.

Microencapsulated Bacteria Soluble Factors Effects on Immune Cells

The microencapsulated *L. acidophilus* LAFTI® L10 CM treated L23pClneo-DsRed2 cell concentrations were the lowest at all time points (Fig. 4.4b); significant differences to the control were observed at 4 and 24 h. The microencapsulated *B. lactis* HN019 (DR10™) CM treated L23pClneo-DsRed2 cell concentrations were higher than the control at all time points. The microencapsulated *S. pyogenes* treated L23pClneo-DsRed2 cells exhibited a higher cell concentration than the control at 4 and 24 h. The 24 h treatment was significantly different to the control.

Comparison between Free and Microencapsulated Bacteria Treatments

The free and microencapsulated *L. acidophilus* LAFTI® L10 treated cells exhibited similar results at 8 and 24 h. The opposite results were observed for the free and microencapsulated *B. lactis* HN019 (DR10™) treated cells at 4 and 8 h. Similarities in total cell concentrations were also observed for the free and microencapsulated *S. pyogenes* at 4 and 8 h.
Figure 4.4. Effects on L23pClneo-DsRed2 cell proliferation at 4, 8 and 24 h from treatments with mammalian cell culture media conditioned with a) free *L. acidophilus* LAFTI® L10 (L10 treatment), *B. lactis* HN019 (DR10™ treatment) and *S. pyogenes* b) microencapsulated *L. acidophilus* LAFTI® L10 (L10 treatment), *B. lactis* HN019 (DR10™ treatment) and *S. pyogenes*. Data represents the mean ± SEM from triplicate experiments where n = 9. Significant difference was determined by the paired sample T-test (SPSS Statistics 18) comparing the means of the controls and treatments (*p < 0.05; **p < 0.01; *** p < 0.001).
4.5 DISCUSSION

4.5.1 Free- and Microencapsulated Bacteria-Conditioned Mammalian Cell Culture Media (CM) Treatment of Porcine Progenitor Immune Cells

4.5.1.1 Free Bacteria CM Treatment of Porcine Progenitor Immune Cells

Soluble factors produced by probiotic bacteria have been proposed to beneficially influence host cells including epithelial and immune cells (Corthesy et al., 2007). In this study, the proliferation of the porcine progenitor immune cells (with fluorescent proteins) was used as a general indicator of immunological response to the bacterial-produced soluble factors. In addition, the responses of the immune cells to the pathogenic bacteria-conditioned media were used to determine if the probiotic bacteria conveyed similar effects to immune cell responses. Previous research has described strain-specific activities by probiotic bacteria (He et al., 2006; Gillingham and Lescheid, 2009; Lopez et al., 2010); such activities were observed in this study as well. The response of the L45hMGFP, L45pCIneo-DsRed2, L23hMGFP and L23pCIneo-DsRed2 cells to the soluble factors in the bacteria CM appeared to be influenced by the probiotic bacterial-strain. For instance, the *L. acidophilus* LAFTI® L10 CM treated L45hMGFP, L45pCIneo-DsRed2 and L23hMGFP cells (Fig. 4.1a, 4.2a and 4.3a) produced higher cell concentrations than the respective controls; these cell counts were significantly different to the control at all time points (except the L45hMGFP treatment at 4 h). On the contrary, the L23pCIneo-DsRed2 CM treated cell concentrations produced significantly different results to the control but with a lower cell concentration (Fig. 4.4a).
Previous research primarily involved \textit{in vivo} or \textit{ex vivo} probiotic bacteria treatment of immune cells; direct interaction between probiotic bacterial-produced soluble factors and immune cells \textit{in vitro} are rarely described. However, the results shown in Section 4.4.1 support previous research that described increased proliferation of lymphocytes in response to lactobacilli treatment. The proliferation of murine splenic lymphocytes was reportedly higher in mice fed \textit{L. acidophilus} (ATCC 4356) than the control (Kirjavainen et al., 1999). The T- and B-cell population in murine Peyer’s patches increased from \textit{L. paracasei} subsp. \textit{paracasei NTU 101} supplementation (Tsaia et al., 2010). Further, enhanced intestinal IgA secreting cell responses were reported for pigs treated with \textit{L. acidophilus NCFM™} and \textit{L. reuteri} (ATCC 23272) (Zhang et al., 2008).

Some studies have described decreased T-cell proliferation in response to Lactobacilli treatment in \textit{in vitro} conditions using human cells (Braat et al., 2004; Smits et al., 2005). The differences observed by Braat et al. (2004) and Smits et al. (2005) to the results presented in this Chapter are due to the experimental design. These authors employed indirect interactions between lactobacilli and T-cells by using dendritic cells and splenocytes as intermediate mediators in \textit{in vitro} conditions. The modulation of dendritic cellular activity by the lactobacilli exposure \textit{in vitro} was shown to induce T-cell hyporesponsiveness (Braat et al., 2004) and encourage Treg cell development (Smits et al., 2005). Therefore, based on the results in Section 4.4.1.1 and those from Braat et al. (2004) and Smits et al. (2005), the type of interaction between lactobacilli and lymphocytes \textit{in vitro} (e.g. direct via conditioned media or indirect using intermediate cells such as dendritic cells) result in different T
cell behaviour as a result of modulatory activities by the intermediate cells (such as DCs).

As shown in Section 4.4.1, differences between the transfected B-cell line (L23hMGFP and L23pCIneo-DsRed2) results for the *L. acidophilus* LAFTI® L10 CM treatment occurred; this discrepancy is potentially a function of the fluorescent protein plasmid. It was established in Chapter 3 that the growth and proliferation of the transfected cells was potentially influenced by the fluorescent protein plasmid composition; it is possible that the plasmid composition and expression of the fluorescent protein has influenced the L23pCIneo-DsRed2-cellular response to the *L. acidophilus* LAFTI® L10 CM treatments. The *B. lactis* HN019 (DR10™) CM treated L45hMGFP and L45pCIneo-DsRed2 cells (T cells) produced lower cell concentrations than the control at all time points (Fig. 4.1 and 4.2a); comparatively, the L23hMGFP and L23pCIneo-DsRed2 (B cells) CM treated cell concentrations exhibited very little differences than the control at all time points (Fig. 4.3a and 4.4a). The underlying mechanisms behind the fluorescent protein expression and subsequent cellular behaviour require further investigation. The possibility that the type of immune cell, i.e. T- or B-cell, may also contribute to the different responses observed for the probiotic strains.

As mentioned previously, most studies described *in vitro* or *ex vivo* treatments of immune cells by probiotic bacteria. However, similar observations to the *B. lactis* HN019 (DR10™) CM treated porcine progenitor immune cells have been reported. Amrouche et al. (2006) described increases in the proliferation of murine spleen-derived lymphocytes following Bifidobacteria treatment; the
lymphocytes were isolated from the spleen and treated with bifidobacterial extracts including cell wall and cytoplasm (Amrouche et al., 2006). Human patients treated with \textit{B. lactis} HN019 (DR10\textsuperscript{TM}) reportedly had increased populations of CD4\textsuperscript{+} and activated (CD25\textsuperscript{+}) T lymphocytes (Gill et al., 2001). Further, the treatment of piglets with rotavirus-associated diarrhoea had a decreased incidence of symptoms (diarrhoea) following treatment with \textit{B. lactis} HN019 (DR10\textsuperscript{TM}) as well as increases in peripheral T lymphocyte populations (Shu et al., 2001). However, conversely to the behaviour of the L23hMGFP and L23pCIneo-DsRed2 cells to the \textit{B. lactis} HN019 (DR10\textsuperscript{TM}) soluble factors (outlined in Section 4.4.1), mice fed \textit{B. breve} reportedly increased B-cell populations within the Peyer’s patches (Yasui and Ohwaki, 1990). It is possible that different responses by T and B cells will be observed for probiotic bacteria treatment in different animal models. The origin of the cells, for example, peripheral vs. spleen, may also influence the response of the lymphocytes to the probiotic bacteria based on priming interactions with other immune components e.g. dendritic cells.

Unlike the \textit{L. acidophilus} LAFTI\textregistered\ L10 and \textit{B. lactis} HN019 (DR10\textsuperscript{TM}) CM treatments, the \textit{S. pyogenes} CM treated cells exhibited varying results for the L45hMGFP, L45pCIneo-DsRed2, L23hMGFP and L23pCIneo-DsRed2 cell lines over the 24 h period. No consistency was observed for the \textit{S. pyogenes} CM treatments in regards to the cell type (B- or T-cell) or the fluorescent protein plasmid (phMGFP or pCIneo-DsRed2). The \textit{S. pyogenes} CM treatment of the four cell lines was conducted to determine if the \textit{L. acidophilus} LAFTI\textregistered\ L10 and \textit{B. lactis} HN019 (DR10\textsuperscript{TM}) CM treated cells behaved similarly to the pathogen-treated cells. The results presented in Section 4.4.1 indicated that soluble factors produced by the two
probiotic strains did not consistently produce similar results to the *S. pyogenes* treated cells. This provides further support for the probiotic bacterial-strain specific effects on the growth of the transfected porcine progenitor immune cells.

### 4.5.1.2 Microencapsulated Bacteria CM Treatment of Porcine Progenitor Immune Cells

Microencapsulation of probiotic bacteria has been proposed to protect the cells in harsh environments, such as stomach acid and bile salts (Kailasapathy, 2002). The stress-related changes and subsequent effects on downstream activities by the probiotic bacteria following microencapsulation (for instance, effects on the proliferation of immune cells) have not been determined to date. As outlined in Chapter 3, the microencapsulated bacteria were shown to survive for at least 24 h in the confined environment with the ability to produce lactic acid (for the *L. acidophilus* LAFTI® L10 and *B. lactis* HN019 (DR10™) strains). The surrounding media (mammalian cell culture media with phenol red indicator) were observed to change colour as an outcome of the acid production. This result indicates that lactic acid and potentially other metabolites/molecules are able to transverse the calcium alginate capsular surface. Similarly, a previous study described no effects on the growth kinetics of *E. coli* DH5 cells that have been encapsulated (Prakash and Chang, 1999).

The conditioning of mammalian cell culture media by the microencapsulated bacteria was conducted similar to the free bacteria; the microencapsulated bacteria were placed in the media for 1 h and any metabolites produced by the bacteria ‘conditioned’ the media. The downstream effects of the confined environment on the
bacteria-production of soluble factors were investigated by applying the microencapsulated bacteria CM to the L45hMGFP, L45pClneo-DsRed2, L23hMGFP and L23pClneo-DsRed2 cell lines. The proliferation of these cell lines was investigated as a general indicator of the immune cell response to the treatments.

It was observed that the total cell concentration of all the cell lines treated with the microencapsulated bacteria CM was lower than the free bacteria CM cell lines at all time points. It is possible that some bacteria metabolites have been ‘blocked’ by the capsular surface and that these metabolites stimulated the proliferation of the immune cells. Therefore, the lower proliferation of the immune cells in response to the conditioned media was due to the blocking of probiotic bacterial-produced soluble factors. In addition, the capsule composition (i.e. alginate, calcium and chitosan) may have affected the proliferation of the immune cells. This is further supported by the lack of proliferation (compared to the control) by L45pClneo-DsRed2 cells treated with microencapsulated *B. lactis* HN019 (DR10™) and *S. pyogenes*; the respective free *B. lactis* HN019 (DR10™) and *S. pyogenes* treatments exhibited different proliferation responses to the control. Similar to the free probiotic and pathogenic bacteria-conditioned media treatments, the immune cell proliferation responses to the microencapsulated probiotic bacteria-conditioned media did not produce consistent results to the microencapsulated pathogenic bacteria-conditioned media.

In some cases, comparisons between the free bacteria and microencapsulated bacteria CM treatments for the same cell line indicated differences in the immune cell proliferation response. For instance, the L45hMGFP cells exhibited higher cell
concentrations than the control at 4 h for both the free and microencapsulated *L. acidophilus* LAFTI® L10 CM; however, differences were observed at 8 and 24 h. The microencapsulated *S. pyogenes* CM treatment of L45hMGFP cells also produced different results comparative to the free *S. pyogenes* CM treatment. This observation was not limited to one immune cell type/fluorescent plasmid. Differences were observed between the free and microencapsulated *B. lactis* HN019 (DR10™) and *S. pyogenes* CM treatments of L45pClneo-DsRed2 cells. The proliferation of L23pClneo-DsRed2 cells also differed for the free and microencapsulated *L. acidophilus* LAFTI® L10 treatments. The dissimilarities in the immune cell proliferation response to the free and microencapsulated probiotics of the same strain are potentially a result of stress-related effects of the microencapsulation environment on the probiotic bacterial activity.

Probiotic stress-response mechanisms enable the bacteria to overcome stressful environments and ensure survival. However, the DNA, RNA and protein of probiotic cells have been reportedly affected by stressful environments (De Angelis and Gobbetti, 2004; Corcoran et al., 2008). Changes at the DNA, RNA and protein level of the probiotic bacteria may have affected the production and/or type of soluble factors. The different immune cell proliferation response to the microencapsulated bacteria CM (comparative to the free bacteria) potentially indicated some stress-related changes to the probiotics activities. However, further experimentation is required to confirm if stress-related changes by the probiotic bacteria to the microencapsulation environment have affected the production of soluble factors.
In some instances, the immune cell proliferation response to the microencapsulated probiotics CM was similar to the free probiotic CM treatments, for example, the free and microencapsulated *L. acidophilus* LAFTI® L10 CM treated L45pClneo-DsRed2 cells. The proliferation response of L23hMGFP cells was also similar for free and microencapsulated *B. lactis* HN019 (DR10™) treatments. Such effects were possibly a function of the immune cell type coupled with the probiotic strain.

A number of factors may influence the response of the porcine progenitor immune cells to the microencapsulated probiotic CM including the probiotic strain, immune cell type and the fluorescent protein. From these results, it has been shown that the microencapsulation of the probiotics has an effect on the downstream responses of the immune cells. This has been indicated by the differences in the proliferation of the immune cells comparative to those treated with free bacteria conditioned media. To date, no studies have described the effects of calcium alginate/chitosan microencapsulated *L. acidophilus* LAFTI® L10 and *B. lactis* HN019 (DR10™) strains on the responses of porcine progenitor immune cells. Nonetheless, previous research has described health benefiting effects from supplementation with microencapsulated probiotics. Microencapsulated *L. acidophilus* was reported to suppress the incidence of colon tumours in mice (Urbanska et al., 2009). Cholesterol reduction was reported for human subjects with hypercholesterol who consumed microencapsulated *L. acidophilus* L1 (Anderson and Gilliland, 1999). Similar observations were observed for hypercholesteric hamsters fed microencapsulated *L. fermentum* 11976 with a reduction in cholesterol levels (Bhathena et al., 2009). Although many studies have described the health benefits
conveyed by probiotics to the host, the health benefits associated with microencapsulated probiotic bacteria requires further investigating.

4.6 CONCLUSION

The free bacteria conditioned mammalian cell culture media influenced the proliferation of the porcine progenitor immune cells (L45hMGFP, L45pClneo-DsRed2, L23hMGFP and L23pClneo-DsRed2) in a strain-dependent manner. The *L. acidophilus* LAFTI® L10 treated immune cells exhibited the most consistent growth. Relationships between the immune cell type and fluorescent protein may have also determined the type of proliferation response to the bacteria CM treatments. The immune cellular proliferation responses to the probiotic bacteria-conditioned media exhibited different results to the pathogenic bacteria-conditioned media treated immune cellular proliferation responses.

The microencapsulated bacteria CM treated porcine progenitor immune cells exhibited lower total cell concentration and proliferation than the corresponding free bacteria CM treated immune cell lines. Potential effects on the activities of the microencapsulated bacteria from the confined environment may be the cause for these differences, in particular, influencing the production of soluble factors by the microencapsulated bacteria. This was determined from the subsequent downstream responses of the porcine progenitor immune cell lines. In some cases, similar trends to the free bacteria CM treated immune cell lines were observed for the corresponding immune cell lines treated with microencapsulated bacteria CM. Strain specific effects were also observed dependent on the relationships between immune cell type, fluorescent protein and bacteria strain.
5. CHAPTER FIVE

Intestinal Cell Co-cultures with Free and Microencapsulated Probiotic Bacteria - Effects on Immune Cell Proliferation

5.1 INTRODUCTION

Intestinal Cell Response to Probiotic Bacteria

Intestinal cells play a critical role in mediating interactions between the GI content and underlying immunological tissues. Cytokines, chemokines and molecules involved in immunological activities are released by intestinal cells in response to bacteria that subsequently influence immunological cells in the underlying tissues (Canny et al., 2006). Probiotic bacterial interactions with intestinal cells have been reported to convey a number of beneficial effects on intestinal cells and immunological activities. Probiotic bacterial physical contact and soluble factors have been shown to enhance intestinal cell protection against infections (Resta-Lenert and Barrett, 2003), improve intestinal barrier integrity (Blum and Schiffrin, 2003) and protect intestinal cells from cytokine-induced apoptosis (Yan and Polk, 2002) as well as effects from radiation (Ciorba et al., 2011).

The intestinal cell line used in this Chapter is a porcine fibroblast intestinal cell line, IPI-1, isolated from the ileum of an adult histocompatible miniature boar and transformed by the SV40 plasmid pSV3-neo (Kaeffer et al., 1993). This cell line has been used as an intestinal cell line model (Kaeffer et al., 1994). Fibroblast intestinal cells reportedly assist in the healing of intestinal epithelial injuries (Rieder et al., 2007) and interact with bacterial lipopolysaccharide via toll-like receptors (TLR)
(Burke et al., 2010). The application of microencapsulated probiotic bacteria to the IPI-1 cells is described in this Chapter. In addition the subsequent effects of conditioned mammalian cell culture media on the porcine progenitor immune cells were investigated. Microencapsulated probiotic bacterial interactions with intestinal cells and the subsequent effects on immunological cells have rarely been described.

The porcine fibroblast intestinal cells, IPI-1, were used as a barrier to determine the indirect effects of the soluble factors produced by the probiotic bacteria and microencapsulated probiotic bacteria on the proliferation of the progenitor immune cells. Similar to the previous Chapter (4), the response of the immune cells to the pathogenic (S. pyogenes) bacteria-conditioned media was used to determine if the probiotic bacteria-conditioned media conveyed similar effects on the immune cell responses.

5.2 AIMS & OBJECTIVES

The aim of this Chapter is:

- To investigate the effects of porcine fibroblast intestinal cells (IPI-1) with free- and microencapsulated bacteria (L. acidophilus LAFTI® L10, B. lactis HN019 (DR10™) and S. pyogenes) conditioned mammalian cell culture media (CM) on the proliferation of the porcine progenitor immune cell lines that have been transfected with the fluorescent proteins phMGFP and pClneo-DsRed2 (i.e. L45hMGFP, L45pClneo-DsRed2, L23hMGFP and L23pClneo-DsRed2 cell lines).
The objectives of this Chapter are:

- To produce mammalian cell culture media (without antibiotics known as cDMEM-AF) conditioned by porcine fibroblast intestinal epithelial cells (IPI-1) with free- microencapsulated bacteria (*L. acidophilus* LAFTI® L10, *B. lactis* HN019 (DR10™) and *S. pyogenes*). This media will be applied to the L45hMGFP, L45pCIneo-DsRed2, L23hMGFP and L23pCIneo-DsRed2 cell lines. The control is the cDMEM-AF media conditioned with IPI-1 cells without bacteria.

- To compare the total cell concentration of the respective controls with the cell concentration of IPI-1/free and microencapsulated bacteria (*L. acidophilus* LAFTI® L10, *B. lactis* HN019 (DR10™) and *S. pyogenes*) conditioned mammalian cell culture media treated L45hMGFP, L45pCIneo-DsRed2, L23hMGFP and L23pCIneo-DsRed2 cells. The statistical significance of each treatment compared to the respective control will be determined for the 4, 8 and 24 h cell counts.

### 5.3 MATERIALS AND METHODS

#### 5.3.1 Treatment of Immune Cells with Mammalian Cell Culture Media Conditioned with Intestinal Cells/Free Bacteria and Intestinal Cells/Microencapsulated Bacteria Co-Cultures

##### 5.3.1.1 Intestinal Cells

The porcine fibroblast intestinal cell line, IPI-1, was sourced from the University of Western Sydney’s cell culture collection. This cell line is an adherent, immortalised, fibroblast derived from the ileum tissue of an adult histocompatible
miniature boar male (European Collection of Animal Cell Cultures No. 93100621). The cells were maintained in T25 flasks (Corning® 25cm$^2$ Rectangular Canted Neck Cell Culture Flask with Vent Cap; 430639, Corning, Australia) in complete DMEM supplemented with FBS (10 %) and bovine pancreas insulin (0.024 IU mL$^{-1}$) (lyophilized, I-1882, Sigma, Castle Hill, Australia). The cells were passaged at 70 to 80 % confluency by decanting the used media and washing the adhered cells with phosphate buffered saline (PBS, pH 7.4) solution (1x, 2 mL) (10010-023, Gibco; Invitrogen, Mulgrave Australia) for approximately 5 min. The PBS solution was removed and the cells were treated with trypsin-EDTA (10X, 0.5 %, 1.5 mL) (15400-054, Invitrogen, Mulgrave, Australia) for 5 min; the flask was tapped to detach any remaining adhered cells. Incomplete DMEM (6 mL) was added to wash the cells/trypsin solution and the entire cell suspension was placed into a 15 mL Falcon™ polypropylene conical tube. The cell suspension was centrifuged with the Universal 32 R centrifuge at 320 g for 5 min. The supernatant was discarded and the pellet resuspended in complete DMEM supplemented with FBS (10 %) and bovine pancreas insulin (0.024 IU mL$^{-1}$).

5.3.1.2 Conditioning of Mammalian Cell Culture Media by IPI-1 Cells and Bacteria Co-Cultures

Soluble factors produced by the IPI-1/bacteria co-cultures were collected in a 24-well plate using the 0.4 µm ThinCert™ tissue culture inserts (662 641, Greiner Bio-one; Interpath Services P/L, West Heidelberg, Australia), as shown in Figure 5.1. The IPI-1 cells were passaged 48 h prior to seeding on the transwell inserts; the cells were un-adhered as outlined in Section 5.3.3.1 and enumerated as outlined in Chapter 3, Section 3.3.1.4. The cells were seeded at a concentration of 5.0 x 10$^5$
cells mL$^{-1}$ on the transwell inserts using a 0.5 mL final volume; complete DMEM (1.0 mL) supplemented with FBS (10%) and insulin (0.024 IU mL$^{-1}$) was placed into the bottom compartment. The cells were incubated at 37 ºC in standard cell culture conditions for 48 h to allow adherence. Following incubation, the cell culture media was removed from both the transwell insert and bottom compartment in preparation for treatments with the bacteria cells suspended in cDMEM-AF.

The *L. acidophilus* LAFTI® L10, *B. lactis* HN019 (DR10™) and *S. pyogenes* cultures were grown in their respective media and conditions for 24 h as outlined in Chapter 2, Section 2.3.1.1. The bacteria cell suspensions were enumerated as outlined in Chapter 2, Section 2.3.1.2, and centrifuged at 3864 g for 10 min (37 ºC) using the Universal 32 R centrifuge. The supernatant was removed. The pellets were washed with cDMEM-AF (1 mL) and re-centrifuged. The supernatant was removed and each pellet was resuspended in cDMEM-AF making a final concentration of 1.0 x 10$^9$ CFU mL$^{-1}$. A 0.5 mL aliquot of bacteria cell suspension was added to each transwell insert; 6 wells were prepared for each bacterium. Control wells were prepared by adding cDMEM-AF (0.5 mL) to the upper compartment containing the adhered IPI-1 cells; cDMEM-AF (1 mL) was added to the bottom compartment of the well. The IPI-1 and bacteria suspensions were incubated at 37 ºC in 5 % CO$_2$ for 1 h. The conditioned media from the bottom compartment of each bacteria and control wells were removed following the 1 h incubation and placed into a respective 50 mL polypropylene tubes; fresh cDMEM-AF (1 mL) was added to the bottom well, this process was repeated 4 times therefore allowing interactions between the IPI-1 cells and bacteria for a total of 4 h. The process of preparing the transwell inserts, preparing the bacteria and conditioning the
media at 1 h intervals for 4 h was repeated twice. The conditioned media was centrifuged at 3864 g for 10 min (37 °C) using the Universal 32 R centrifuge. The conditioned media supernatant was passed through a 0.20 µm sterile, non-pyrogenic filter and placed into 9 mL aliquots in a 15 mL Falcon™ polypropylene conical tube. The IPI-1/bacteria-conditioned media was stored at -20 °C.

Figure 5.1. Conditioning of mammalian cell culture media using a tranwell insert. The intestinal epithelial cells (IPI-1) are allowed to adhere and grown on the insert microporous membrane for 48 h. The bacteria are added to the top compartment once the IPI-1 cells have adhered and grown a layer of cells. The soluble factors produced by the bacteria and IPI-1 cells diffuse to the bottom compartment conditioning the media.

5.3.1.3 Treatment of Progenitor Immune Cells with IPI-1 cells and Bacteria Co-culture Conditioned Mammalian Cell Culture Media

The progenitor immune cell lines, L45hMGFP, L45pClneo-DsRed2, L23hMGFP and L23pClneo-DsRed2 (developed as described in Chapter 3), were used to evaluate the immune cellular proliferation in response to the intestinal cell (IPI-1)/bacterial-conditioned cDMEM-AF. The progenitor immune cell suspensions were prepared as outlined in Chapter 4, Section 4.3.1.2 and the immune cells were treated with IPI-1-conditioned cDMEM-AF (control), IPI-1 and \textit{L. acidophilus}-
conditioned cDMEM-AF, IPI-1 and B. lactis-conditioned cDMEM-AF or IPI-1 and S. pyogenes-conditioned cDMEM-AF. Cell counts were conducted as outlined in Chapter 4, Section 4.3.1.2. Treatments of each progenitor immune cell lines with intestinal cell/bacteria-conditioned cDMEM-AF were repeated in triplicate.

5.3.1.4 Conditioning of Mammalian Cell Culture Media by IPI-1 Cells and Microencapsulated Bacteria Co-Cultures

Soluble factors produced by the IPI-1/microencapsulated bacteria co-cultures were collected in a 6-well plate using the 0.4 µm ThinCert™ tissue culture inserts.

Preparation of IPI-1 Cells on Inserts

The IPI-1 cells were passaged 48 h prior to seeding on the transwell inserts; the cells were un-adhered as outlined in Section 5.3.1.1 and enumerated as outlined in Chapter 3, Section 3.3.1.4. The cells were seeded at a concentration of 5.0 x 10^5 cells mL\(^{-1}\) on the transwell inserts using a 1.5 mL final volume; complete DMEM (3.0 mL) supplemented with FBS (10 %) and insulin (0.024 IU mL\(^{-1}\)) was placed into the bottom compartment. The cells were incubated at 37 ºC in standard cell culture conditions for 48 h to allow adherence. Following incubation, the cell culture media was removed from both the transwell insert and bottom compartment in preparation for treatments with the bacteria cells suspended in cDMEM-AF.

Microencapsulation of Bacteria Cultures

The bacteria cultures (L. acidophilus LAFTI® L10, B. lactis HN019 (DR10™) and S. pyogenes) were prepared and microencapsulated as outlined in Chapter 4, Section 4.3.1.3.
Conditioning of Mammalian Cell Culture Media by IPI-1 and Microencapsulated Bacteria

The complete media in the upper and lower compartments of the transwell inserts (with adhered IPI-1 cells) were removed. The upper compartment was resuspended in cDMEM-AF (1.5 mL) and approx. 20 capsules and the lower compartment was filled with cDMEM-AF (3.0 mL). The IPI-1/microencapsulated bacteria were allowed to condition the media for 1 h. Following the 1 h incubation, the conditioned media in the lower compartment were removed and fresh cDMEM-AF (3.0 mL) was added; this was performed for 4 h. The control well was prepared with empty capsules and adhered IPI-1 cells. The process of preparing the transwell inserts, preparing the bacteria and conditioning the media at 1 h intervals for 4 h was repeated twice. The conditioned media were stored in a 50 mL polypropylene tube. The conditioned media were centrifuged at 3864 g for 10 min (37 °C) using the Universal 32 R centrifuge. The conditioned media supernatant was passed through a 0.20 µm sterile, non-pyrogenic filter and placed into 9 mL aliquots in a 15 mL Falcon™ polypropylene conical tube. The IPI-1/microencapsulated bacteria-conditioned media was stored at -20 °C.

5.3.1.5 Treatment of Progenitor Immune Cells with IPI-1/Microencapsulated Bacteria Co-culture Conditioned Mammalian Cell Culture Media

The progenitor immune cell lines, L45hMGFP, L45pCIneo-DsRed2, L23hMGFP and L23pCIneo-DsRed2 (developed as described in Chapter 3), were used to evaluate the immune cellular proliferation in response to the intestinal cell
(IPI-1)/microencapsulated bacterial-conditioned cDMEM-AF. The progenitor immune cell suspensions were passaged and treated using the IPI-1/microencapsulated bacteria conditioned cDMEM-AF media as outlined in Section 5.3.1.3.

5.3.2 Statistical Analysis

The mean and standard error of the mean (SEM) of the total cell counts from the triple experiments were calculated using Microsoft Excel 2003. Comparisons between the control and treatment means were conducted using the paired-samples T test with the SPSS Statistics 18 Program (2010).

5.4 RESULTS

5.4.1 Treatment of Immune Cells with Mammalian Cell Culture Media Conditioned by Intestinal Cells (IPI-1) Co-Cultured with Free- and Microencapsulated Bacteria

5.4.1.1 Treated L45hMGFP progenitor immune cells

Intestinal Cells/Free Bacteria Soluble Factors Effects on Immune Cells

The L45hMGFP cell concentration following treatment with porcine fibroblast intestinal cells (IPI-1) and free bacteria co-culture CM is shown in Figure 5.2a. The IPI-1/L. acidophilus LAFTI® L10 CM treated cells exhibited similar concentrations to the control. The IPI-1/B. lactis HN019 (DR10™) CM treated L45hMGFP cell concentration was lower than the control at 4 and 8 h (the 8 h treatment was significantly different to the control). The IPI-1/S. pyogenes CM
treated L45hMGFP cells exhibited a lower cell concentration than the control at all time points (4 h treatment was significantly different to the control).

**Intestinal Cells/Microencapsulated Bacteria Soluble Factors Effects on Immune Cells**

The total cell concentration of L45hMGFP cells treated with IPI-1/microencapsulated bacteria CM is shown in Figure 5.2b. At 24 h, the IPI-1/microencapsulated *L. acidophilus* LAFTI® L10 CM treated cell concentration was significantly higher than the control; the 4 and 8 h treatments were lower with the 8 h treatment significantly different to the control. The IPI-1/microencapsulated *B. lactis* HN019 (DR10™) and IPI-1/microencapsulated *S. pyogenes* CM treated L45hMGFP cells concentrations were significantly different to the control at 4 and 8 h exhibiting a lower cell concentration than the control at all time points. The 24 h IPI-1/microencapsulated *S. pyogenes* CM treated cells were significantly lower than the control.

**Comparison between IPI-1/Free and Microencapsulated Bacteria Treatments**

The IPI-1/free and microencapsulated *L. acidophilus* LAFTI® L10 CM treated L45hMGFP total cell concentration exhibited similar results at 4 and 8 h with a lower concentration than the control. In addition, the total cell concentrations of IPI-1/free and microencapsulated *B. lactis* HN019 (DR10™) and IPI-1/free and microencapsulated *S. pyogenes* CM L45hMGFP cells was lower than the control at all time points.
Figure 5.2. Effects on L45hMGFP cell proliferation at 4, 8 and 24 h from treatments with mammalian cell culture media conditioned with intestinal cells (IPI-1) and a) free \textit{L. acidophilus} LAFTI® L10 (L10 treatment), \textit{B. lactis} HN019 (DR10™ treatment) and \textit{S. pyogenes}, and b) microencapsulated \textit{L. acidophilus} LAFTI® L10 (L10 treatment), \textit{B. lactis} HN019 (DR10™ treatment) and \textit{S. pyogenes}. Data represents the mean ± SEM from triplicate experiments where n = 9. Significant difference was determined by the paired sample T-test (SPSS Statistics 18) comparing the means of the controls and treatments (**p < 0.01; *** p < 0.001).
5.4.1.2 Treated L45pCI neo-DsRed2 progenitor immune cells

Intestinal Cells/Free Bacteria Soluble Factors Effects on Immune Cells

The IPI-1/L. acidophilus LAFTI® L10 CM treated cell concentration was higher than the control at 4 and 8 h but lower than the control at 24 h; the 8 and 24 h results were significantly different to the control (Fig. 5.3a). The IPI-1/B. lactis HN019 (DR10™) CM treated cells exhibited a higher cell concentration than the control at 4 h with lower concentrations at 8 and 24 h (both significantly different to the control). The IPI-1/S. pyogenes CM treated L45pCIneo-DsRed2 cell concentration was higher than the control at all time points (8 h cell concentration was significantly different to the control).

Intestinal Cells/Microencapsulated Bacteria Soluble Factors Effects on Immune Cells

The IPI-1/microencapsulated L. acidophilus LAFTI® L10 CM treated L45pCIneo-DsRed2 cell concentration was higher than the control at 4 and 8 h with a similar cell concentration at 24 h (Fig. 5.3b). The IPI-1/microencapsulated B. lactis HN019 (DR10™) and IPI-1/microencapsulated S. pyogenes CM treated L45pCIneo-DsRed2 cell concentration was similar to the control at all time points.

Comparison between IPI-1/Free and Microencapsulated Bacteria Treatments

Similar trends were observed for the IPI-1/free and microencapsulated L. acidophilus LAFTI® L10 CM treated L45pCIneo-DsRed2 cells at 4 and 8 h (higher than the control). The IPI-1/free and microencapsulated B. lactis HN019 (DR10™) treated L45pCIneo-DsRed2 cell concentrations were similar at 4 h but differed at 8 and 24 h with lower cell concentrations. The IPI-1/free and microencapsulated S. pyogenes treated cells exhibited similar trends at 4 and 24 h.
Figure 5.3. Effects on L45pCIneo-DsRed2 cell proliferation at 4, 8 and 24 h from treatments with mammalian cell culture media conditioned with intestinal cells (IPI-1) and a) free *L. acidophilus* LAFTI® L10 (L10 treatment), *B. lactis* HN019 (DR10™ treatment) and *S. pyogenes*, and b) microencapsulated *L. acidophilus* LAFTI® L10 (L10 treatment), *B. lactis* HN019 (DR10™ treatment) and *S. pyogenes*. Data represents the mean ± SEM from triplicate experiments where n = 9. Significant difference was determined by the paired sample T-test (SPSS Statistics 18) comparing the means of the controls and treatments (* p < 0.05; **p < 0.01; *** p < 0.001).
5.4.1.3 Treated L23hMGFP progenitor immune cells

Intestinal Cells/Free Bacteria Soluble Factors Effects on Immune Cells

The IPI-1/bacteria CM treated L23hMGFP cell concentration is shown in Figure 5.4a. The control and all the IPI-1/bacteria CM treated L23hMGFP cell concentrations were approximately the same (approx. $4.9 \times 10^5$ cells mL$^{-1}$) at 4 h, however at 8 and 24 h, the control cell concentration exceeded the three IPI-1/bacteria CM treated cell concentrations. The IPI-1/L. acidophilus LAFTI® L10 exhibited similar to total cell concentrations as IPI-1/B. lactis HN019 (DR10™) at 8 h and IPI-1/S. pyogenes 24 h.

Intestinal Cells/Microencapsulated Bacteria Soluble Factors Effects on Immune Cells

The IPI-1/microencapsulated bacteria CM treated L23hMGFP cell concentrations are shown in Figure 5.4b. At 4 h, the control cell concentration was higher than the IPI-1/microencapsulated bacteria CM treated cell concentrations (all significantly different to the control). The IPI-1/microencapsulated L. acidophilus LAFTI® L10 CM treatment was significantly lower than the control at 24 h. The IPI-1/microencapsulated B. lactis HN019 (DR10™) CM treated L23hMGFP cell concentration was significantly higher at 8 and 24 h. The IPI-1/microencapsulated S. pyogenes CM treatments were significantly lower than the control at all time points.

Comparison between IPI-1/Free and Microencapsulated Bacteria Treatments

The IPI-1/free and microencapsulated bacteria CM treatments of L23hMGFP cells exhibited different responses at all time points. The IPI-1/microencapsulated B. lactis HN019 (DR10™) treated cells were the only treatments to produce a higher cell concentration than the control (at 8 and 24 h).
Figure 5.4. Effects on L23hMGFP cell proliferation at 4, 8 and 24 h from treatments with mammalian cell culture media conditioned with intestinal cells (IPI-1) and a) free *L. acidophilus* LAFTI® L10 (L10 treatment), *B. lactis* HN019 (DR10™ treatment) and *S. pyogenes*, and b) microencapsulated *L. acidophilus* LAFTI® L10 (L10 treatment), *B. lactis* HN019 (DR10™ treatment) and *S. pyogenes*. Data represents the mean ± SEM from triplicate experiments where n = 9. Significant difference was determined by the paired sample T-test (SPSS Statistics 18) comparing the means of the controls and treatments (* p < 0.05; **p < 0.01; *** p < 0.001).
5.4.1.4 Treated L23pCl neo-DsRed2 progenitor immune cells

Intestinal Cells/Free Bacteria Soluble Factors Effects on Immune Cells

As shown in Figure 5.5a, the IPI-1/L. acidophilus LAFTI® L10 CM treated L23pClneo-DsRed2 cell concentration was approximately the same as the control at 4 h and 8 h, and significantly lower than the control at 24 h. The IPI-1/B. lactis HN019 (DR10™) CM treated cell concentration was the highest at 4 and 8 h but lower than the control at 24 h. The IPI-1/S. pyogenes CM treated L23pClneo-DsRed2 cell concentration was lower than the control at all time points; the 24 h result was significantly different to the control.

Intestinal Cells/Microencapsulated Bacteria Soluble Factors Effects on Immune Cells

The IPI-1/microencapsulated L. acidophilus LAFTI® L10 CM treated cell concentration was higher than the control at all time points; the 8 h treatment was significantly different to the control (Fig. 5.5b). The IPI-1/microencapsulated B. lactis HN019 (DR10™) CM treated cell concentration was significantly lower than the control at all time points. The IPI-1/microencapsulated S. pyogenes CM treated cell concentration was higher than the control at all time points.

Comparison between IPI-1/Free and Microencapsulated Bacteria Treatments

The opposite effect was observed for the IPI-1/free and microencapsulated bacteria treatments. The IPI-1/free and microencapsulated L. acidophilus LAFTI® L10 exhibited a different result at 24 h. The IPI-1/free and microencapsulated B. lactis HN019 (DR10™) exhibited different results at 4 and 8 h. The IPI-1/free and microencapsulated S. pyogenes treated cells exhibited different results at all time points.
Figure 5.5. Effects on L23pCIneo-DsRed2 cell proliferation at 4, 8 and 24 h from treatments with mammalian cell culture media conditioned with intestinal cells (IPI-1) and a) free *L. acidophilus* LAFTI® L10 (L10 treatment), *B. lactis* HN019 (DR10™ treatment) and *S. pyogenes*, and b) microencapsulated *L. acidophilus* LAFTI® L10 (L10 treatment), *B. lactis* HN019 (DR10™ treatment) and *S. pyogenes*. Data represents the mean ± SEM from triplicate experiments where n = 9. Significant difference was determined by the paired sample T-test (SPSS Statistics 18) comparing the means of the controls and treatments (* p < 0.05; ** p < 0.01; *** p < 0.001).
5.5 DISCUSSION

5.5.1 Treatment of Immune Cells with Mammalian Cell Culture Media Conditioned by Intestinal Cells (IPI-1) Co-Cultured with Free- and Microencapsulated Bacteria

5.5.1.1 Intestinal Cells/Free Bacteria CM Treatment of Porcine Progenitor Immune Cells

As outlined in the previous Chapter, immune cell activity is modulated in response to probiotic bacteria and metabolite exposure. Intestinal cells also have a role in modulating immunological responses to probiotics by performing a number of regulatory activities that influence underlying immune cells in the GI tract. For this study, fibroblast intestinal cells from a porcine ileum (IPI-1) were used as the intestinal cell model to evaluate the effects of intestinal/bacteria co-culture-produced soluble factors on the response of the T- and B-cell lines. It should be noted that the T- and B-cells were derived from the same pig as the IPI-1 cells.

The IPI-1 porcine fibroblast intestinal cell line has been used as a small intestine model in previous studies (Kaeffer et al., 1994). Fibroblast intestinal cells reportedly interact with bacterial lipopolysaccharide via TLRs resulting in the activation of the fibroblast intestinal cellular activity (Burke et al., 2010) and assist in intestinal epithelial cell wound healing (Rieder et al., 2007). Fibroblast intestinal cells release a number of cytokines and chemokines to promote the healing of damaged intestinal cells (Rieder et al., 2007); therefore chemical signalling by the fibroblast intestinal cells would influence the underlying intestinal epithelial cells...
and immunological tissues in the GI tract. To date, no studies have described the effects of probiotics on fibroblast intestinal cells.

The results presented in Section 5.4.1 indicate that the porcine fibroblast intestinal cells (IPI-1) influenced the response of the porcine progenitor immune cell proliferation. This is demonstrated by comparing the responses of the immune cells to the IPI-1/bacteria CM (Section 5.4.1) and the free bacteria CM treatments (Chapter 4, Section 4.4.1). For instance, the IPI-1/L. acidophilus LAFTI® L10 and IPI-1/B. lactis HN019 (DR10™) CM treated L45hMGFP, L45pCIneo-DsRed2 (at 24 h) and L23hMGFP cell proliferation results exhibited opposite results to the free L. acidophilus LAFTI® L10 and B. lactis HN019 (DR10™) CM treatments. However, the proliferation response of the L23pCIneo-DsRed2 cells did not follow this pattern. The IPI-1/L. acidophilus LAFTI® L10 and IPI-1/B. lactis HN019 (DR10™) (Fig. 5.5a) CM treated L23pCIneo-DsRed2 cells exhibited similar proliferation responses to the L. acidophilus LAFTI® L10 and B. lactis HN019 (DR10™) CM (Fig. 4.4a) treatments. Therefore similar to the bacteria CM treated immune cells, the immune cell proliferation response to the IPI-1/bacteria CM may be influenced by the immune cell type coupled with the fluorescent protein (i.e. phMGFP and pCIneo-DsRed2).

The response of the immune cells to the IPI-1/S. pyogenes did not follow a similar proliferation pattern to the IPI-1/probiotic bacteria CM treatments. This indicates that the intestinal cells were responding differently to the pathogenic bacteria comparative to the probiotic bacteria with the IPI-1 potentially identifying S. pyogenes as a pathogen and responding accordingly. Such differences indicate
that the porcine fibroblast intestinal (IPI-1) cells interact with the bacteria via physical contact in addition to responding to the bacterial-produced soluble factors. The physical interactions between the bacteria and porcine fibroblast intestinal cells are possibly via TLRs. Intestinal epithelial cells interact with probiotics by TLRs resulting in the activation and subsequent secretion of chemical signals (e.g. cytokines) that primes the underlying immunological cells (Walker, 2008). It is a possibility that the physical interactions and influence of the bacteria soluble factors on the fibroblast intestinal cells resulted in the production of cytokines by the fibroblast intestinal cells that subsequently influenced the L45hMGFP, L45pClneo-DsRed2, L23hMGFP and L23pClneo-DsRed2 cells upon treatment with the IPI-1/bacteria CM. Many studies have investigated the effects of probiotic bacteria-produced soluble factors on intestinal epithelial cells. For instance, two proteins isolated from *Lactobacillus GG* culture supernatant were reported to protect murine intestinal epithelial cells that had cytokine-induced apoptosis *in vitro* (Tao et al., 2006). Conditioned media by *B. infantis* reportedly enhanced the intestinal epithelial barrier function in mice with colitis by normalizing gut permeability (Ewaschuk et al., 2008). Direct contact between probiotics and intestinal cells has been reported to directly affect epithelial barrier function in mice by protecting against infections (Madsen et al., 2001). It is possible that the *L. acidophilus* LAFTI® L10, *B. lactis* HN019 (DR10™) and *S. pyogenes* have influenced the activity and integrity of the fibroblast intestinal cells.

Intestinal epithelial cells also interact with the underlying immunological tissues and cells comprising the Peyer’s patches. It has been proposed that intestinal epithelial cells generate the first line of response to probiotic bacteria and
subsequently convey signals to the underlying immunological cells in the lamina propria (Vinderola et al., 2005; Oswald, 2006). A study using an *in vitro* co-culture model employing the human intestinal cell lines CaCO-2 and HT-29 with non-pathogenic lactobacilli and CD4+ T cells, CD8+ T cells, CD19+ B cells and CD14 monocytes described monocyte development being influenced by the intestinal epithelial cells; the authors stated that the gut epithelium contributes to mucosal immunological homeostasis through the regulation of interactions with commensal bacteria (Haller et al., 2002). Therefore, the IPI-1/probiotic conditioned media potentially assisted in the development of the immune cells. However, based on the low proliferation of most cell lines (comparative to the control), it is possible that regulatory signals produced by the IPI-1 cells influenced the immune cell proliferation response to the IPI-1/bacteria CM.

### 5.5.1.2 Intestinal Cells/ Microencapsualted Bacteria CM Treatment of Porcine Progenitor Immune Cells

To date, only one study has described the effects of microencapsulated probiotics on immunological cells. The effects of microencapsulated probiotic yogurt on intestinal inflammation of C57BL/6J-Apc^{Min}/J mice were evaluated by screening the levels of pro-inflammatory cytokines (IL-1β, IL-6, L-12, TNF-α and IFN-γ) produced by small intestinal cells (Urbanska et al., 2010). The levels of the pro-inflammatory cytokines, IL-1β, IL-6, TNF-α and IFN-γ in the small intestine of the mice receiving the probiotic yogurt were lower than the control animals; however, the small intestinal and serum levels of IL-12 were higher in the treated mice comparated to the controls (Urbanska et al., 2010).
Similar to the IPI-1/free bacterial CM, microencapsulated bacteria- and free bacteria-CM treated immune cells, trends in the proliferation response of the immune cells to the IPI-1/microencapsulated bacteria were observed. The relationship between the bacteria strain, cell type (T- or B-cell) and fluorescent protein (phMGFP or pCIneo-DsRed2) potentially exist for these treatment conditions. As shown for the IPI-1/bacteria conditioned media treated immune cells, the response of the fibroblast intestinal cells (IPI-1) to the microencapsulated bacteria may have influenced the treated immune cell responses in addition to the microencapsulated environment.

It was observed that the intestinal cells affected the subsequent downstream proliferation response of the immune cells. For instance, the IPI-1/microencapsulated *L. acidophilus* LAFTI® L10 and IPI-1/microencapsulated *B. lactis* HN019 (DR10™) CM treated L45hMGFP cells exhibited opposite results to the microencapsulated *L. acidophilus* LAFTI® L10 and microencapsulated *B. lactis* HN019 (DR10™) CM treatments. However, the fluorescent protein was also observed to influence the response of the immune cells to the IPI-1/microencapsulated probiotic CM. For example, the IPI-1/microencapsulated *L. acidophilus* LAFTI® L10 and IPI-1/*B. lactis* HN019 (DR10™) CM treated L45pClneo-DsRed2 cells exhibited a similar proliferation pattern to the microencapsulated *L. acidophilus* LAFTI® L10 and microencapsulated *B. lactis* HN019 (DR10™) CM treated L45pClneo-DsRed2 cells. The pathogenic bacteria strain produced differing results dependent on the immune cell type. The IPI-1/microencapsulated *S. pyogenes* CM compared to the microencapsulated *S. pyogenes* CM treated L23hMGFP and L23pClneo-DsRed2 cells proliferation rate
exhibited similar results whereas the proliferation rates of the L45hMGFP and L45pClneo-DsRed2 cell lines exhibited different results. Therefore in addition to the effects of the microencapsulated environment on the probiotic bacteria, the immune cell type, fluorescence protein, intestinal cells and bacteria strain also influence the subsequent downstream responses of the immune cells.

5.6 CONCLUSION

The porcine fibroblast intestinal cells (IPI-1) were observed to affect the proliferation response of the porcine progenitor immune cells to the free and microencapsulated bacteria CM treatments. In terms of the proliferation response by the immune cells, regulatory responses were observed to the IPI-1/free and microencapsulated bacteria co-culture CM; different responses were observed for the IPI-1/S.pyogenes CM comparative to the probiotic bacteria co-culture CM. Similarly to the free and microencapsulated bacteria CM treatments, the proliferation response of the immune cells exhibited relationships between the immune cell type, fluorescent protein, if the bacteria were free or microencapsulated, presence of the fibroblast intestinal cells and bacteria strain.

To gain a further understanding of the immune cell response to the free, microencapsulated and IPI-1 co-cultures with free and microencapsulated bacteria, the gene expression of various cytokines, chemokines and CD markers were investigated (Chapter 6).
6. CHAPTER SIX

Free and Microencapsulated Probiotic Bacteria Effects on Cytokine & CD Marker Gene Expression by Immune Cells

6.1 INTRODUCTION

In the previous Chapters, the effects of bacteria-produced soluble factors on the porcine progenitor immune cell proliferation responses were investigated. The porcine progenitor immune cells were treated with mammalian cell culture media conditioned (CM) by free bacteria, microencapsulated bacteria, and fibroblast intestinal cell co-cultures with free and microencapsulated bacteria. It was established that the immune cell responses were influenced by relationships between immune cell types, fluorescent protein, bacteria strain, presence of intestinal cells and if the bacteria were free or microencapsulated. This Chapter explores the type of immune cell response to the different CM treatments by investigating the immune cell gene expression of cytokines and cluster of differentiation (CD) markers.

Cytokine Responses to Probiotics

The production of cytokines in response to microencapsulated probiotics has rarely been described. Further, only one study to date has outlined the production of cytokines by intestinal cells in response to microencapsulated probiotics (Urbanska et al., 2010). Pro-inflammatory, anti-inflammatory and regulatory cytokine production by a number of immune cells from the innate and adaptive immune system in response to probiotic bacteria have been described (Christensen et al., 2002; Di Giacinto et al., 2005; Smits et al., 2005; Torii et al., 2007). In addition, the production of pro- and anti-inflammatory cytokines by intestinal cells in response to
probiotic bacteria has been reported as a strain-dependent event (Haller et al., 2000). However, to determine the effects of the confined microencapsulated environment on probiotic bacterial activities, the cytokine response of immunological cells may be used as a downstream indicator. Comparative analysis of free- and microencapsulated probiotic bacterial produced soluble factors may provide information on how this environment has affected the activities of the probiotic bacteria.

As outlined previously, the proliferation response of the porcine progenitor immune cells were investigated in Chapter 4 to determine if any patterns existed in response to the free- and microencapsulated probiotic bacterial produced soluble factors. Strain, immune cell type (T- or B-cell), the fluorescent protein type (phMGFP and pCIneoDsRed2) and the bacterial treatment type (free or microencapsulated) were determined to influence the proliferation of the immune cells. In this Chapter, the production of cytokines and CD markers by the immune cells in response to microencapsulated bacteria and intestinal cells/microencapsulated bacteria co-cultured conditioned media was compared to the responses of the immune cells treated with free bacteria- and intestinal cells/free bacteria co-cultured conditioned media. These results were used to determine if any differences were observed for direct interactions by microencapsulated bacterial produced soluble factors and indirect interactions via the intestinal cells/microencapsulated co-culture-produced soluble factors on the immune cell production of cytokines and CD markers. Such responses can be used to determine the type of immunological response to the bacteria soluble factors.
6.2 AIMS & OBJECTIVES

The aims of this Chapter are:

- To identify the gene expression of various cytokines and CD markers by the L45hMGFP, L45pClneo-DsRed2, L23hMGFP and L23pClneo-DsRed2 cell lines in response to treatments with conditioned mammalian cell culture media. The media has been conditioned with free- and microencapsulated bacteria.

- To identify the gene expression of various cytokines and CD markers by the L45hMGFP, L45pClneo-DsRed2, L23hMGFP and L23pClneo-DsRed2 cell lines in response to treatments with conditioned mammalian cell culture media. The media has been conditioned with porcine fibroblast intestinal cells (IPI-1)/bacteria and IPI-1/microencapsulated bacteria.

The objectives of this Chapter are:

- To perform real-time polymerase chain reaction (RT-PCR) to determine the gene expression of various cytokines and cluster of differentiation (CD) markers by the L45hMGFP, L45pClneo-DsRed2, L23hMGFP and L23pClneo-DsRed2 cell lines in response to free- and microencapsulated bacteria conditioned media treatments.

- To perform real-time polymerase chain reaction (RT-PCR) to determine the gene expression of various cytokines and cluster of differentiation (CD) markers by the L45hMGFP, L45pClneo-DsRed2, L23hMGFP and L23pClneo-DsRed2 cell lines in response to IPI-1/free- and IPI-1/microencapsulated bacteria conditioned media treatments.
6.3 MATERIALS & METHODS

6.3.1 Preparation of Porcine Progenitor Immune Cells Treated with Free- and Microencapsulated Bacteria-Conditioned Media

6.3.1.1 Treatment of Porcine Progenitor Immune Cells with Free Bacteria Conditioned Media

The cells (L45hMGFP, L45pCIneo-DsRed2, L23hMGFP and L23pCIneo-DsRed2) were prepared for treatments with the bacterial-conditioned cDMEM-AF media (CM) as outlined in Chapter 4, Section 4.3.1.2 with the modification of preparing one well in a 6-well plate for each treatment with approximately $1.5 \times 10^6$ cells mL$^{-1}$ in bacterial-conditioned cDMEM-AF media (3 mL). The cells were incubated in standard cell culture conditions for 24 h.

6.3.1.2 Treatment of Porcine Progenitor Immune Cells with Microencapsulated Bacteria Conditioned Media

The cells (L45hMGFP, L45pCIneo-DsRed2, L23hMGFP and L23pCIneo-DsRed2) were prepared for treatments with the microencapsulated bacterial-conditioned cDMEM-AF media (CM) as outlined in Chapter 4, Section 4.3.1.4 with the modification of preparing one well in a 6-well plate for each treatment with approximately $1.5 \times 10^6$ cells mL$^{-1}$ in bacterial-conditioned cDMEM-AF media (3 mL). The cells were incubated in standard cell culture conditions for 24 h.
6.3.2 Preparation of Porcine Progenitor Immune Cells Treated with Conditioned Media by Intestinal Cells with Free- and Microencapsulated Bacteria

6.3.2.1 Treatment of Porcine Progenitor Immune Cells with Intestinal Cells/ Free Bacteria Conditioned Media

The cells (L45hMGFP, L45pCIneo-DsRed2, L23hMGFP and L23pCIneo-DsRed2) were prepared for treatments with the IPI-1/bacterial-conditioned cDMEM-AF media (CM) as outlined in Chapter 5, Section 5.3.1.2.

6.3.2.2 Treatment of Porcine Progenitor Immune Cells with Intestinal Cells/ Microencapsulated Bacteria Conditioned Media

The cells (L45hMGFP, L45pCIneo-DsRed2, L23hMGFP and L23pCIneo-DsRed2) were prepared for treatments with the bacterial-conditioned cDMEM-AF media (CM) as outlined in Chapter 5, Section 5.3.1.4 with the modification of preparing one well in a 6-well plate for each treatment with approximately $1.5 \times 10^6$ cells mL$^{-1}$ in 3 mL of bacterial-conditioned cDMEM-AF media. The cells were incubated in standard cell culture conditions for 24 h.

6.3.3 Gene Expression of Conditioned Mammalian Cell Culture Media Treated Porcine Progenitor Immune Cells

6.3.3.1 RNA Extraction

Extraction of RNA was performed using TRIzol® Reagent (15596-026, Invitrogen, Mulgrave, Australia) as per the manufacturer’s instructions. At 24 h, the cells were enumerated and centrifuged at 98.92 g for 5 min using the Hettich
Universal 32 R centrifuge (HD Scientific Supplies Pty. Ltd.). The supernatant was removed and the pellet resuspended in TRIzol® Reagent (1 mL per 5.0 x 10^6 cells mL^-1); repetitive pipetting was conducted to assist in the lysis of cells and incubated at room temperature for 5 min. Chloroform (C2432-500ML, Sigma-Aldrich, Castle Hill, Australia) was added to the TRIzol® reagent/lysate at 0.2 mL per 1 mL of TRIzol® Reagent and mixed by shaking for 15 s. The solution was incubated at room temperature for 2 - 3 min and was centrifuged at 12 000 g (4 ºC) for 15 min using the Hettich Universal 32 R centrifuge. The solution separated into three phases – the upper colourless aqueous phase (contained RNA) was removed by pipetting and placed into a sterile 1.5 mL centrifuge tube (616 201, Greiner Bio-one; Interpath Services, Heidelberg West, Australia). Precipitation of RNA was conducted using isopropanol (100 %) (I9516, Sigma-Aldrich, Castle Hill, Australia) that was added to the RNA solution at 0.5 mL per 1 mL of TRIzol® Reagent and incubated at room temperature for 10 min. The solution was centrifuged at 12 000 g (4 ºC) for 10 min using the Eppendorf 5415R Bench Centrifuge (Serial No. 0011439). The supernatant was removed and the RNA was washed using ethanol (70 %, 1 mL per 1 mL of TRIzol® Reagent). The solution was briefly vortexed and centrifuged at 7 500 g (4 ºC) for 5 min using the Eppendorf 5415R Bench Centrifuge. The supernatant was removed and the pellet was allowed to air dry for approximately 2 min. The pellet was resuspended in nuclease free water (100 µL) and stored at –80 ºC.

6.3.3.2 Determination of RNA Concentration and Quality

Quantification of RNA was determined by measuring the absorbance of RNA with the Genequant (Pharmacia). A 12.5 dilution of RNA was prepared by adding
RNA (8 µL) to sterile Tris-HCl (10 mM, 92 µL) (T1378-1KG, Sigma-Aldrich, Castle Hill, Australia). Absorbance at 260 nm was recorded and the 260 nm/280 nm ratio was used to determine RNA quality. The RNA quantity was calculated by the following formula (outlined in Invitrogen Purelink™ RNA mini kit instructions):

$$\text{Total RNA (µg)} = \text{OD}_{260} \times (40 \, \mu g/1 \, \text{OD}_{260} \times 1 \, \text{mL}) \times \text{Dil. Factor} \times \text{Total Vol. (mL)}$$

### 6.3.3.3 Generation of cDNA from Genomic RNA

The Reverse Transcription System (A3500; Promega, Sydney, Australia) was used to generate cDNA from the genomic RNA as per the manufacturer’s instructions. All RNA samples were incubated at 70 ºC for 10 min, centrifuged briefly and placed on ice. The reverse transcription reactions were prepared at a final volume of 55 µL for 1 µg of RNA. Reagents were added together in the following order on ice - magnesium chloride (final concentration 5 mM), reverse transcription buffer (1 x final concentration), dNTP mix (final concentration 1 mM per dNTP), recombinant RNasin® ribonuclease inhibitor (1 u µL⁻¹) and AMV reverse transcriptase (15 u µg⁻¹). The reverse transcription mix was distributed into individual microcentrifuge tubes. To each tube, RNA (1 µg) and random primers (0.5 µg mL⁻¹, 1 µL of) were added. Nuclease free water (NFW) was added making a final volume of 55 µL. The mixture was initially incubated at room temperature for 10 min. Using the Thermolyne heating block (17600, Model DB17610-26), the samples were incubated at 42 ºC for 15 min and then at 95 ºC for 5 mins. The final incubation was conducted at 4 ºC for 5 mins. All samples were stored at -20 ºC.
6.3.3.4 PCR Primers

The primers used to determine the gene expression of 16 genes by the treated porcine progenitor immune cells are listed in Table 6.1. The GAPDH primer was adapted from (Duvigneau et al., 2005). Primers for IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p40, IFN-γ, TNF-α and TNF-β were adapted from (Dozois et al., 1997). Primers for IL-5, CD3, CD19, CD62L, CD69 and MHC Class II were designed using Primer 3 (version 0.4.0.) and Primer BLAST (NCBI). All primers were synthesized by Sigma-Aldrich (Castle Hill, Australia). The plasmid sequences (for phMGFP and pClneo-DsRed2) and all the primers were analysed using Primer3 software to check for complementary sequences. No complementary sequences were detected.
Table 6.1 Primers of cytokines and CD markers used to identify the gene expression of treated porcine progenitor immune cells.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sense Primer (5' → 3')</th>
<th>Antisense Primer (5' → 3')</th>
<th>Anneal. Temp.</th>
<th>Cycle No.</th>
<th>Ex. prod. size</th>
<th>Accession No.</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>ACATGGCCTCCAAGGAGTAAGA</td>
<td>GATCGAGTTGGGGCTGTGACT</td>
<td>62 ºC</td>
<td>30</td>
<td>106 bp</td>
<td>X94251.1</td>
<td>Duvigneau et. al. (2005)</td>
</tr>
<tr>
<td>IL-1α</td>
<td>TGCCAGCTATGAGCCACTTCC</td>
<td>TGACGGGTCTCGAATGATGCT</td>
<td>65 ºC</td>
<td>40</td>
<td>337 bp</td>
<td>NM_214029.1</td>
<td>Dozois et al. (1997)</td>
</tr>
<tr>
<td>IL-1β</td>
<td>AAAGGGGACTTGAGAGAGAG</td>
<td>CTGCTTGAGAGGTGCTGTGATGT</td>
<td>57 ºC</td>
<td>40</td>
<td>286 bp</td>
<td>NM_001005149.1</td>
<td>Dozois et al. (1997)</td>
</tr>
<tr>
<td>IL-2</td>
<td>GATTTACAGTGTCTTTTGA</td>
<td>GTTGAGTAGATGCTTTGACA</td>
<td>51 ºC</td>
<td>40</td>
<td>338 bp</td>
<td>NM_213861.1</td>
<td>Dozois et al. (1997)</td>
</tr>
<tr>
<td>IL-4</td>
<td>TACCAGCAACTTCTGCCAC</td>
<td>ATCGTCTTTAGCCTTTCCAA</td>
<td>57 ºC</td>
<td>40</td>
<td>324 bp</td>
<td>NM_214123.1</td>
<td>Dozois et al. (1997)</td>
</tr>
<tr>
<td>IL-5</td>
<td>CCTTGACACTGCTTCCATTTC</td>
<td>TCTCCATCTTTCCCTCCAC</td>
<td>60 ºC</td>
<td>40</td>
<td>236 bp</td>
<td>NM_214205.1</td>
<td>NA</td>
</tr>
<tr>
<td>IL-6</td>
<td>ATGAAACTCCCTTCACAAGAC</td>
<td>TGGCTTTGTGCTTGGATTTCTTC</td>
<td>59 ºC</td>
<td>40</td>
<td>493 bp</td>
<td>NM_214399.1</td>
<td>Dozois et al. (1997)</td>
</tr>
<tr>
<td>IL-8</td>
<td>TTTCTGACAGCTCTCTGTAGG</td>
<td>CTGCTGTGGTTGTGCTTTCTC</td>
<td>59 ºC</td>
<td>40</td>
<td>266 bp</td>
<td>NM_213867.1</td>
<td>Dozois et al. (1997)</td>
</tr>
<tr>
<td>IL-10</td>
<td>GCATCCACTCCCAACC</td>
<td>CTCCCTCATCTCTCATCGATCAT</td>
<td>59 ºC</td>
<td>40</td>
<td>446 bp</td>
<td>NM_214041.1</td>
<td>Dozois et al. (1997)</td>
</tr>
<tr>
<td>IL-12p40</td>
<td>GATGCTGGCCGCTACACC</td>
<td>TCCAGCAGACCTCAATG</td>
<td>58 ºC</td>
<td>40</td>
<td>377 bp</td>
<td>NM_214013.1</td>
<td>Dozois et al. (1997)</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>GTTTTTCTGCTCTTCTAGC</td>
<td>TCTCCGCTTTTCTAGTTAG</td>
<td>53 ºC</td>
<td>40</td>
<td>410 bp</td>
<td>NM_213948.1</td>
<td>Dozois et al. (1997)</td>
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<tr>
<td>TNF-α</td>
<td>ATCGGCCGCCAGGAGGAAGAG</td>
<td>GATGCGAGAGAGGAGTGGAC</td>
<td>64 ºC</td>
<td>40</td>
<td>351 bp</td>
<td>X54859.1</td>
<td>M29079.1</td>
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<tr>
<td>TNF-β</td>
<td>CCCATCCCTCCCTCCTCTGT</td>
<td>GCTCCAAGAGACGCTACTG</td>
<td>59 ºC</td>
<td>40</td>
<td>874 bp</td>
<td>X54859.1</td>
<td>Dozois et al. (1997)</td>
</tr>
<tr>
<td>TGF-β</td>
<td>CCACCTCAGCCCTCTCTGCT</td>
<td>TGGGTCTCGGTATCTCAG</td>
<td>58 ºC</td>
<td>40</td>
<td>165 bp</td>
<td>NM_214015.1</td>
<td>NA</td>
</tr>
<tr>
<td>CD62L</td>
<td>GGTGGAAGGGGTATTGACCTT</td>
<td>ATGACCTGCGGTGACAGAAGAA</td>
<td>59 ºC</td>
<td>40</td>
<td>215 bp</td>
<td>NM_001112678.1</td>
<td>NA</td>
</tr>
<tr>
<td>CD69</td>
<td>TCCACACAGAGACCAACTCC</td>
<td>GACCACATTGCCACAGCAGC</td>
<td>59 ºC</td>
<td>40</td>
<td>129 bp</td>
<td>NM_214091.1</td>
<td>NA</td>
</tr>
</tbody>
</table>

GAPDH – Glyceraldehyde-3-phosphate dehydrogenase; IL – Interleukin; IFN – Interferon; TGF – Transforming growth factor; MHC – Major histocompatibility complex; bp – base pairs; IL-12p40 – interleukin 12B cytotoxic lymphocyte maturation factor 2, p40;
6.3.3.5 Real-Time Polymerase Chain Reaction (RT-PCR)

All RT-PCR samples consisted of PCR master mix (12.5 µL) (M7505; Promega, Sydney, Australia), primer (10 µM final concentration, 1.25 µL), SYBR® Green I (1 µL) (S-7563, Invitrogen, Mulgrave Australia), cDNA (2 µL) and NFW (7 µL). The negative controls consisted of NFW (2 µL) in place of cDNA. The PCR master mix (2x) contained Taq DNA polymerase (50 units mL⁻¹), reaction buffer (pH 8.5), dATP (400 µM), dGTP (400 µM), dCTP (400 µM), dTTP (400 µM) and MgCl₂ (3 mM). Amplifications were carried out using the Corbett Research Rotor-Gene 3000 with an initial denaturation at 95 °C for 2 min. Each cycle consisted of denaturation at 95 °C for 45 s, annealing for 45 s and extension for 60 s at 74 °C. A final extension was conducted at 74 °C for 5 min. A melt analysis was also conducted following the PCR cycling from 74 - 99 °C with a hold for 30 s on the first step and hold for 5 s for all other steps. The PCR products were stored at -20 °C. The PCR products were confirmed by gel electrophoresis using an agarose (2.0 %) and TBE (0.5x) gel. The PCR product (7 µL) was mixed with SYBR® Green I (1 µL) (S-7563, Invitrogen, Mulgrave Australia) and loading dye (2 µL) (G190A; Promega, Sydney, Australia). A 100 bp ladder was used for band comparison with the PCR product and prepared by adding the 100 bp ladder (2 µL) (G2101, Promega, Sydney, Australia) to loading dye (2 µL) (G190A; Promega, Sydney, Australia) and SYBR® Green I (1 µL) (S-7563, Invitrogen, Mulgrave Australia). The gel was run for 45 min at 120 volts by the PowerPac™ Basic Power Supply (164-5050, Bio-Rad, Gladesville, Australia). All gels were imaged by the Bio-Rad Molecular Imaging Gel Doc™ XR+ using the Quantity 1-D Analysis Software (170-9600, Bio-Rad, Gladesville, Australia).
6.4 RESULTS

6.4.1 Gene Expression of Porcine Progenitor Immune Cells Treated with Free- and Microencapsulated Bacteria-Conditioned Media

The gene expression of the transfected progenitor immune cell lines (i.e. L45hMGFP, L45pClneo-DsRed2, L23hMGFP and L23pClneo-DsRed2) treated with free and microencapsulated bacteria CM is shown in Table 6.2. The gene expression of cytokines/CD markers expressed by the immune cells for both free- and microencapsulated bacteria CM treatments are shown in yellow. Gene expression by only free-bacteria CM treatments are shown in green with the microencapsulated CM treatments shown in blue. No gene expression is denoted by red colouring.

The gene expression of GAPDH (house keeper gene) was observed for both free- and microencapsulated bacteria-CM treated immune cells (Table 6.2). Other various cytokine expressions were observed by the immune cells treatments for both free- and microencapsulated bacteria-CM that appeared depended on the immune cell type (T- or B-cell), bacteria strain and fluorescent protein. Gene expression for both free- and microencapsulated bacteria CM-treated immune cells was observed for IL-1β by *S. pyogenes* CM treated L45pClneo-DsRed2 cells, IL-2 by *L. acidophilus* LAFTI® L10 CM treated L23hMGFP and TNF-β by *S. pyogenes* L23pClneo-DsRed2 cells. The majority of expression by both free- and microencapsulated-bacteria was observed for the pro-inflammatory cytokine, IL-8. The expression of IL-8 was observed for all immune cells treated by the free- and microencapsulated *L. acidophilus* LAFTI® L10 CM, the free- and microencapsulated *B. lactis* HN019 (DR10™) CM treated L23hMGFP, L23pClneo-DsRed2 and L45pClneo-DsRed2 cells, control L23pClneo-DsRed2 and
L45pClneo-DsRed2 cells and free- and microencapsulated-\textit{S. pyogenes} CM treated L23hMGFP cells. No gene expression was observed for IL-4 by any of the cell lines treated with free- or microencapsulated CM. Gene expression of other cytokines appeared dependent if the bacteria were free or microencapsulated. For instance, IL-1α, IL-2, IL-6, IL-8 and TGF-β were observed for cell lines treated with all free bacteria CM. A few samples exhibited gene expression for only microencapsulated bacteria CM treated cell lines; this included microencapsulated \textit{L. acidophilus LAFTI® L10} CM treated L23hMGFP (IL-12p40) and L45hMGFP (IL-12p40 and IFN-γ), microencapsulated \textit{B. lactis} HN019 (DR10™) CM treated L23hMGFP (TNF-β), L23pClneo-DsRed2 (TNF-β) and L45hMGFP (IL-12p40), and microencapsulated \textit{S. pyogenes} CM treated L23hMGFP (IL-12p40 and TNF-β). The majority of gene expression was observed for the free-bacteria CM treatment of immune cells. Expression of IL-10 was only observed for free-bacteria CM treated L45pClneo-DsRed2 cells (not the control) with TNF-β expression observed for the L45pClneo-DsRed2 (not \textit{S. pyogenes} CM treatments) and L23pClneo-DsRed2 cells (not the control and \textit{B. lactis} HN019 (DR10™) CM treatments). The CD marker gene expression for both CD62L and CD69 was observed for all cell lines treated with free-bacteria CM with the exception of \textit{S. pyogenes} CM treated L23pClneo-DsRed2 cells. The gene expression of IFN-γ was observed in the L45 cell lines (i.e. L45hMGFP and L45pClneo-DsRed2) but not the L23 cell lines (L23hMGFP and L23pClneo-DsRed2). No gene expression for IL-1α, IL-4, IL-5, IL-6, IL-10, TNF-α, TGF-β, CD62L and CD69 was observed for any of the transfected progenitor cells lines treated with the microencapsulated bacteria-conditioned media (or the controls).
Table 6.2. Gene expression of cytokines and CD markers from free- and microencapsulated bacteria CM treated immune cells

<table>
<thead>
<tr>
<th>Gene Expression from Free Bacteria Treatment (TM)</th>
<th>Gene Expression from Microencapsulated Bacteria TM</th>
<th>No Gene Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gene</td>
<td>L23hMGFP</td>
<td>C</td>
</tr>
<tr>
<td>GAPDH</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>IL-1α</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>IL-1β</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>IL-2</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>IL-4</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>IL-5</td>
<td>+</td>
<td>-</td>
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<tr>
<td>IL-6</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>IL-8</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>IL-10</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>IL-12p40</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>TNF-α</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>TNF-β</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>TGF-β</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CD62L</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CD69</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

C – Control; L10 – *L. acidophilus* treated; DR10 – *B. lactis* treated; SP – *S. pyogenes* treated; GAPDH – Glyceraldehyde-3-phosphate dehydrogenase; IL – Interleukin; IFN – Interferon; TGF – Transforming growth factor; IL-12p40 – interleukin 12B cytotoxic lymphocyte maturation factor 2p40
6.4.2 Gene Expression of Progenitor Immune Cells Treated with IPI-1/Free- and IPI-1/Bacteria-Conditioned Mammalian Cell Culture Media

The gene expression of the transfected progenitor immune cells (i.e. L45hMGFP, L45pCIneo-DsRed2, L23hMGFP and L23pCIneo-DsRed2) treated with IPI-1/free and microencapsulated bacteria CM are shown in Table 6.3. Both IPI-1/free and IPI-1/microencapsulated bacteria CM treated immune cell lines were observed with gene expression for GAPDH, IL-1α, IL-8, TGF-β, TNF-α (except the IPI-1/microencapsulated L. acidophilus LAFTI® L10 CM treated L45pCIneo-DsRed2), CD62L and CD69 (except all the L45pCIneo-DsRed2 CM treatments). The gene expression of IL-10 was also observed for both IPI-1/free- and IPI-1/microencapsulated L. acidophilus LAFTI® L10 and B. lactis HN019 (DR10™) treated L45pCIneo-DsRed2 cells. Similar to the free- and microencapsulated CM treated immune cells (Section 6.4.1), the expression of certain cytokines appeared to be influenced by the treatment type, i.e. IPI-1/free- or IPI-1/microencapsulated bacteria CM. For instance, IL-2 and IL-6 gene expression was observed for only IPI-1/free bacteria CM treated immune cells with IL-1β gene expression for only IPI-1/microencapsulated bacteria CM treated immune cells (except B. lactis HN019 (DR10™) treated L23pCIneo-DsRed2 and L45pCIneo-DsRed2 and S. pyogenes treated L23pCIneo-DsRed2). Expression of IL-5 was observed only for IPI-1/free bacteria CM treated immune cells; however this expression also appeared to be influenced by the bacteria strain and fluorescent protein with no expression observed for IPI-1/B. lactis HN019 (DR10™) and IPI-1/S. pyogenes treated L45pCIneo-DsRed2 and L23pCIneo-DsRed2 cells. No gene expression for IL-4 and TNF-β was observed for either IPI-1/free- or IPI-1/microencapsulated treated immune cells. The
expression of IFN-γ was observed for only two samples – the IPI/free *B. lactis* HN019 (DR10™) treated L23hMGFP and L45pClneo-DsRed2 cells. Only the IPI-1/free bacteria CM treatments exhibited IL-12p40 gene expression by all immune cells except L45hMGFP control, *L. acidophilus* LAFTI® L10 and *B. lactis* HN019 (DR10™) treated cells. As outlined previously, the gene expression of cytokines appeared dependent on the treatment state of the bacteria i.e. free vs. microencapsulated. No gene expression was observed for IL-2, IL-4, IL-5, IL-6, IL-12p40, IFN-γ and TNF-β by any of the IPI-1/microencapsulated bacteria CM immune cellular controls and treatments.
Table 6.3. Gene expression of cytokines and CD markers from IPI-1/free- and microencapsulated bacteria-CM treated immune cells

<table>
<thead>
<tr>
<th>Gene Expression from IPI-1/Free Bacteria Treatment (TM)</th>
<th>Gene Expression from IPI-1/Microencap. Bacteria TM</th>
<th>No Gene Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>L23hMGFP</td>
<td>L23pClneo-DsRed2</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>L10</td>
<td>DR10</td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>GAPDH</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>IL-1α</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>IL-1β</td>
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<td>IL-2</td>
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<tr>
<td>IL-10</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>IL-12p40</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>TNF-α</td>
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<td>TNF-β</td>
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<tr>
<td>TGF-β</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CD62L</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CD69</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

C – Control; L10 – *L. acidophilus* treated; DR10 – *B. lactis* treated; SP – *S. pyogenes* treated; GAPDH – Glyceraldehyde-3-phosphate dehydrogenase; IL – Interleukin; IFN – Interferon; TGF – Transforming growth factor; IL-12p40 – interleukin 12B cytotoxic lymphocyte maturation factor 2p40
6.4.3 Comparison between the Gene Expression of Immune Cells Treated with Free and Microencapsulated Bacteria, and Intestinal Cell Co-cultures with Free and Microencapsulated Bacteria

The intestinal cell and microencapsulated bacteria co-cultures were shown to induce gene expression of a number of cytokines that the microencapsulated bacteria CM alone did not induce. For instance, gene expression of IL-1β, IL-10, TNF-α, TGF-β, CD62L and CD69 were observed to be expressed by immune cells treated with IPI-1/free and microencapsulated bacteria (Table 6.3) whereas only the free bacteria-treated immune cells (Table 6.2) expressed these cytokines (varied expression depending on immune cell type and bacteria). However, in some cases, cytokine gene expression was observed for both microencapsulated bacteria CM treated and IPI-1/microencapsulated CM treated immune cells including IL-8 and IL-12p40. The gene expression of IL-1α was observed for immune cells treated with free bacteria CM (Table 6.2) and IPI-1/free as well as IPI-1/microencapsulated bacteria CM (Table 6.3). Similarities in the gene expression were observed for IL-2 and IL-6 with only free bacteria CM and IPI-1/free bacteria CM treated immune cells expressing these cytokines. The expression of IL-4 by immune cells was not observed for all treatments types. Although variable gene expression was observed for IL-5 (depending on immune cell type and bacteria strain), only free bacteria and IPI-1/free bacteria CM treated immune cells expressed this cytokine (no microencapsulated bacteria treated cells). Further, no gene expression for TNF-β by immune cells treated with the IPI-1/free and microencapsulated bacteria CM were observed; variable gene expression for the free and microencapsulated bacteria CM treated immune cells was observed for TNF-β.
6.5 DISCUSSION

6.5.1 Gene Expression of Porcine Progenitor Immune Cells Treated with Free- and Bacteria-Conditioned Media

6.5.1.1 Gene Expression of Porcine Progenitor Immune Cells Treated with Free Bacteria-Conditioned Media

The gene expression of the cytokines IL-1α, IL-2, IL-6, IL-8, TNF-α, TGF-β and the CD markers CD62L and CD69 were observed for the control, *L. acidophilus* LAFTI® L10 and the *B. lactis* HN019 (DR10™) treated L45hMGFP, L45pCIneo-DsRed2, L23hMGFP and L23pCIneo-DsRed2 cells (Section 6.4.1). The *S. pyogenes* treated cells lines also exhibited the gene expression of these cytokines with the exception of L23pCIneo-DsRed2 *S. pyogenes* treated cells, gene expression for TNF-α, CD62L or CD69 was not observed (Table 6.2).

Pro-inflammatory and Regulatory Cytokine Gene Expression by Immune Cells in Response to Bacteria-Conditioned Media

The cytokines, IL-1α, IL-2, IL-6, IL-8 and TNF-α, have been characterized as pro-inflammatory cytokines and are associated with T<sub>H1</sub> immune responses. The T<sub>H1</sub> immune response directs cell-mediated immunity that targets intracellular pathogens including viruses, certain bacteria, yeast, fungi, protozoans and prevents tumour cell progress (Gillingham and Lescheid, 2009). The expression of IL-2, IL-6 and TNF-α has been predominately reported for T lymphocytes. However, IL-2 secretion by rabbit B cells was reported by Lagoo et al. (1990); the authors proposed the B cell IL-2 secretions acted as an autocrine proliferation-inducing lymphokine (Lagoo et al., 1990). Endogenous secretion of IL-6 by human B cells was reported by Oshiba and Gelfand who proposed it was a cellular mechanism to counter
negative regulatory effects of antigens on IgE production (Oshiba and Gelfand, 1999). The endogenous expression of IL-6 and TNF-α by human B-cells was also reported; the investigation was to determine the factors that promote B-cell proliferation and differentiation (Burdin et al., 1995). It should be noted that IL-6 has also been associated with Th2 immunological activities. Production of the IL-1α, IL-2, IL-6, IL-8 and TNF-α cytokines by immunological cells treated with strains of *L. acidophilus* has been reported in previous studies; however, the majority of these studies describe Th1 immune responses *in vivo* or as outlined previously, Th1 immune responses from dendritic cellular treatment of lymphocytes *ex vivo* and *in vitro*. For instance, the up-regulation of IFN-induced chemokines and responsive genes in the small intestine mucosa of human volunteers from *L. acidophilus* LAFTI® L10 consumption was suggested to promote Th1 immune responses (van Baarlen et al., 2011).

Regulation of Th1 and Th2 cytokine responses was reported for *L. acidophilus* strain L-92 consumed by BALB/c mice with an associated suppression of serum ovalbumin (OVA)-specific IgE; the mice had initially been immunized with OVA to induce an IgE response (Torii et al., 2007). Human monocyte-derived immature dendritic cells treated with various concentrations of *L. rhamnosus* Lcr35 reportedly produced gene expression associated with immune responses at high doses, this was determined from DNA microarray and qRT-PCR; cytokine secretion by the dendritic cells exhibited increases in pro-Th1/Th17 (regulatory T cells) levels (Evrard et al., 2011). Profiling of dendritic cellular cytokine production is used to predict the resulting immunological responses by lymphocytes, in particular, whether a Th1, Th2 or regulatory (Treg) immune response will be encouraged. As an
example, bone marrow-derived murine dendritic cells were exposed to non-viable Lactobacillus spp.; the authors reported that the cytokine profiles of treated dendritic cells were strain dependent and the potential exists to modulate T\textsubscript{H}1/T\textsubscript{H}2/T\textsubscript{H}3 responses based on the gut microflora and consumed probiotics (Christensen et al., 2002). Further, Treg cells were primed by human monocyte-derived dendritic cells treated with \textit{L. reuteri} and \textit{L. casei} \textit{in vitro} (Smits et al., 2005).

The majority of studies researching the immunological responses to Bifidobacteria sp. treatments are usually associated with down-regulation of pro-inflammatory cytokines in models of intestinal inflammatory diseases (Medina et al., 2008; Philippe et al., 2011). A number of studies have also described regulatory immunological responses to probiotics. However, very little research has outlined the immunological responses of T and B cells to probiotic bacterial soluble factors without mediators (such as dendritic cells) priming the response. The cytokine, TGF-β, has been associated with immune regulation with the ability to modulate T\textsubscript{H}1 and T\textsubscript{H}2 responses in the mucosa (Ohtsuka and Sanderson, 2000). All the control, \textit{L. acidophilus} LAFTI® L10, \textit{B. lactis} HNO19 (DR10\textsuperscript{TM}) and \textit{S. pyogenes} treated cell lines expressed the genes for TGF-β in this Chapter. Although a number of T\textsubscript{H}1 cytokines were produced by the control and treatments, the detection of TGF-β gene suggests the cells were potentially participating in immunoregulatory activities. Previous studies have described the production of T\textsubscript{H}1 cytokines and TGF-β in response to LAB treatment. As previously mentioned, the regulation of T\textsubscript{H}1 and T\textsubscript{H}2 cytokine responses occurred when \textit{L. acidophilus} strain L-92 was consumed by BALB/c mice (Torii et al., 2007). The authors (Torri et al. 2007) also described TGF-β production in the Peyer’s patches of the mice noting that TGF-β activates
Treg cells (possibly regulating T\(_h\)1 and T\(_h\)2 cytokine response) and associated the presence of TGF-\(\beta\) expression with potential immunomodulatory activities of LAB (Torii et al., 2007). Also, dendritic cells treated with LAB/intestinal cell conditioned media primed naive T cells by the increased production of TGF-\(\beta\) and decreased levels of IFN-\(\gamma\) (Zeuthen et al., 2007). The number of regulatory CD4\(^+\) T cells with TGF-\(\beta\) surface receptors was reported to increase in mice with induced colitis following supplementation with the probiotic VSL\#3 (contains a number of different LAB including Bifidobacteria and Lactobacillus); the authors proposed that administration of VSL\#3 induces immunoregulatory responses and reduces the severity of colitis (Di Giacinto et al., 2005).

**Gene Expression of CD Markers by Treated Immune Cells**

The expression of CD69 is used as an indicator of T cell stimulation by mitogens or antigens (Lindsey et al., 2007) as well as B cell activation (Lauzurica et al., 2000). The gene expression of T cell mRNA in mice has been reported to occur within 30 to 60 min following stimulation and the CD69 cell-surface marker is observed at 3 h (Ziegler et al., 1994). As noted previously, the gene expression of CD69 was observed by all the treated cell lines except the \(S.\) pyogenes treated L23pCIneo-DsRed2 cells. This result supports previous research describing CD69 expression for T- and B cells treated by probiotic strains *in vitro*. For instance, *in vitro* treatment with *L. casei* Shirota induced the expression of CD69 on T cells as well as the production of the pro-inflammatory cytokines IL-1\(\beta\), IL-6, TNF-\(\alpha\) and IL-12 and the anti-inflammatory cytokine IL-10 (Dong et al., 2010). Strain specific immune modulation was described for a number of Lactobacilli and Bifidobacteria strains treatment of human peripheral blood mononuclear cells (PBMC) *in vitro* with
increased expression of CD69 on lymphocytes; the Lactobacilli was described to promote T_{H1} cytokines with Bifidobacterial strains encouraging anti-inflammatory cytokine production (Dong et al., 2011). Inactivated *L. acidophilus* was described to modulate allergic immune responses to grass pollen with an up-regulation of CD69 expression on T lymphocytes (Rasche et al., 2007).

The cell surface marker, CD62L (also known as L-selectin), is a receptor found on leukocytes that signals for these cells to enter secondary lymphoid tissues; the CD62L binds to ligands on the endothelial cells that facilitates entry into the lymphoid organs (Cotran et al., 1999). The gene expression for CD62L was observed for all control and bacteria cell lines with the exception of the *S. pyogenes* treated L23pClneo-DsRed2 cell line. The cell surface expression of CD62L has been used as a selective criterion for T lymphocytes to be treated *ex vivo*; these cells have been isolated from murine models with induced colitis and treated with probiotics (Schultz et al., 2004). The expression of CD62L from probiotic bacterial treatment with soluble factors has rarely been described. The gene expression of CD62L of the bacteria treated cell lines indicates that these cells are potentially being primed for further immunological interactions.

**Anti-Inflammatory Cytokine Gene Expression by Treated Immune Cells**

The expression of the anti-inflammatory cytokines, IL-4, IL-5 and IL-10, by the bacteria CM treated cell lines produced varied results (Section 6.4.1). These cytokines are associated with T_{H2} immune responses that drive humoral immunity and the production of antibodies (Gillingham and Lescheid, 2009). No gene expression of IL-4 was observed for any of the control and bacteria CM treated cell
lines. This cytokine (IL-4) induces the maturation and differentiation of naïve T cells into mature T cells and antibody production by B cells (Kidd, 2003). The absence of IL-4 expression by the control and treated cell lines suggests that the resulting immunological responses are favouring Th1 and even regulatory T cell responses as opposed to Th2. The bacteria treated L45pClneo-DsRed2 cells were observed to express IL-10; however, the control and other cell line treatments did not express this cytokine. A number of studies have reported the expression of IL-10 by lymphocytes upon treatment with probiotics. Up-regulation of IL-10 secretion from LAB treatments was reported for PBMCs, monocyte-derived dendritic cells and dendritic cell/T cell cocultures (Hua et al., 2010). The production of IL-10 by regulatory T cells from probiotic bacteria treatment has also been reported (Magny, 2011). The expression of IL-5 was observed for the control and S. pyogenes treated L23hMGFP, B. lactis HN019 (DR10™) treated L23pClneo-DsRed2, and all three bacteria treated L45pClneo-DsRed2 cell lines. The varied results are potentially a bacteria strain and/or T- or B-cell associated response to the soluble factors. The production of IL-5 by PBMCs was reportedly decreased when treated with various lactobacilli and allergens (Pochard et al., 2002). Similarly, IL-4 and IL-5 secretion by PBMCs was inhibited by LAB treatment with an associated enhancement of IFN-γ stimulation (Ghadimi et al., 2008).

Bacteria Strain Effects on the Cytokine & CD Marker Gene Expression by Treated Immune Cells

The L45pClneo-DsRed2 treated cells were observed to express the majority of the cytokines tested. In particular, the L. acidophilus LAFTI® L10 treated L45pClneo-DsRed2 cells were observed to produce the most cytokines with gene
expression for IL-1α, IL-1β, IL-2, IL-5, IL-6, IL-8, IL-10, IL-12p40, IFN-γ, TNF-α, TNF-β and TGF-β. The control L45pCIneo-DsRed2 cells did not express IL-1β or IL-10. The B. lactis HN019 (DR10™) and S. pyogenes treated L45pCIneo-DsRed2 cells did not express IFN-γ and TNF-β, respectively. Based on these results, it is possible that the T-lymphocyte response to the probiotic treatments is potentially strain dependent and associated with T₉₁ and regulatory immunological responses; T₉₂ immune responses cannot be discounted with the expression of IL-5 and IL-10 detected. It should be noted that IL-12p40 was expressed by L45pCIneo-DsRed2 and L23pCIneo-DsRed2 but not the L45hMGFP and L23hMGFP cell lines; it is possible that the fluorescent protein has influenced the immune cell lines response to the CM that subsequently affected the expression of IL-12p40. However, to confirm this effect on IL-12p40, further experimentation is required.

The gene expression of IFN-γ was detected for only the T cell lines (L45hMGFP and L45pCIneo-DsRed2). The control and B. lactis HN019 (DR10™) treated L45hMGFP and the control, L. acidophilus LAFTI® L10 and S. pyogenes treated L45pCIneo-DsRed2 cells expressed IFN-γ genes. Therefore, the production of this cytokine by the T cell line was independent of the bacteria strain. Previous research has described increased IFN-γ production by T cells in human athletes that have been treated with L. acidophilus LAFTI® L10 relieving symptoms of fatigue (Clancy et al., 2006). Based on the results presented in Section 6.4.1, the response by the control, L. acidophilus LAFTI® L10, B. lactis HN019 (DR10™) and S. pyogenes treated cell lines appear to produce pro-inflammatory cytokines therefore indicating a T₉₁ immunological response. A T₉₂ and regulatory immune response was also detected; potential modulation of the immunological response to the
treatments could have occurred by the cell lines. In addition, the cell type, transfected fluorescent plasmid and type of bacteria treatment could have influenced the immunological response to the treatments supporting the results presented in Chapter 4 for the proliferation responses of the immune cells.

6.5.1.2 Gene Expression of Porcine Progenitor Immune Cells Treated with Microencapsulated Bacteria-Conditioned Media

The gene expression of the microencapsulated bacteria conditioned media treated L45hMGFP, L45pCIneo-DsRed2, L23hMGFP and L23pCIneo-DsRed2 cells produced different cytokine and CD marker profiles comparative to the free bacteria conditioned media treated cells. The majority of the cytokines (IL-1α, IL-4, IL-5, IL-6, IL-10, TNF-α and TGF-β) and both CD markers (CD62L and CD69) were not expressed by the microencapsulated bacteria conditioned media treated immune cells. However, gene expression for IL-8 and IL-12p40 was observed for all four cell lines and appeared dependent on the bacteria strain. For instance, microencapsulated *L. acidophilus* LAFTI® L10 treated L45hMGFP, L45pCIneo-DsRed2, L23hMGFP and L23pCIneo-DsRed2 cells expressed IL-8 and IL-12p40. The microencapsulated *B. lactis* HN019 (DR10™) treated L23hMGFP, L23pCIneo-DsRed2 and L45pCIneo-DsRed2 cells expressed IL-8 with the treated L23pCIneo-DsRed2, L45hMGFP and L45pCIneo-DsRed2 treated cells expressing IL-12p40. The microencapsulated *S. pyogenes* treated L23hMGFP cells were observed to express IL-8, no expression was observed for the other cell lines. Expression of IL-12p40 was also observed from the microencapsulated *S. pyogenes* treated L23hMGFP and L45hMGFP cells.
The gene expression of IFN-γ and IL-2 was also observed for microencapsulated *L. acidophilus* LAFTI® L10 treated L45hMGFP and L23hMGFP cells (Table 6.2). The gene expression of these pro-inflammatory cytokines (predominately IL-8 and IL-12p40) indicates a T_H1 immunological response by the porcine progenitor immune cells to the microencapsulated bacteria conditioned media particularly for the microencapsulated *L. acidophilus* LAFTI® L10 and *B. lactis* HN019 (DR10™) treatments. As outlined for the free bacteria conditioned media treatment of the porcine progenitor immune cells, previous studies have described the secretion of pro-inflammatory cytokines by immune cells in response to probiotic strains (Evrard et al., 2011; van Baarlen et al., 2011). Increases in IL-12p40 secretion by dendritic cells was observed following treatment with *L. rhamnosus* Lcr35 (Evrard et al., 2011).

The gene expression of the anti-inflammatory cytokines (i.e. IL-4, IL-5, IL-10 and TGF-β) was not observed for any of the cell line treatments indicating that T_H2 or regulatory cytokines were not observed for any of the microencapsulated bacteria CM treated immune cells. The gene expression of the pro-inflammatory cytokines by the microencapsulated bacteria CM treated cells is similar to the free bacteria CM treatment of the porcine progenitor immune cells. It is also possible that strain-dependent effects on the gene expression of the cytokines by the progenitor immune cells occurred. The microencapsulated *L. acidophilus* LAFTI® L10 CM treated cells were observed to produce IL-8 and IL-12p40 regardless of the cell line or the fluorescent protein. The type of cell was also observed to be influenced at the gene expression level. For instance, TNF-β was expressed for the microencapsulated
B. lactis HN019 (DR10™) and S. pyogenes L23hMGFP and L23pCIneo-DsRed2 treated cells; the L45hMGFP and L45pCIneo-DsRed2 did not express this cytokine.

From the cytokine expression profile produced by microencapsulated bacteria CM treated L45hMGFP, L45pCIneo-DsRed2, L23hMGFP and L23pCIneo-DsRed2 cell lines, the microencapsulated L. acidophilus LAFTI® L10 and B. lactis HN019 (DR10™) treated cells appeared to produce a Th1 immunological response similar to the free bacteria CM treated cell lines. The production of Th2 and Treg cytokines was absent from the gene expression profiles of the microencapsulated bacteria CM treated cells. As outlined previously, it is possible that the microencapsulated environment has induced stress-related changes that have affected the production of soluble factors. However, the underlying mechanisms associated with the changes in bacteria activities and the response of the immune cells (such as intracellular signalling) requires further investigations.

6.5.2 Gene Expression of Porcine Progenitor Immune Cells Treated with IPI-1/Free- and IPI-1/Bacteria-Conditioned Media

6.5.2.1 Gene Expression of Porcine Progenitor Immune Cells Treated with IPI-1/Free Bacteria-Conditioned Media

The gene expression of the IPI-1/free bacteria conditioned media treated L45hMGFP, L45pCIneo-DsRed2, L23hMGFP and L23pCIneo-DsRed2 exhibited similar results to the bacteria CM treated immune cell lines. The gene expression of the pro-inflammatory cytokines, IL-1α, IL-2, IL-6 and IL-8, and the immunoregulatory cytokine TGF-β, was observed for all IPI-1/bacteria CM and bacteria CM treated porcine immune cells lines.
Previous Studies Describing Cytokine Production by Intestinal Cells to Probiotic Treatments

Interactions between intestinal cells and underlying immune cells in the GI tract occur via a number of interactive events including cytokine secretion by intestinal cells. A number of studies have described cytokine production by intestinal cells in response to probiotic bacteria. For instance, pro-inflammatory cytokines were produced by rat intestinal epithelial cells following bifidobacterium treatment (Ruiz et al., 2005). The secretion of IL-6 by murine primary intestinal epithelial cells (from the small intestine) following lactobacilli stimulation was proposed by the authors to encourage B cell differentiation (Vinderola et al., 2002). Immunoregulation by TGF-β at the intestinal level assists in the maintenance of barrier integrity and stimulates oral tolerance to GI antigens (Blum and Schiffrin, 2003). Human intestinal epithelial cells treated with lactobacilli were reported to produce TGF-β (Blum and Schiffrin, 2003). Different bifidobacteria species were investigated to determine the effects on murine intestinal cell cytokine production; the authors reported TGF-β secretion by the murine intestinal cells in response to a B. longum strain and noted that cytokine production was influenced by the bifidobacteria strain (Menard and Waligora-Dupriet, 2008). Therefore, the production of cytokines by intestinal cells in response to probiotic bacteria subsequently primes immune cells in the underlying immunological tissue in the GI tract.

Gene Expression of Cytokines & CD Markers by Immune Cells in Response to IPI-1 Co-cultures with Free and Microencapsulated Bacteria
A number of similarities were observed for the cytokine and CD marker gene expression of immune cells treated with IPI-1/free bacteria co-cultures and free bacteria CM treatments. The gene expression of CD62L and CD69 was observed for all IPI-1/free bacteria conditioned media treated immune cell lines (Table 6.3); these results were similar to the respective free bacteria conditioned media immune cell lines with the exception of the *S. pyogenes* treated L23pClneo-DsRed2 cells (Table 6.2). Similarly, the expression of TNF-α was observed for all IPI-1/bacteria conditioned media treated immune cell lines (Table 6.3) as was observed for the bacteria conditioned media treated immune cell lines with the exception of the *S. pyogenes* L23pClneo-DsRed2 cells (Table 6.2). As described for the proliferation response of immune cells to the bacteria and IPI-1/bacteria co-culture CM treatments (Chapter 5), relationships between the fluorescent protein and immune cell type possibly exist. For instance, the gene expression of IL-12p40 by L23pClneo-DsRed2 and L45pClneo-DsRed2 cells was observed for the IPI-1/bacteria CM treated and bacteria CM treatments. In some cases, cytokine gene expression was observed for the bacteria CM treatments but not the control e.g. IL-10 expression by L45pClneo-DsRed2 was observed for all bacteria CM treatments and not for the control. These results indicate that the gene expression of cytokines and CD markers by the immune cells were not influenced by the presence of the fibroblast intestinal cells in some cases. It is also possible that the intestinal cells produced chemical signals that encouraged these similar gene expression profiles by the immune cells.

The gene expression of IL-5 was observed in more IPI-1/bacteria CM samples comparative to the bacteria CM treated immune cells. The control and all three IPI-1/bacteria conditioned media treated L23hMGFP and L45hMGFP cells
were observed to genetically express IL-5 as well as the IPI-1/control and IPI-1/L. *acidophilus* LAFTI® L10 treated L23pCIneo-DsRed2 cells, and the IPI-1/control L45pCIneo-DsRed2 cells. These results suggest that in addition to the T<sub>H1</sub> immunological response by the treated immune cells, the IPI-1/bacteria conditioned media treated cells are undergoing a T<sub>H2</sub> and regulatory immunological response. It is possible that the fibroblast intestinal cells (IPI-1) encourage immunomodulatory activities by the immune cells. However, similar to the observations for the bacteria CM treated immune cells, the gene expression of the cytokines by the IPI-1/bacteria treated immune cells appeared to be dependent on the immune cell type, bacteria strain and fluorescent protein (phMGFP and pCIneo-DsRed2). For instance, the control and IPI-1/bacteria CM treated L45hMGFP and L23hMGFP cells expressed IL-5. Interferon (IFN)-γ was expressed by the IPI-1/B. *lactis* HN019 (DR10™) treated L23hMGFP and L45pCIneo-DsRed2 and IL-10 was expressed by IPI-1/L. *acidophilus* LAFTI® L10 CM treated L23pCIneo-DsRed2 and IPI-1/B. *lactis* HN019 (DR10™) CM treated L45hMGFP. Unlike the bacteria CM treated immune cellular gene expression, the IPI-1/bacteria CM treated L45hMGFP, L45pCIneo-DsRed2, L23hMGFP and L23pCIneo-DsRed2 cells did not exhibit gene expression for IL-1β and TNF-β. Similar to the free and microencapsulated bacteria CM treatments, the gene expression of IL-4 was not observed for the IPI-1/bacteria CM treated immune cells.

Therefore, from these results, it is possible that the fibroblast intestinal cells (IPI-1) are releasing cytokines, chemokines and other soluble factors in response to the bacteria exposure that regulates the porcine progenitor immune cellular activities; these activities appear to be strain dependent coupled with the immune cell type.
Also, a shift in the \( \text{T}_\text{H}2 / \text{regulatory} \) cytokine gene expression was observed for the IPI-1/free bacteria CM treatments of the immune cells whereas the free bacteria CM treatments exhibited a \( \text{T}_\text{H}1 \) cytokine gene expression response. From these results, it appears that the fibroblast intestinal cells interacted with the bacteria cells potentially via physical contact as well as in response to the bacterial-produced soluble factors. Reports have described modified cytokine secretions by intestinal epithelial cells following interactions with probiotic bacteria; the intestinal epithelial cellular signal transduction pathways were modified by the probiotic bacterial exposure (Neish, 2004; Otte and Podolsky, 2004; Ruiz et al., 2005). Such interactions resulted in the production of cytokines, chemokines and other soluble factors by the IPI-1 cells; these chemical signals along with the soluble factors produced by the bacteria influenced the porcine progenitor immune cells and encouraged the genetic expression of \( \text{T}_\text{H}1 \), \( \text{T}_\text{H}2 \) and regulatory cytokines.

6.5.2.2 Gene Expression of Porcine Progenitor Immune Cells Treated with IPI-1/Microencapsulated Bacteria-Conditioned Media

As outlined previously, only one study to date has reported the influence microencapsulated probiotic bacteria has on immunological responses. This study described decreased small intestinal responses of IL-1\( \beta \), IL-6, TNF-\( \alpha \) and IFN-\( \gamma \) in mice fed microencapsulated probiotic yogurt (comparative to the control) (Urbanska et al., 2010). The responses of immunological cells (both innate and adaptive), to microencapsulated bacteria co-cultured with intestinal cells has not been reported at this time.
The immunological responses of the IPI-1/microencapsulated bacteria CM treated L45hMGFP, L45pClneo-DsRed2, L23hMGFP and L23pClneo-DsRed2 cell lines exhibited less cytokine gene expression comparative to the IPI-1/bacteria CM treated cell lines and more cytokine gene expression than the microencapsulated CM cell lines (Table 6.3). Therefore, the porcine fibroblast intestinal cells have potentially influenced the porcine immunological responses to the IPI-1 and microencapsulated bacteria CM.

The IPI-1/microencapsulated bacteria CM treated cell lines expressed genes for IL-1α, IL-8, TNF-α (except IPI-1/microencapsulated L. acidophilus LAFTI® L10 treated L45pClneo-DsRed2), TGF-β and CD62L. These results were similar to the IPI-1/bacteria conditioned media treated cell lines. The expression of the pro-inflammatory cytokines (IL-1α, IL-8 and TNF-α) have been reported for probiotic bacteria (Ruiz et al., 2005). These results indicate that the microencapsulated probiotics potentially produced soluble factors similar to the free probiotics and this was reflected in the downstream response of the immune cells. The gene expression of IL-1β was observed for some of the IPI-1/microencapsulated bacteria conditioned media treated cell lines; the IPI-1/bacteria conditioned media treated cell lines did not express the IL-1β gene (Table 6.3). The expression of IL-1β appeared dependent on the bacteria strain and the fluorescent protein with the control and all the IPI-1/microencapsulated bacteria CM treated L45hMGFP and L23hMGFP expressing this cytokine; the IPI-1/microencapsulated B. lactis HN019 (DR10™) did not induce IL-1β gene expression for the L23pClneo-DsRed2 and L45pClneo-DsRed2 cell lines. The type of immune cell appeared to influence the gene expression of IL-10 with the IPI-1/microencapsulated S. pyogenes treated L45hMGFP, the control, IPI-
1/microencapsulated *L. acidophilus* LAFTI® L10 and IPI-1/microencapsulated *B. lactis* HN019 (DR10™) treated L45pCIneo-DsRed2 also genetically expressing this cytokine.

The gene expression of CD69 was observed for all control and IPI-1/microencapsulated bacteria CM treated cell lines with the exception of L45pCIneo-DsRed2 cells. Based on these results, it appears that the IPI-1/microencapsulated bacterial CM treated immune cells lines are producing a T$_{H1}$ and immunoregulatory reaction to the treatments. The cytokine and CD marker gene expression of the IPI-1/microencapsulated bacteria CM treated immune cell lines significantly differs from the microencapsulated bacteria CM treated immune cell lines that produced only pro-inflammatory cytokines (T$_{H1}$ immunological response) (Table 6.3). Therefore, the fibroblast intestinal cells potentially encouraged immunomodulatory activities by the porcine immune cells through the production of soluble factors in response to the microencapsulated bacteria conditioned media. However, the underlying mechanism behind these reactions requires further research. Identification of the pathways involved in the intracellular signalling by the immune cells in response to the soluble factors may provide information relevant to the activity of microencapsulated probiotic bacterial-produced soluble factors and also the cytokine and chemokine production of the fibroblast intestinal cells. Such information could be used to increase the understanding of intestinal inflammatory diseases e.g. Crohn’s disease, and possible result in the development of treatment options using microencapsulated probiotic bacteria.
6.6 **CONCLUSION**

The gene expression of the microencapsulated bacteria CM treated immune cells exhibited different cytokine profiles compared to the free bacteria CM treated immune cells. The majority of tested cytokines were not expressed by the microencapsulated bacteria CM treated immune cells. The microencapsulated *L. acidophilus* LAFTI® L10 and *B. lactis* HN019 (DR10™) treated cell lines produced gene expression for IL-8 and IL-12p40, as well as other pro-inflammatory cytokines. The free bacteria CM treated immune cells expressed genes for pro-inflammatory and regulatory cytokines. The expression was dependent on the immune cell line and the bacteria strain. The immunological response of the cell lines to the microencapsulated *L. acidophilus* LAFTI® L10 and *B. lactis* HN019 (DR10™) conditioned media indicated a T\(_H\)1 immunological response.

The gene expression of the IPI-1/bacteria CM treated porcine immune cells exhibited a T\(_H\)1, T\(_H\)2 and regulatory immunological response. The probiotic bacteria induced the gene expression of T\(_H\)2 associated cytokines dependent on the cell type. The pro-inflammatory cytokines, IL-1\(\alpha\), IL-2, IL-6, IL-8 and TNF-\(\alpha\), were observed for all treatments and immune cell types. The gene expression of the regulatory cytokine, TGF-\(\beta\), was also observed for all treatments and cell types. Comparatively, less cytokine gene expression was observed for the IPI-1/microencapsulated bacteria CM treated immune cells with IL-1\(\alpha\), IL-8 and TGF-\(\beta\) being expressed for all treatments and immune cell types. The expression of IL-10 (anti-inflammatory cytokine) was observed for only the transfected L45 cells. Similar to the observations for the IPI-1/free bacteria CM treated immune cells, the immunological response appears to encompass a T\(_H\)1 and regulatory response to the IPI-
More cytokines were expressed by the immune cells treated with the intestinal cells/microencapsulated bacteria CM compared to the microencapsulated bacteria CM treated immune cells. This result is potentially a function of regulatory signals produced by the intestinal cells in response to the microencapsulated bacteria.

The porcine fibroblast intestinal cells may have responded to the microencapsulation components and bacteria soluble factors resulting in the production of soluble factors by the intestinal cells that affected the downstream responses of the immune cells. Changes to the internal environment of the immune cells in response to the soluble factors produced by free, microencapsulated and IPI-1 co-cultures were investigated in Chapter 7 by calculating the diffusion rates of the intracellularly expressed fluorescent proteins using LSCM and the image analysis software Raster Image Correlation Spectroscopy (RICS).
7. CHAPTER SEVEN
Immune Intracellular Fluorescent Protein Diffusion Responses to Free and Microencapsulated Probiotic Bacteria

7.1 INTRODUCTION

The gene expression of cytokines and CD markers by porcine progenitor immune cells in response to soluble factors produced by free, microencapsulated and intestinal cell co-cultures with free and microencapsulated bacteria was investigated in Chapter 6. Similar to the proliferation responses of the immune cells (Chapter 4), the gene expression of cytokines and CD markers were dependent on relationships between the immune cell type, fluorescent protein, if the bacteria were free or microencapsulated and in some instances, if intestinal cells were present. This Chapter investigates the diffusion responses of the immune intracellular fluorescent proteins to the soluble factors produced by the bacteria using LSCM and image analysis software. The immune cells expressing fluorescent proteins were developed as outlined in Chapter 3. The diffusion rates may provide information regarding the intracellular environment in terms of responses to the bacterial-produced soluble factors.

Fluorescent Protein Applications

Fluorescent proteins are commonly tagged to genes to track the activities of a protein of interest (De Giorgi et al., 1996). A number of applications have utilised fluorescent proteins as bio-indicators of mammalian intracellular activities including monitoring pH changes (Kneen et al., 1998), calcium signalling (Romoser et al., 1997) and intracellular redox changes (Dooley et al., 2004). In this study, the
fluorescent proteins, pHMGFP and pCIneo-DsRed2, were transfected into the porcine progenitor immune cells, L45 and L23 (T- and B-cells, respectively) to provide a bio-indicator of intracellular activities in response to the different probiotic bacterial treatments. The fluorescent proteins were not tagged to a particular gene but were expressed randomly throughout the cells. As described in Chapter 3, the expression of the fluorescent proteins appeared dependent on the vector, fluorescent protein and the viable state of the cell; apoptotic cells were observed to express almost entire cellular fluorescence. Viable cells expressed a few fluorescent vesicles. The expression of the two fluorescent proteins appeared to be localized in the cytosol and nuclear regions of the cells as determine from co-localization studies using fluorescent probes specific for DNA, RNA and cytosol.

Image Analysis Software – Raster Image Correlation Spectroscopy

Raster Image Correlation Spectroscopy (RICS) is used to measure molecular dynamics and concentrations from confocal-obtained images over time (Brown et al., 2008). The RICS program has been applied to measure the diffusion rates of the p53 protein in live HeLa cells (Hong et al., 2010), as well as distinguishing between bound and unbound intracellular fluorescent proteins (Digman and Gratton, 2009). In this Chapter, the RICS program was used to analyse the intracellular fluorescent protein responses of the porcine progenitor immune cells (L45hMGFP, L45pCIneo-DsRed2, L23hMGFP and L23pCIneo-DsRed2) following treatment with the free- and microencapsulated bacteria mammalian cell culture conditioned media (CM), and the co-cultured porcine fibroblast intestinal cellular (IPI-1) with free- and microencapsulated CM. Differences in the diffusion rate of the intracellular fluorescent proteins were used to determine if the intracellular environment of the
immune cells behaved differently to the probiotic/pathogenic bacteria strains, the free- and microencapsulated bacteria as well as to evaluate if the fluorescent protein itself influenced the cellular responses (i.e. the efficiency of each fluorescent protein as an intracellular bio-indicator). To date, no study has described the intracellular responses of immunological cell to probiotic CM treatment using fluorescent proteins and RICS.

7.2 **AIMS & OBJECTIVES**

The aims of this Chapter are:

- To investigate the effects of free bacteria and microencapsulated bacteria conditioned mammalian cell culture media on the fluorescence expression of the L45hMGFP, L45pCIneo-DsRed2, L23hMGFP and L23pCIneo-DsRed2 cell lines using Raster Image Correlation Spectroscopy (RICS) analysis.

- To investigate the effects of intestinal cells (IPI-1)/free bacteria and IPI-1/microencapsulated bacteria conditioned mammalian cell culture media on the fluorescence expression of the L45hMGFP, L45pCIneo-DsRed2, L23hMGFP and L23pCIneo-DsRed2 cell lines using Raster Image Correlation Spectroscopy (RICS) analysis.

The objectives of this Chapter are:

- To perform RICS analysis to compare the diffusion rate of the intracellular fluorescent proteins expressed by immune cells (L45hMGFP, L45pCIneo-DsRed2, L23hMGFP and L23pCIneo-DsRed2) treated with free- and microencapsulated bacteria-conditioned mammalian cell culture media.
To perform RICS analysis to compare the diffusion rate of the intracellular fluorescent proteins expressed by immune cells (L45hMGFP, L45pClneo-DsRed2, L23hMGFP and L23pClneo-DsRed2) treated with IPI-1/free- and IPI-1/microencapsulated bacteria-conditioned mammalian cell culture media.

7.3 MATERIALS AND METHODS

7.3.1 Raster Image Correlation Spectroscopy (RICS) Analysis of the Intracellular Fluorescence Expression by Immune Cells Treated Bacteria Conditioned Media

7.3.1.1 Data Acquisition using Fluorescence Correlation Spectroscopy (FCS) and ISS-Vista Software

Calibration using Fluorescein for RICS Analysis

The Leica TCS-SP5 and FCS software was used to obtain a calibration of fluorescein for further analysis using RICS. A solution of fluorescein (30 nM) was prepared in sodium hydroxide (0.1 M) (S8045, Sigma-Aldrich, Castle Hill, Australia). The scan speed for data acquisition was set to 500 Hz, the 256 x 256 format was selected and the pinhole was set to 1 airy unit in the Leica software. Excitation of fluorescein was performed using the Argon-488 laser (20 % maximum power, 3 % intensity) and fluorescence was detected using the dual avalanche photo diodes (APDs) and the 500 – 550 and 607- 683 nm filter. The XI port was selected for the mirror and the FCS Wizard was used to obtain FCS readings of the fluorescein solution. The 63 x 1.4 water immersion objective was used and a drop of de-ionised water was placed on the objective. A 22 mm x 40 mm cover slip was placed onto the water droplet/objective and approximately 3 drops of 30 nm fluorescein was placed...
onto the cover slip. The FSC software was calibrated for 100 ms and the calibration was set. The ISS Vista software was set to 500 KHz with an acquired time of 60 s and the “acquire raw data only for single point option” was selected. The “Run FCS Test” in the Leica FCS software was selected concurrently with the acquire option in the ISS Vista software; consecutive images were obtained. The raw data was saved and the SimFCS software was opened to determine the waist (Wo) of the fluorescein solution for analysis of samples using RICS software. The huge vector analysis was selected in the SimFCS software and the raw data file acquired from the ISS Vista software was opened. A large vector correlation was performed on the raw data; the fast forum transfer length was set to 128k, the plot each segment was selected and a sampling frequency of 500 000 was set, the channel 1 autocorrelation function was selected and the correlation plot was calculated. The fit of the data was selected and the waist (Wo) was allowed to fluctuate to obtain a value for further RICS analysis. The radiculate waist/axial waist (Wz/Wo) was set to 3 (for single photon ratio) and the diffusion (D1) was set to 300 μm s$^{-1}$, both values were fixed. The 2-photon formula option was de-selected and the “perform fit” was conducted. The Wo value was recorded for the RICS analysis of the fluorescence intensity of the cell lines in response to the bacteria treatments. The calibration of fluorescein was conducted if the confocal system was shut down.

**Acquisition of ISS Vista Data for the fluorescence expression of bacteria-conditioned media treated porcine progenitor immune cells**

The L45hMGFP, L45pClneo-DsRed2, L23hMGFP and L23pClneo-DsRed2 cells were treated with the free- and microencapsulated bacteria-CM in a final volume of 1 mL as outlined in Chapter 4, Section 4.3.1.2 and Section 4.3.1.4
(respectively) for 24 h. The transfected immune cells were also treated with IPI-1/free- and IPI-1/microencapsulated bacteria-CM in a final volume of 1 mL as outlined in Chapter 5, Section 5.3.1.2 and Section 5.3.1.3, for 24 h. The Leica TCS-SP5 software was used to image the cells and the ISS Vista software collected the FCS data for further analysis with the RICS software. The Argon-488 laser was used to excite the MGFP and pClneo-DsRed2 fluorescent plasmids; the maximum power was set to 20 % and the intensity was set to 3 %.

The data sets were acquired using the XYT time scan analysis; the “time between frames” was minimised and 150 acquisition frames was selected. The fluorescence was detected using the APD channels and the scan speed was set to 100 Hz. The mirror was set to the XI port and the 63 x 1.4 water immersion objective was used. A drop of de-ionised water was placed onto the objective; 10 μL of each cell sample (4 in total) was placed onto the 42 x 0.17 mm circular slides and covered with the 32 x 0.17 mm circular cover slip. The zoom was set to 14.9. The 500 – 550 and 607-683 nm filter was used for L45hMGFP and L23hMGFP cells; the data for the MGFP emission was collected in Channel 1. The 470 – 500 and 535 – 580 filter was used for L45pClneo-DsRed2 and L23pClneo-DsRed2 cells; the data for the pClneo-DsRed2 fluorescence emission was collected in Channel 3. The ISS Vista software was set to 100 KHz with an acquired time of 240 s, the external trigger was applied and the software collected data following the start of imaging by the Leica TCS-SP5 software. Data for each control and bacteria-treated cells were collected in triplicate.
7.3.1.2 Raster Image Correlation Spectroscopy (RICS) Analysis of the Fluorescence Expression by the Bacteria-Conditioned Media Treated Porcine Progenitor Immune Cells

The data acquired by the ISS Vista software was analysed using the RICS software developed by Professor Enrico Gratton and Dr. Michelle Digman in 2005 (Laboratory for Fluorescence Dynamics, University of California, Irvine) to determine the diffusion of the intracellular fluorescent proteins in response to the bacteria-conditioned media treatment.

The ISS Vista data for the control and bacteria-conditioned media treated cell lines was opened in the RICS software. The moving average of 10 was subtracted and fit of the data was performed. The Wo value determined from the fluorescein calibration was entered and the value was fixed. The pixel size was set at 0.05 and the G1 (intensity) and D1 (diffusion in μm² s⁻¹) was allowed to fluctuate. The “perform fit” option was selected and the G1 and D1 values were recorded.

7.4 RESULTS

7.4.1 Raster Image Correlation Spectroscopy (RICS) Analysis of the Intracellular Fluorescence Expression by Free- and Microencapsulated CM Media Treated Immune Cells

The diffusion rates of the intracellular fluorescent protein expression by the L45hMGFP, L45pCIneo-DsRed2, L23hMGFP and L23pCIneo-DsRed2 cells following treatment with the free- and microencapsulated-bacteria conditioned mammalian cell culture media (CM) is shown in Table 7.1. The intracellular fluorescent protein diffusion rate for all free- and microencapsulated bacteria CM
treated immune cells did exhibit significant differences comparative to the respective controls with the exception of the microencapsulated *S. pyogenes* CM treated L23pCIneo-DsRed2 cells. However, the controls, free- and microencapsulated bacteria CM treated L45hMGFP cells exhibited similar diffusion rates ranging from 0.00 to 1.55 µm$^2$ s$^{-1}$. The free- and microencapsulated-bacteria CM treated L23hMGFP and L23pCIneo-DsRed2 cells also exhibited similar diffusion rates with the exception of the free- and microencapsulated *B. lactis* HN019 (DR10™) treated L23pCIneo-DsRed2 cells. The free- control, *L. acidophilus* LAFTI® L10 and *B. lactis* HN019 (DR10™) treated L45pCIneo-DsRed2 cells exhibited different intracellular fluorescent protein diffusion rates to the microencapsulated CM treatments; a slower diffusion rate of 0.26 to 1.32 µm$^2$ s$^{-1}$ was observed for the free bacteria CM treatments with the microencapsulated CM treatments exhibiting a diffusion rate of 98.28 to 135.55 µm$^2$ s$^{-1}$ (Table 7.1). The microencapsulated *S. pyogenes* CM treated L45pCIneo-DsRed2 diffusion rate differed from the control and probiotics strains with a slower rate of 0.52 µm$^2$ s$^{-1}$. Examples of the RICS images are shown in Figure 7.1.
Table 7.1. The average diffusion coefficient (µm$^2$ s$^{-1}$) of the porcine progenitor immune cellular fluorescent protein following 24 h treatment with free- and microencapsulated bacteria-conditioned mammalian cell culture media ($n = 3$).

<table>
<thead>
<tr>
<th></th>
<th>L45hMGFP Free</th>
<th>L45pCIneo-DsRed2 Free</th>
<th>L23hMGFP Free</th>
<th>L23pCIneo-DsRed2 Free</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>D</td>
<td>SEM</td>
<td>D</td>
<td>SEM</td>
</tr>
<tr>
<td>Control</td>
<td>0.41</td>
<td>0.16</td>
<td>0.93</td>
<td>0.92</td>
</tr>
<tr>
<td>L10</td>
<td>0.86</td>
<td>0.21</td>
<td>0.00</td>
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<td>0.68</td>
<td>1.08</td>
<td>0.58</td>
</tr>
<tr>
<td>SP</td>
<td>0.80</td>
<td>0.55</td>
<td>0.18</td>
<td>0.05</td>
</tr>
</tbody>
</table>

D – diffusion of fluorescent protein in µm$^2$ s$^{-1}$; SEM – Standard Error of Mean; * p < 0.05; L10 – L. acidophilus LAFTI® L10; DR10™ – B. lactis HN019 (DR10™); SP – S. pyogenes. † Results differing from the control/other bacteria treatments.

Figure 7.1. Raster Image Correlation Spectroscopy Images for some of the progenitor immune cell treatments a) Control L23hMGFP cells, b) L23hMGFP treated with free S. pyogenes, c) Control L45pCIneo-DsRed2 cells, d) L45pCIneo-DsRed2 cells treated with microencapsulated S. pyogenes
7.4.2 Raster Image Correlation Spectroscopy (RICS) Analysis of the Intracellular Fluorescence Expression by the IPI-1/Free- and IPI-1/Microencapsulated Conditioned Media Treated Immune Cells

The intracellular fluorescent protein diffusion rates of the immune cells treated with the IPI/free- and microencapsulated bacteria CM are shown in Table 7.2. No significantly differences were observed for the IPI-1/free- and bacteria CM treatments comparative to the respective controls with the exception of the IPI-1/free *S. pyogenes* treated L45hMGFP cells. The diffusion rates of the IPI-1/free bacteria CM treated immune cells were observed to be similar for the majority of the IPI-1/microencapsulated CM treated immune cells. However, the IPI-1/free *S. pyogenes* treated L45hMGFP and L45pCIneo-DsRed2 cells exhibited higher diffusion rates comparative to the IPI-1/microencapsulated *S. pyogenes* treatments with the opposite result observed for the L23hMGFP and L23pCIneo-DsRed2 cells. The L45pCIneo-DsRed2 cells treated with the IPI-1/free *L. acidophilus* LAFTI® L10 CM exhibited a lower intracellular fluorescent protein diffusion rate compared to the IPI-1/microencapsulated *L. acidophilus* LAFTI® L10 CM treated cells. The IPI-1/free control CM treated L45hMGFP cells exhibited a higher intracellular fluorescent protein diffusion rate than the IPI-1/microencapsulated control cells. The intracellular fluorescent protein diffusion rates of the immune cells treated with the IPI-1/free- and microencapsulated bacteria CM (Table 7.2) exhibited higher rates than most of the the free- and bacteria CM treated cells (Table 7.1). Examples of the RICS images are shown in Figure 7.2.
Table 7.2. The average diffusion coefficient ($\mu$m$^2$ s$^{-1}$) of the porcine progenitor immune cellular fluorescent protein following 24 h treatment with intestinal cells (IPI-1)/free- and IPI-1/microencapsulated bacteria-conditioned mammalian cell culture media ($n = 3$).

<table>
<thead>
<tr>
<th></th>
<th>L45hMGFP</th>
<th>L45pClneo-DsRed2</th>
<th>L23hMGFP</th>
<th>L23pClneo-DsRed2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>D</td>
<td>SEM</td>
<td>D</td>
<td>SEM</td>
</tr>
<tr>
<td>Control</td>
<td>7.74</td>
<td>3.19</td>
<td>0.91</td>
<td>0.77</td>
</tr>
<tr>
<td>L10</td>
<td>25.66</td>
<td>13.52</td>
<td>32.08$^\dagger$</td>
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</tr>
<tr>
<td>DR10™</td>
<td>12.27</td>
<td>5.56</td>
<td>9.17</td>
<td>4.40</td>
</tr>
<tr>
<td>SP</td>
<td>40.66$^*$</td>
<td>7.81</td>
<td>4.69</td>
<td>4.45</td>
</tr>
</tbody>
</table>

D – diffusion of fluorescent protein in $\mu$m$^2$ s$^{-1}$; SEM – Standard Error of Mean; $^*$ p < 0.05; L10 – *L. acidophilus* LAFTI® L10; DR10™ – *B. lactis* HN019 (DR10™); SP – *S. pyogenes*. $^\dagger$ Results differing from the control/other bacteria treatments.

![Raster Image Correlation Spectroscopy Images](image-url)

**Figure 7.2.** Raster Image Correlation Spectroscopy Images for some of the progenitor immune cell treatments with intestinal cell co-cultures a) Control L45pClneo-DsRed2 cells, b) L45pClneo-DsRed2 treated with free *B. lactis* HN019 (DR10™) c) Control L45hMGFP cells, d) L45hMGFP cells treated with microencapsulated *S. pyogenes*
7.5 **DISCUSSION**

7.5.1 **Raster Image Correlation Spectroscopy (RICS) Analysis of the Intracellular Fluorescence Expression by Porcine Progenitor Immune Cells Treated with Conditioned Mammalian Cell Culture Media**

The RICS analysis used in this Chapter has provided a global measurement of the progenitor immune intracellular fluorescent protein diffusion over time (240 s) in response to the bacteria-conditioned media. The measurement of intracellular fluorescent proteins is taken in a 2D plane; the movement of the fluorescent protein in that 2D plane can be monitored over time even if the protein moves in and out of the frame of reference. Therefore, the diffusion of the proteins in that particular plane can be measured by RICS should these proteins move across in a latitude direction (the x axis), longitudinally (y axis) or up/down (z axis) within the cell.

Since the internal environment of cells is continuously undergoing dynamic activities involving cytoskeletal components, movement of proteins and RNA, etc. any changes in such activities, particularly on a large scale, would affect the intracellular dynamics. As outlined in Chapter 3, the fluorescent proteins (phMGFP and pClneo-DsRed2) transfected in the L45 (T cell) and L23 (B cell) cell lines were found to be expressed in parts of the nucleus and cytosol; therefore any changes of the intracellular environment in response to an external compound/stimulus may potentially influence the expression, location and/or diffusion of the fluorescent proteins.
Fluorescent Protein Diffusion Rates of Immune Cells Treated with Free and Microencapsulated Bacteria Conditioned Media (CM)

The diffusion rates of the immune cell fluorescent proteins treated with free and microencapsulated bacteria CM (Section 7.4.1) exhibited trends with a relationship between the immune cell type and the bacteria strain. For instance, the fluorescent protein diffusion rates for the free and microencapsulated *B. lactis* HN019 (DR10™) treated L45hMGFP and L45pCIneo-DsRed2 cells were higher than the control, *L. acidophilus* LAFTI® L10 and *S. pyogenes* treatments (Table 7.1). The L23hMGFP and L23pCIneo-DsRed2 cells did not exhibit the same response. Therefore, this observation supports the results found in Chapters 4 and 5 (proliferation response of immune cells) and Chapter 6 (gene expression of cytokines and CD markers) in which relationships exist between two factors (i.e. bacteria strain and immune cell type). It is possible that the probiotic treatments are influencing the development of the immune cells, for example, clonal selection or deletion of immune cells (an immunological process that determines if cells proceed to be competent, functional cells or eliminated).

Another example of the changes observed include the L45pCIneo-DsRed2 cells treated with microencapsulated bacteria CM. The fluorescent protein diffusion rates of the control and microencapsulated probiotics CM treated L45pCIneo-DsRed2 cells exhibited similar results (high diffusion rates) whereas the microencapsulated *S. pyogenes* treated cells was observed with a slow diffusion rate. This effect is possibly due to the pathogenic nature of the bacteria strain. Further, the microencapsulated *S. pyogenes* CM treated L23pCIneo-DsRed2 cells (Table 7.1) differed to the control and microencapsulated probiotic CM fluorescent protein
diffusion rates (significantly different to the control). This also is potentially a result of the pathogenic nature of *S. pyogenes*. From these results, the fluorescent protein diffusion rates of immune cells treated with microencapsulated bacteria were not different except the L45pCIneo-DsRed2 and L23pCIneo-DsRed2 cells. A relationship between the fluorescent protein and response of microencapsulated bacteria may also exist.

**Fluorescent Protein Diffusion Rates of Immune Cells Treated with Intestinal Cells and Free and Microencapsulated Bacteria Co-Cultured Conditioned Media (CM)**

Similar to the free and microencapsulated CM treated immune cells, the fluorescent protein diffusion rates of immune cells treated by intestinal cell co-cultures with free and microencapsulated bacteria CM exhibited relationships between the immune cell type, fluorescent protein type and bacteria strain (Table 7.2). Comparisons between the free and microencapsulated bacteria CM treated cells (Table 7.1) with the IPI-1/free and IPI-1/microencapsulated bacteria co-cultured CM (Table 7.2) reveals that the overall diffusion rates for the IPI-1 co-cultured CM treated cells were higher than the cells treated with the free- and microencapsulated bacteria CM. This could potentially be a regulatory function of the intestinal cells that subsequently influence the response of the immune cells as indicated by the differences in the fluorescent proteins diffusion rates. The L45hMGFP cells treated with IPI-1/free and IPI-1/microencapsulated probiotics CM produced similar fluorescent protein diffusion rates. This suggests that the microencapsulation environment may not affect the probiotic bacteria activities as indicated by the downstream response of the immune intracellular fluorescent protein diffusion rates. However, the IPI-1/free *S. pyogenes* treated L45hMGFP cells exhibited a high
diffusion rate comparative to the control and probiotic treatments that was significantly different to the control (Table 7.1). This result provides further support for immune cell responses to pathogenic bacteria strains.

7.6 CONCLUSION

The pathogenic strain, *S. pyogenes*, was observed to influence the diffusion rates of the immune intracellular fluorescent proteins in both free and microencapsulated states. The immune intracellular fluorescent protein diffusion rates were not strongly influenced by the probiotic strains. Such effects are potentially indicative of immunological intracellular events that are responding to the pathogen for further immunological attention. However, similar to the immune cell proliferation and gene expression responses to the bacteria CM treatments, trends were observed with a relationship between the immune cell type, bacteria strain, presence of intestinal cells and if the bacteria was microencapsulated. A relationship between immune cell type and bacteria strain was observed for the free and microencapsulated bacteria CM treatments in terms of the immune intracellular fluorescent protein diffusion rates. The intestinal cell co-cultures produced an overall higher diffusion rate comparative to the non-co-culture treatments potentially due to regulatory activities conveyed by the intestinal cells to the immune cells.

Tagging of specific intracellular proteins with fluorescent proteins enables the response of immune cells to the probiotic bacterial-produced soluble factors to be determined. The diffusion coefficient of the fluorescent proteins by the RICS program may provide information that increases the understanding of the
mechanisms involved in such a response and assist in treating intestinal diseases such as Crohn's disease.
8. CHAPTER EIGHT

General Discussion & Future Directions

This thesis investigated the responses of porcine progenitor T and B cells to soluble factors produced by probiotic bacteria under a number of conditions (Figure 8.1). Previous research has revealed the responses of immune and intestinal cells to probiotic bacteria are dependant on the strain of bacteria. To some extent, distinguished responses by the immune and intestinal cells also occur relative to the species of the cell’s host from probiotic bacteria treatment. The majority of research has involved innate immune cells (i.e. dendritic cells) from human and mouse hosts. Very little research has described the responses of porcine adaptive immune cells to probiotic bacteria. However, cells derived from pigs can be used as a potential indicator of both animal and human responses to probiotics. Therefore, the L45 (T cell) and L23 (B cell) porcine progenitor immune cell lines utilised in this thesis provide a suitable model for \textit{in vitro} analysis of immune cell responses to the soluble factors produced by probiotic bacteria.

Immune cell responses to probiotics reportedly occur in a strain specific manner. The findings presented in this thesis support this notion. Other trends in adaptive immune cell responses to the probiotic strains are presented in this thesis. One example of this was observed with intestinal cells (porcine cell line IPI-1) appearing to perform a regulatory activity subsequently influencing the downstream response of the immune cells. Microencapsulation also affected the activities and secretions of the probiotic bacteria and the subsequent exposure of the immune cells to these soluble factors elicited unique responses.
**Figure 8.1.** Project overview of the model development to determine the intracellular milieu responses of immune cells to probiotic bacteria using fluorescent progenitor adaptive immune cells, laser scanning confocal microscopy and image analysis software, Raster Image Correlation Spectroscopy (RICS).

**Microencapsulation of Probiotics – Fluorescence Microscopy Analysis of Viability**

The drive behind the microencapsulation of probiotics is to maintain the viability and health-promoting characteristics of probiotics, particularly those included in food products. The survival of microencapsulated probiotics in *in vitro* stomach
conditions has been reported including maintaining a higher viability than free bacteria treated in similar conditions (Chandramouli et al., 2004). In this thesis, the growth of microencapsulated probiotics was observed over 24 h. Determination of microencapsulated probiotics viability was conducted using fluorescence microscopy. To date, fluorescent bacterial viability kits have been applied to develop standard curves for free bacteria cells. In this thesis, viability standard curves of microencapsulated bacteria were developed using a fluorescence bacteria viability kit, LSCM and image analysis software (Bitplane Imaris). The development of the viability standard curve for microencapsulated probiotic bacteria was successfully conducted in this study. Such standard curves will have future applications for other investigations using microencapsulated probiotics including those incorporated into food products and provide real-time viability data.

Potential stress-related activities by the probiotic bacteria occur due to the confined environment. To date, no research has described the effects of microencapsulation on probiotic bacteria including changes in activities. In this study, any stress-related changes experienced by the microencapsulated probiotics due to the confined environment were evaluated by the downstream responses of the porcine immune cells. The responses evaluated included the proliferation, cytokine gene expression and intracellular fluorescent protein diffusion coefficients; the microencapsulated treatments were compared to the free bacteria treatments. Differences in the immune cell responses to the microencapsulated probiotic strain were observed. However, further investigations to fully elucidate the effects of microencapsulation effects on probiotic bacteria are required. Biochemical analysis
of the probiotic bacteria activities may provide further insight into the effects of microencapsulation.

**Immune Cell Model Expressing an Intracellular Fluorescent Protein**

To study the intracellular responses of immune cells to the probiotic bacterial-produced soluble factors (from free and microencapsulated bacteria), the porcine progenitor immune cells were transfected with two different fluorescent proteins (Monster green® fluorescent protein and DsRed2). The expression of these fluorescent proteins were not linked to any particular gene product or protein and occurred randomly throughout the cells. The diffusion rate of the intracellular fluorescent proteins was measured to determine any changes in the intracellular milieu to probiotic bacteria treatment.

The porcine immune cells were observed to produce fluorescence within 24 h of transfection indicating successful production of the fluorescent protein. Cells stimulated with mitogens (Con A and leupeptin treatment) exhibited similar fluorescence to control cells independent of the type of fluorescent protein. However, the opposite effect was observed for cells undergoing induced apoptosis by anisomycin, with almost the entire cell exhibiting fluorescent protein localisation. Previous research has described similar effects with several different GPF plasmids inducing apoptosis in several different cell lines (either fibroblast, carcinoma and hepatocytes origin) regardless of the fluorescent protein expression levels (Liu et al., 1999). Other reports have identified GFP as toxic to adult stem cells with cyan fluorescent protein and yellow fluorescent protein as non-toxic and able to produce long-term, stable fluorescence (Sherley and Taghizadeh, 2008). Two different
fluorescent proteins were used in this study, a GFP and red fluorescent protein. However, both were observed to express almost entire cell fluorescence in apoptotic porcine progenitor immune cells. The fluorescence expression of the cell lines was used as an indicator of apoptosis by the probiotic bacteria treatments.

**Downstream Responses of Porcine Immune Cells to Probiotic Bacterial-Produced Soluble Factors**

Probiotic bacterial strain-related activities were observed from the proliferation, cytokine gene expression and intracellular fluorescent protein diffusion responses of the porcine immune cells. The immune cell type, presence of intestinal cells and whether the probiotic bacteria were free or microencapsulated also influenced the responses of the porcine immune cells to the bacterial-produced soluble factors. This was reflected in the proliferation responses of the immune cells to the conditioned media.

The induction of pro-inflammatory, anti-inflammatory and regulatory cytokine gene expression in the porcine immune cells was observed following treatment with media conditioned by free and microencapsulated bacteria. The induction of these genes was also observed when the porcine immune cells were incubated in media that had been conditioned by co-cultures of intestinal cells with free and microencapsulated bacteria (outlined in Chapter 6). However, immune cells that were incubated with the microencapsulated probiotic bacteria treated media exhibited less pro-inflammatory, anti-inflammatory and regulatory cytokine gene expression compared to immune cells incubated with the free probiotic bacteria conditioned media.
Gene expression profiles consistent with a T\textsubscript{H}1 immunological response was observed for both the \textit{L. acidophilus} LAFTI® L10 and \textit{B. lactis} HN019 (DR10\textsuperscript{TM}) treated immune cells. This observation supports previous research that describes the upregulation of T\textsubscript{H}1-associated genes from \textit{L. acidophilus} LAFTI® L10 treatment of \textit{in vivo} human mucosa (van Baarlen et al., 2011). In addition, upregulation of the T\textsubscript{H}1 associated cytokine, IFN-\gamma, in PMBC was reported for \textit{B. lactis} HN019 (DR10\textsuperscript{TM}) treatment of elderly subjects (Arunachalam et al., 2000). However, the presence of intestinal cells influenced the cytokine gene expression profile of the immune cells treated with microencapsulated probiotic bacteria.

Similar to the immune cells treated with the microencapsulated probiotic bacteria, the intestinal cells/microencapsulated probiotic co-cultures produced less cytokine gene expression comparative to the intestinal cells/free probiotic co-culture treated immune cells. A T\textsubscript{H}1, T\textsubscript{H}2 and regulatory immune response was observed for the immune cells incubated with the intestinal cells/free bacteria co-culture media. It is possible that the intestinal cells are responding to the free bacteria by producing a number of soluble factors that subsequently influences the type of response by immune cells. Although the gene expression of cytokines by the immune cell encompass T\textsubscript{H}1, T\textsubscript{H}2 and regulatory cytokines, the actual production of cytokines for further immunological signalling would be dependent on the threshold of a particular cytokine. It has been reported that the immune cell activation threshold differs for each cytokine (Chen et al., 2000). Therefore the type of immunological response would also be dependent on the threshold of the cytokine and if the probiotic bacteria stimulus is sufficient to result in further signalling by the immune cells. Further research involving the secretion of cytokines will provide more information about the
type of immunological response to the intestinal cells/free bacteria co-cultures. This was not addressed in this study as the focus was to investigate the immune intracellular molecular events associated with probiotic bacteria soluble factors.

The results presented in this thesis indicate that IPI-1 intestinal cells interact with the probiotic bacteria via physical contact, in a manner possibly involving mammalian TLRs and the bacteria PAMPs. This is reflected in the cytokine gene expression by the immune cells treated with the intestinal cells/probiotic co-culture conditioned media. The physical contact between the probiotic bacteria and the intestinal cells results in the production of soluble factors (e.g. cytokines and chemokines). The immune cell response to these soluble factors indicates the responses intestinal cells may have to the probiotic bacteria. The response of the immune cells to the intestinal cells/microencapsulated probiotic conditioned media differs to the intestinal cells/free probiotic conditioned media treatments. This may indicate that the physical interactions between the intestinal cells and probiotic bacteria are important in downstream inducement of immunological responses by adaptive immune cells. However, the possibility that the confined microencapsulation environment affects the activities of probiotic bacteria should not be discounted.

Intracellular responses produced by immune cells to the probiotic bacteria soluble factors were analysed using the intracellular diffusion rates of fluorescent proteins (Chapter 7). To our knowledge this is the first report of intracellular responses by immune cells to probiotic bacteria using fluorescent proteins as a bio-indicator. Trends were observed for the diffusion rates of the immune cell
intracellular fluorescent proteins in response to the conditioned media. In most cases, the diffusion rates for the probiotic treatments (including free, microencapsulated and intestinal cell co-cultures) were similar to the respective controls. The immune cells treated with pathogen (*S. pyogenes*)-conditioned media produced diffusion rates that differed from the probiotic and control treated immune cells and was independent of the immune cell type and fluorescent protein used. Therefore the internal milieu of the immune cells is influenced by the type of bacteria strain the cells are exposed to as reflected by the diffusion rates of the immune intracellular fluorescent protein. In addition, when intestinal cells were co-cultured with the bacteria and immune cells a higher diffusion rate of fluorescent proteins was observed compared to the non-co-culture treatments. This may be due to regulatory signals conveyed by the intestinal cells to the immune cells.

The tagging of specific intracellular proteins with fluorescent proteins may assist in identifying the protein and signal transduction pathway involved in immunological responses to probiotic bacteria. Advancements in fluorescence microscopy technologies now allow whole body imaging of mice/rats and the *in vivo* tracking of fluorescent molecules. Such technology can be applied to track and monitor probiotic bacteria *in vivo* in animal models. However, experimentation with such equipment is limited at this time due to high costs and limited availability. Nonetheless, the development of image analysis software, such as the SIMfcs program used for RICS analysis, allows for more information to be extracted from fluorescence microscopy. Ultimately, an increased understanding of the intracellular mechanisms involved in the adaptive immune cell responses to probiotic bacteria may assist in treating intestinal diseases such as Crohn's disease.
Future Directions

The majority of probiotic bacteria research has encompassed *ex vivo* and *in vivo* experimentation. This provides information about the final stages of immunological responses. However, diet, illness, environment and stress may influence immunological responses in *in vivo* conditions. Although *ex vivo* provides a model that is not influenced by as many factors, variation in experimental design may influence the results. Testing of probiotic strains with *in vitro* models can be used to identify potential probiotic strains based on a selection criteria outlined for probiotic characteristics (Tuomola et al., 2001). The concept of *in vitro* models can also encompass immunological cell line models to identify the type of immunological responses occurring to probiotic strains.

This study involved the identification of adaptive immune cell responses to free bacteria soluble factors, microencapsulated bacteria soluble factors, and the influence of intestinal cells on the response of immune cells to the free and microencapsulated probiotics. The intracellular molecular response to the different probiotic treatments revealed probiotic strain-specific responses for the free and microencapsulated bacteria. Further research involving immune intracellular molecular events in response to probiotics may be used to determine differences in probiotic dose responses including a minimal dose amount, the cell signalling pathways involved in immune responses, and the upregulation and downregulation of specified genes that potentially influences downstream protein production.

The model presented in this thesis also provides the opportunity to evaluate the effects of probiotic bacterial strains on immune cell responses; in this case
alginate microencapsulation effects were investigated. A number of different types of microencapsulating chemicals are used to protect probiotics and producing an immunological profile of responses to these different types may identify chemicals that compliment the activities of the probiotic strains. In addition, the testing of different probiotic strains will allow for an immunological profile to be developed.

Evaluation of intracellular molecular events in response to probiotic bacteria can be further applied to intestinal cell models, in particular those of diseased intestine conditions such as IBD and Crohns Disease. As mentioned previously, the dose response of immune cells to probiotics may be determined using intracellular molecular tracking techniques such as fluorescence microscopy and image analysis software. This can also be applied to intestinal cell lines. Responses of diseased intestinal cells to pathogenic bacteria can be compared to the responses by intestinal cell to probiotic strains; differences identified for the pathogenic and probiotic bacteria treatments may assist in increasing the understanding of how to treat/prevent intestinal diseases.
APPENDIX

A.1 Chapter Two – Growth of Probiotic Bacteria in Mammalian Cell Culture Conditions & Confocal Microscopy Analysis of Microencapsulated Probiotic Bacteria

A.1.1 Bacteria Standard Curves

Standard curves were prepared for *L. acidophilus* LAFTI L10, *B. lactis* HN019 (DR10™) and *S. pyogenes* for enumeration purposes during this project. Standard curves were prepared as outlined in the University of Western Sydney Microbiology 1 Laboratory Manual (Markham, 2005). The cultures were incubated for approximately 18 h at 37 °C, conditions as outlined above. McCartney bottles were prepared with 4 mL of either sterile MRS or BHI broth and a serial dilution of $\frac{1}{2}$, $\frac{1}{4}$, $\frac{1}{8}$, $\frac{1}{16}$ and $\frac{1}{32}$ was prepared for each bacteria. Each dilution was added to a flat-bottom, clear 96-well plate at a final volume of 200 µL in 9 replicates. Blanks were also prepared by adding 200 µL of MRS or BHI broth (also 9 replicates). The absorbance was measured at 600 nm by the Bio-Rad Benchmark Plus Microplate Spectrophotometer (Model No. Benchmark Plus, Serial No. 10687, Bio-Rad, Australia). A serial dilution at $10^{-5}$, $10^{-6}$ and $10^{-7}$ were prepared in sterile 0.85% NaCl (S5886, Sigma-Aldrich, Australia) solution for each bacterium and 0.1 mL of each dilution was added to either MRS or BHI agar plates. Spread plates for all serial dilutions were prepared in 9 replicates. The plates were incubated at 37 °C for 24 h in aerobic conditions for *S. pyogenes*, and anaerobic conditions for *L. acidophilus* LAFTI L10 and *B. lactis* HN019 (DR10™). The colony forming units per mL (CFU mL$^{-1}$) were calculated from the spread plates using the $10^{-5}$ dilution for *S. pyogenes*, $10^{-6}$ dilution for *L. acidophilus* LAFTI L10 and *B. lactis* HN019 (DR10™). The log$_{10}$ CFU mL$^{-1}$ were calculated for the $\frac{1}{2}$, $\frac{1}{4}$, $\frac{1}{8}$, $\frac{1}{16}$ and $\frac{1}{32}$ serial
dilutions and was plotted against the absorbance values. These standard curves were used to enumerate the bacteria cultures in Chapters 4, 5, 6 and 7.

**Figure A.1.1** – Standard curve for *L. acidophilus* LAFTI® L10

**Figure A.1.2** – Standard curve for *B. lactis* HN019 (DR10™)

**Figure A.1.3** – Standard curve for *S. pyogenes*
### Table A.1.1 – MRS Broth Formula (CM0359, Oxoid Ltd.)

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<th>Ingredient</th>
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<tbody>
<tr>
<td>Peptone</td>
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<td>Manganese sulphate 4H₂O</td>
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pH 6.2 ± 0.2 @ 25°C

### Table A.1.2 – BHI Broth Formula (CM1135, Oxoid Ltd.)

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pH 7.4 ± 0.2 @ 25°C

### A.2 Chapter Two – Development of a fluorescent immune cell model to indicate intracellular immune responses to probiotic bacteria

#### A.2.1 Dulbecco’s Modified Eagle’s Media (DMEM) Preparation

Stock DMEM (refer to Appendix Table A.1.1 for media formulation details) was prepared by adding 13.48 g of high glucose DMEM powder (12800-082, Gibco®, Invitrogen; Mulgrave, Australia) to 3.7 g of Sodium Bicarbonate Hybrid-Max® (S4019-500G, Sigma, Castle Hill, Australia) and dissolved in 800 mL of MilliQ water. The pH was adjusted to 7.6 and MilliQ water was added making a final volume of 1 L. The media was sterilised using a sterile 0.22 µm Steritop™ Filter Unit (SCGPT10RE, Millipore, Kilsyth, Australia) that was connected to a sterile 1 L Schott bottle aseptically in the Clyde-Apac BH2000 series laminar flow hood (class 2 safety cabinet). This stock media was stored at 4 °C.
Incomplete DMEM was prepared by adding 10 mL of 100 mM sodium pyruvate (Trace 21-154-0100V), 10 mL of non-essential amino acids (NEAA) (Trace 21-145-0100V), 10 mL of 5000 µg mL$^{-1}$ penicillin/streptomycin (Trace 21-145-0100V) and 10 mL of 250 µg mL$^{-1}$ amphostat B (Thermo Trace 21-144-0050V) to stock DMEM making a final volume of 1 L. β-Mercaptoethanol (M 6250; Sigma-Aldrich, Sydney, Australia) was added to the incomplete DMEM by preparing a diluted solution; 4 µL of β-mercaptoethanol was added to 1 mL of sterile stock. This 1 mL solution was transferred to make up the 1 L of incomplete media; the incomplete DMEM was stored at 4 ºC (refer to Appendix Table A.1.2 for non-essential amino acid details).

Complete media was prepared by adding heat inactivated (60 ºC; 30 min) foetal bovine serum (FBS) (10100-147, Gibco; Invitrogen, Mulgrave, Australia) to incomplete DMEM. The FBS was added at a final concentration of 10 % and the complete DMEM was stored at 4 ºC. The complete media was incubated at 37 ºC prior to use.

**Figure A.2.1** – The vector diagram of phMGFP. Source: Promega [Accessed 06.11.09]

http://www.promega.com/figurespopup.asp?partno=e6421&product=monster+green%3csup%3e%26%23x00ae%3b%3c%2fsup%3e+fluorescent+protein+phmgfp+vector&fn=3898ma
Figure A.2.2 – The vector diagram of pDsRed2. Source: Clontech [Accessed 06.11.09]

Figure A.2.3 – The vector diagram of pClneo. Source: Promega [Accessed 06.11.09]
Table A.2.1 – Media formulation of DMEM powder (Cat. No. 12800, Invitrogen)

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<td>Glycine</td>
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<tr>
<td>L-Isoleucine</td>
<td>131</td>
<td>105</td>
<td>0.802</td>
</tr>
<tr>
<td>L-Leucine</td>
<td>131</td>
<td>105</td>
<td>0.802</td>
</tr>
<tr>
<td>L-Lysine hydrochloride</td>
<td>183</td>
<td>146</td>
<td>0.798</td>
</tr>
<tr>
<td>L-Methionine</td>
<td>149</td>
<td>30</td>
<td>0.201</td>
</tr>
<tr>
<td>L-Phenylalanine</td>
<td>165</td>
<td>66</td>
<td>0.4</td>
</tr>
<tr>
<td>L-Serine</td>
<td>105</td>
<td>42</td>
<td>0.4</td>
</tr>
<tr>
<td>L-Threonine</td>
<td>119</td>
<td>95</td>
<td>0.798</td>
</tr>
<tr>
<td>L-Tryptophan</td>
<td>204</td>
<td>16</td>
<td>0.0784</td>
</tr>
<tr>
<td>L-Tyrosine disodium salt dihydrate</td>
<td>261</td>
<td>104</td>
<td>0.398</td>
</tr>
<tr>
<td>L-Valine</td>
<td>117</td>
<td>94</td>
<td>0.803</td>
</tr>
<tr>
<td><strong>Vitamins</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Choline chloride</td>
<td>140</td>
<td>4</td>
<td>0.0286</td>
</tr>
<tr>
<td>D-Calcium pantothenate</td>
<td>477</td>
<td>4</td>
<td>0.00839</td>
</tr>
<tr>
<td>Folic Acid</td>
<td>441</td>
<td>4</td>
<td>0.00907</td>
</tr>
<tr>
<td>Niacinamide</td>
<td>122</td>
<td>4</td>
<td>0.0328</td>
</tr>
<tr>
<td>Pyridoxine hydrochloride</td>
<td>204</td>
<td>4</td>
<td>0.0196</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>376</td>
<td>0.4</td>
<td>0.00106</td>
</tr>
<tr>
<td>Thiamine hydrochloride</td>
<td>337</td>
<td>4</td>
<td>0.0119</td>
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<tr>
<td>i-Inositol</td>
<td>180</td>
<td>7.2</td>
<td>0.04</td>
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<tr>
<td><strong>Inorganic Salts</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calcium Chloride (CaCl₂) (anhyd.)</td>
<td>111</td>
<td>200</td>
<td>1.8</td>
</tr>
<tr>
<td>Ferric Nitrate (Fe(NO₃)₃·9H₂O)</td>
<td>404</td>
<td>0.1</td>
<td>0.000248</td>
</tr>
<tr>
<td>Magnesium Sulfate (MgSO₄) (anhyd.)</td>
<td>120</td>
<td>97.67</td>
<td>0.814</td>
</tr>
<tr>
<td>Potassium Chloride (KCl)</td>
<td>75</td>
<td>400</td>
<td>5.33</td>
</tr>
<tr>
<td>Sodium Chloride (NaCl)</td>
<td>58</td>
<td>6400</td>
<td>110.34</td>
</tr>
<tr>
<td>Sodium Phosphate monobasic (NaH₂PO₄·H₂O)</td>
<td>138</td>
<td>125</td>
<td>0.906</td>
</tr>
<tr>
<td><strong>Other Components</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D-Glucose (Dextrose)</td>
<td>180</td>
<td>4500</td>
<td>25</td>
</tr>
<tr>
<td>Phenol Red</td>
<td>376.4</td>
<td>15</td>
<td>0.0399</td>
</tr>
<tr>
<td>Sodium Pyruvate</td>
<td>110</td>
<td>110</td>
<td>1</td>
</tr>
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</table>
### Table A.2.2 – Non-essential amino acids (Cat. No. 12800, Invitrogen)

<table>
<thead>
<tr>
<th>COMPONENTS</th>
<th>Molecular Weight</th>
<th>Concentration (mg/L)</th>
<th>mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycine</td>
<td>75</td>
<td>750</td>
<td>10</td>
</tr>
<tr>
<td>L-Alanine</td>
<td>890</td>
<td>∞</td>
<td></td>
</tr>
<tr>
<td>L-Asparagine</td>
<td>1320</td>
<td>∞</td>
<td></td>
</tr>
<tr>
<td>L-Aspartic acid</td>
<td>1330</td>
<td>∞</td>
<td></td>
</tr>
<tr>
<td>L-Glutamic Acid</td>
<td>1470</td>
<td>∞</td>
<td></td>
</tr>
<tr>
<td>L-Proline</td>
<td>1150</td>
<td>∞</td>
<td></td>
</tr>
<tr>
<td>L-Serine</td>
<td>1050</td>
<td>∞</td>
<td></td>
</tr>
</tbody>
</table>


### A.3 Publications

#### Books


#### Book Chapters


A.4 Presentation/ Awards

Presentations

2010  
*Australian Institute of Food Science and Technology John Christian Young Scientist Food Microbiology Award Presentations*  
- Oral presentation: “Cellular interactions of probiotic bacterial with progenitor immune cells”

2010  
*International Probiotics and Prebiotics Conference - Slovakia*  
- Oral presentation: “Cellular interactions of probiotic bacterial with progenitor immune cells”

2010  
*UWS Annual Postgraduate Conference*  
- Oral presentation: “Biochemical and physiological interactions of probiotic bacteria with mammalian cells”

2009  
*AusBiotech & GlaxoSmith Kline Student Excellence Awards*  
- Oral presentation: “Biochemical and physiological interactions of probiotic bacteria with mammalian cells”

2009  
*UWS Annual Postgraduate Conference*  
- Oral presentation: “Biochemical and physiological interactions of probiotic bacteria with mammalian cells”

2008  
*UWS Annual Postgraduate Conference*  
- Oral presentation: “Biochemical and physiological interactions of probiotic bacteria with mammalian cells”

Awards

2010  
*International Probiotic and Prebiotics Conference - Slovakia*  
- Nominated as a candidate for the Young Scientist Award

2010  
*John Christian Young Scientist Food Microbiology Award (AIFST)*  
- 1st Prize Oral Presentation

2009  
*AusBiotech & GlaxoSmith Kline Student Excellence Awards*  
- 2009 NSW Finalist

2008  
*UWS Annual Postgraduate Conference*  
- 1st Prize Oral Presentation (College of Health and Science Research Futures Postgraduate Forum Postgraduate Award - Centre for Plant and Food Science section)
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