

**Probiotic-Derived Factors for the Treatment and Prevention
of 5-Fluorouracil-Induced Intestinal Mucositis**

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Declaration

This thesis contains no material that has been accepted for the award of any other degree or diploma in any University or other tertiary institution and to the best of my knowledge and belief contains no material previously published or written by another person except where due reference has been made in the text.

I give consent for this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

Date

Luca Prisciandaro

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Dedication

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Abstract

5-fluorouracil (5-FU) is one of the most commonly prescribed anti-neoplastic drugs in modern cancer treatment. Although the drug is effective at destroying cancer cells, its administration is accompanied by serious, dose-limiting side effects, amongst the most prevalent of which is intestinal mucositis. This disorder is characterised by ulceration and inflammation of the small intestine, and sufferers often experience severe abdominal pain, nausea and diarrhoea. Despite its predominance, there are currently no definitive treatments for intestinal mucositis.

Probiotics are defined as live bacteria which are able to exert beneficial physiological or therapeutic effects. Strains can be sourced from either food or the human microbiota, but must meet specific requirements prior to being officially recognised as probiotics. The mechanisms of probiotic action are highly species and strain specific, however, a number of strains have been shown to exert beneficial effects which may be suited to the treatment of intestinal mucositis. These include; inhibition of pathogenic bacterial growth and inflammation, maintenance of cell cycling and strengthening of the intestinal barrier. While the majority of probiotic research has focused on the use of live bacteria, there has been a recent interest in bioactive factors that are secreted by the bacterial cells into the cell-free supernatant (SN). There are a range of benefits to using SNs in preference to live bacteria, such as reduced risk of sepsis and greater quality control during production. This thesis represents the first detailed examination into the efficacy of probiotic-based SNs in the treatment of 5-FU-induced intestinal mucositis.

Firstly, four different probiotic SNs were investigated *in vitro* for their ability to maintain cell growth following administration of 5-FU. The two strains deemed most effective were then assessed in an *in vivo* model of intestinal mucositis. Rats were treated with SNs both before and after 5-FU administration. Improvement was reported in some indicators of intestinal damage in rats following SN administration. However, the overall effects were less pronounced than expected, given the extent of improvement reported in the *in vitro* model. These findings suggested that a different screening method was required prior to *in vivo* examination, and that the current *in vivo* treatment protocol required review.

As mucositis occurs only following chemotherapy administration, there is opportunity to administer therapeutic compounds prior to the onset of the disorder with the aim of preventing its development, rather than treating the damage. Two strains, *Lactobacillus rhamnosus* GG (LGG) and *Escherichia coli* Nissle 1917 (EcN), were examined for their ability to prevent 5-FU-induced reduction in intestinal barrier function and increased epithelial cell apoptosis in an *in vitro* model. Both SNs inhibited 5-FU-induced changes to barrier function and apoptosis. The success of these strains in a preventative treatment regime warranted further investigation *in vivo*. However, in the rat model of 5-FU-induced mucositis, no significant protective effects were observed. These findings highlighted inconsistencies between *in vitro* and *in vivo* models. One reason for this disagreement may have been due to the degradation of active compounds during gut transit. In order to determine if acidic or proteinase-rich conditions (two characteristics of the gastric environment) altered the efficacy of LGG and EcN SNs, a small *in vitro* pilot study was performed. All SNs not exposed to either acidic

or proteinase-rich conditions were effective in maintaining cell proliferation following 5-FU administration, but the efficacy of LGG SN was significantly reduced following protease- and acid-treatment. However, neither treatment diminished the efficacy of EcN SN. These results suggested a requirement for new administration techniques to allow the SNs to reach their target area.

In summary, this thesis explores the potential use of probiotic-derived factors to treat 5-FU-induced intestinal mucositis. It describes the capacity for LGG and EcN SNs to improve parameters of chemotherapy-induced damage *in vitro*. These strains were less effective *in vivo*, however, further investigations into effective delivery methods are warranted to ensure that the active compounds reach the small intestine. This thesis provides support for future investigations into the use of probiotic SNs for the treatment of intestinal mucositis.

Abbreviations

5-FU	5-fluorouracil
ANOVA	Analysis of variance
BR11	<i>Lactobacillus fermentum</i> BR11
CEC	CytoScan electron carrier
CM	Culture media
DMEM	Dulbecco's modified eagle medium
DPBS	Dulbecco's phosphate buffered saline
DSS	Dextran Sulphate Sodium
EcN	<i>Escherichia coli</i> Nissle 1917
EGCG	Epigallocatechin gallate
EGF	Epidermal growth factor
GLP-2	Glucagon-like peptide-2
HID-AB	High iron diamine-alcian blue
IFN	Interferon
IL	Interleukin
JI	Jejunum-ileum junction
LGG	<i>Lactobacillus rhamnosus</i> GG
LPS	Lipopolysaccharide
MCP	Monocyte chemotactic protein
MPO	Myeloperoxidase
MRS	de Man rogosa and sharpe
MTX	Methotrexate

NF- κ B	Nuclear factor- κ B
PAS	Periodic acid-schiff
ROS	Reactive oxygen species
SN	Supernatant
TEER	Transepithelial electrical resistance
TH-4	<i>Streptococcus thermophilus</i> TH-4
TNBS	Trinitrobenzene sulphonic acid
TNF	Tumour necrosis factor
TSB	Tryptic soy broth
ZO	Zonula occluden

Chapter 1. Evidence supporting the use of probiotics for the prevention and treatment of chemotherapy-induced intestinal mucositis

1.1 Intestinal mucositis

Chemotherapy and radiotherapy are common and effective treatments for many forms of cancer; however, the cytotoxic effect of these treatments presents a major oncological problem (Gibson and Keefe, 2006; Logan *et al.*, 2007). Radiotherapy and chemotherapy target rapidly dividing neoplastic cells, but can also affect the progenitor cell populations located in various sites throughout the human body (Duncan and Grant, 2003). The epithelia of the gastrointestinal tract are particularly susceptible; hence, radiotherapy and chemotherapy can often lead to the development of mucositis (Duncan and Grant, 2003; Gibson and Keefe, 2006; Triantafyllou *et al.*, 2008). Mucositis is a familiar disorder, with approximately 40% of patients receiving standard-dose chemotherapy, and almost 100% of patients receiving high-dose chemotherapy, being diagnosed with the condition (Keefe *et al.*, 1997). Symptoms of gastrointestinal mucositis include severe inflammation, ulceration, abdominal bloating, diarrhoea, nausea and intense abdominal pain (Logan *et al.*, 2007; Sonis *et al.*, 2004).

Our understanding of the pathogenesis of intestinal mucositis remains incomplete. It was originally hypothesised that intestinal damage occurred solely as a result of increased intestinal epithelial cell apoptosis due to chemotherapy treatment; however, more recent theories suggest an important role for pro-inflammatory cytokines in the development of the disorder (Sonis, 1998). This proposed mechanism comprises five overlapping stages (Scully *et al.*, 2003; Sonis, 1998). The first stage begins immediately following treatment with cytotoxic agents, involving indirect tissue damage as a result of the production of reactive oxygen species (ROS). The second stage is associated with the activation of transcription factors,

most importantly nuclear factor- κ B (NF- κ B; Chang *et al.*, 2012). NF- κ B activation promotes the up-regulation of genes which disrupt mucosal integrity, including the pro-inflammatory cytokines, tumour necrosis factor (TNF)- α , interleukin (IL)-1 β and IL-6 (Sonis, 2002). In the third stage, pro-inflammatory cytokines act via positive feedback to induce further activation of NF- κ B and hence, further pro-inflammatory cytokine production. Recent investigations have shown that other biologically active proteins or pro-inflammatory mediators are also up-regulated during this stage, resulting in an inflammatory cascade and the activation of matrix metalloproteinases, which leads to further epithelial damage (Sonis, 2002). It is only at the fourth stage of mucositis that the condition becomes clinically evident, as damage to the epithelial wall facilitates bacterial colonisation, resulting in significant pain. This stage also involves the loosening of tight junctions in the epithelial wall and the subsequent loss of barrier function, allowing the transfer of harmful luminal antigens into the surrounding intestinal tissue (Keefe *et al.*, 2000). The final phase only occurs following discontinuation of cancer therapy, and is associated with reepithelialisation of the mucosa and the gradual return to typical mucosal appearance and function.

Recently, there has been a proliferation of studies which suggest that the gastrointestinal microbiota and mucins are altered by chemotherapeutic agents and may be involved in the development of mucositis (Stringer *et al.*, 2007a; Stringer *et al.*, 2009a; Stringer *et al.*, 2009b). Changes to the composition of the microbiota can have serious implications for the host as it is involved in a number of important functions including maintenance of immunity, protection from pathogenic invasion, and nutrient processing all of which may be compromised by chemotherapy. Chemotherapy-induced alterations to the gut microbiota are yet to be investigated in

great detail; however, recent literature suggests that effects occur in an agent-and organ-specific manner (Stringer *et al.*, 2009a). For example, 5-fluorouracil (5-FU) administration has been reported to decrease the total number of *Clostridium spp.*, *Staphylococcus spp.* and *Escherichia spp.* in the stomach, whereas in the jejunum, a decrease in *Lactobacillus spp.* and *Clostridium spp.* coincided with an increase in *Escherichia spp.* Colonic *Lactobacillus spp.* decreased with 5-FU, whereas both *Escherichia spp.* and *Clostridium spp.* were increased (Stringer *et al.*, 2009b). In contrast, irinotecan administration decreased *Enterococcus spp.*, *Serratia spp.* and *Peptostreptococcus spp.* in the stomach, and increased jejunum *Enterococcus spp.*, *Serratia spp.* (perhaps due to bacterial overflow from the stomach), *Lactobacillus spp.* and *Clostridium spp.*; and increased colonic *Escherichia spp.* and *Clostridium spp.* (Stringer *et al.*, 2007b).

In rodent models, treatment with 5-FU has been shown to decrease goblet cell numbers, and increase mucin secreting cavitated cell numbers in the crypts of the jejunum (Stringer *et al.*, 2009b). These changes could inhibit the protective capabilities of the mucosal barrier following the depletion of stored mucins (Stringer *et al.*, 2009b), thereby rendering the gut more susceptible to chemotherapy damage and subsequent pathogenic invasion.

1.2 Probiotics

With identification of an altered microbial environment and increased pro-inflammatory cytokine expression as key components of intestinal mucositis, probiotics represent a promising therapeutic option. Probiotics can be defined as live bacteria which, when administered in sufficient numbers, are able to exert beneficial

physiologic or therapeutic activities (Sartor, 2004). Bacteria can be derived from sources such as cultured food and the normal human microbiota, but must meet certain criteria including complete identification at genus, species and strain level (Borchers *et al.*, 2009). Probiotic bacteria are most commonly of the *Lactobacillus* or *Bifidobacterium* genera, although strains have also been identified from *Enterococcus*, *Streptococcus* and *Lactococcus* species, while certain non-pathogenic *Escherichia* strains are also classified as probiotics (Borchers *et al.*, 2009). Furthermore, probiotic strains can be genetically engineered to secrete specific bioactive compounds such as anti-inflammatory IL-10 (Pang *et al.*, 2009, Steidler *et al.*, 2000).

A high degree of species and strain specificity is associated with the beneficial effects exerted by probiotics, and as such, the mechanisms underlying these effects are not completely understood. In addition to strain or species, mechanisms are also dependent on factors such as the bacterial environment and the disease setting under investigation (Shanahan, 2004). Common mechanisms of action for probiotics include inhibition of pathogenic enteric bacteria, improvement of epithelial barrier function and manipulation of host immunoregulation (Sartor, 2004).

1.3 Rationale for use of probiotic-based therapies

Intestinal mucositis is characterised by a spectrum of deleterious effects on the gastrointestinal tract, including but not limited to, uncontrolled inflammation (Sonis, 2002), increased intestinal permeability (Keefe *et al.*, 2000), pathogen load (Stringer *et al.*, 2009a) and pro-inflammatory cytokine expression (Logan *et al.*, 2007, Sonis *et al.*, 2004), reduction of mucin levels (Stringer *et al.*, 2009b), oxidative damage

(Sonis, 1998) and increased cell apoptosis (Keefe *et al.*, 2000). Interestingly, there is evidence, based largely on data from other intestinal disorders, to suggest that probiotics may be an effective method of treating each individual component (Table 1) and thus, possibly mucositis overall.

1.3.1 Anti-inflammatory effects

Inflammation plays an important role in the development of intestinal mucositis. Treatment with anti-neoplastic agents activates NF- κ B, which in turn triggers a number of pro-inflammatory cytokines such as TNF- α (Logan *et al.*, 2007). Certain probiotic strains have anti-inflammatory properties and present a viable option for counteracting this component of intestinal mucositis. Sokol and colleagues isolated *Faecalibacterium prausnitzii*, a strain found in the microbiota of Crohn's disease patients associated with reduced risk of postoperative recurrence (Sokol *et al.*, 2008). This strain was investigated for anti-inflammatory properties both *in vivo* and *in vitro*. Stimulation of peripheral blood mononuclear cells with *F. prausnitzii* significantly reduced pro-inflammatory IL-12 and interferon (IFN)- γ levels, and increased release of the anti-inflammatory cytokine, IL-10. Furthermore, in Caco-2 cells with a reporter gene for NF- κ B activity, treatment with live *F. prausnitzii* had no effect; however, treatment with the bacterial supernatant (SN) completely inhibited NF- κ B expression (Sokol *et al.*, 2008). With the paramount role of NF- κ B in the development of intestinal mucositis, probiotics capable of reducing NF- κ B expression represent promising therapeutic candidates. Petrof and colleagues treated a mouse colon cell line with *Lactobacillus plantarum* conditioned media and reported reduced NF- κ B and monocyte chemoattractant protein-1 (MCP-1), an inflammatory chemokine involved in leukocyte recruitment (MacDermott, 1999),

binding activity in areas of inflammation following activation by a TNF-receptor (Petrof *et al.*, 2004). Conditioned media from *Bifidobacterium breve* Yakult and *Bifidobacterium bifidum* Yakult, administered individually, reduced pro-inflammatory IL-8 secretion in human HT-29 epithelial cells stimulated with TNF- α (Imaoka *et al.*, 2008). Interestingly, only the *B. bifidum* Yakult conditioned media inhibited IL-8 gene expression in the cells, and as such the mechanism of action for *B. breve* Yakult remains obscure, but could be associated with production of anti-inflammatory factors by probiotic bacteria. The ability to suppress expression of IL-8 (or other pro-inflammatory cytokines) represents a key mechanism by which probiotics may reduce the severity of intestinal mucositis.

Anti-inflammatory effects of probiotics have also been reported *in vivo*. Liu *et al.* (2011) demonstrated the ability of *Lactobacillus plantarum* K68 to reduce the development of dextran sulphate sodium (DSS)-colitis in BALB/c mice by inhibiting the expression of pro-inflammatory IL-1 β and TNF- α . This model of intestinal inflammation was also employed by Zakostelka *et al.* (2011) to determine the ability of *Lactobacillus casei* 114001 to diminish TNF- α and IFN- δ expression in colitis. In the trinitrobenzene sulphonic acid (TNBS) model of colitis, *F. prausnitzii* also demonstrated anti-inflammatory effects (Sokol *et al.*, 2008). A six-day pre-treatment with either the live strain or its secreted compounds led to a reduction in colonic pro-inflammatory TNF- α and IL-12 levels, and an increase in anti-inflammatory IL-10. *Saccharomyces boulardii* has also been shown to increase *in vivo* levels of IL-10 in germ-free mice (Martins *et al.*, 2009). Increasing levels of anti-inflammatory cytokines may be effective in the treatment of mucositis. However, a strain such as *F. prausnitzii* which can influence both pro- and anti-inflammatory pathways, may

have more pronounced effects. Future studies comparing probiotics acting through different anti-inflammatory mechanisms for their capacity to treat or prevent intestinal mucositis should be investigated.

Roselli and colleagues tested two probiotic formulations: Mix 1 consisted of *Lactobacillus acidophilus* Bar13 and *Bifidobacterium longum* Bar33, while Mix 2 comprised *L. plantarum* Bar10, *Streptococcus thermophilus* Bar20 and *Bifidobacterium animalis* subsp. *lactis* Bar 30 (Roselli *et al.*, 2009). Once again, the TNBS model of colitis was employed, and both combinations significantly increased levels of regulatory T cells and inhibited expression of TNF- α and MCP-1. Furthermore, Mix 1 inhibited IL-12 and IFN- γ expression. However, individual strains were not investigated to determine if one particular strain was primarily responsible for the observed effects.

1.3.2 Maintenance of intestinal permeability

Damage to the epithelial wall is the first clinical sign of mucositis resulting in significant morbidity for the patient (Sonis *et al.*, 2004). Increased epithelial permeability allows the transfer of harmful pathogens into the surrounding tissue, and an overall loss of intestinal function (Keefe *et al.*, 2000). A number of probiotic strains have been shown to improve the integrity of the epithelium, allowing the intestinal barrier to maintain normal function.

Treatment with the live probiotic *Escherichia coli* Nissle 1917 (EcN) resulted in an up-regulation of the tight junction molecule zonula occluden (ZO)-1 at both the mRNA and protein levels, and reduced intestinal barrier permeability in mice

following DSS-induced damage (Ukena *et al.*, 2007). The same effect was observed in an identical model of colitis following administration of *L. casei* 114001 (Zakostelka *et al.* 2011). The probiotic combination VSL#3 has also been successfully employed to prevent DSS-induced increases in intestinal permeability and decreases in expression of the tight junction proteins occludin and claudin-1, -3, -4 and -5 in BALB/c mice (Mennigen *et al.*, 2009). While the active bacterial strains in VSL#3 were not determined in this study, the results of Ewaschuk and colleagues suggest that the *Bifidobacterium infantis* strain may have played an important role. Culture media (CM) from *B. infantis* increased transepithelial electrical resistance (TEER, an indicator of intestinal integrity) and ZO-1 expression in T84 cells (Ewaschuk *et al.*, 2008). Furthermore, *B. infantis* CM prevented TNF- α and IFN- γ induced reduction of TEER and rearrangements of tight junction proteins. These findings were confirmed *in vivo*, as probiotic treatment normalised colonic permeability in IL-10-deficient mice (Ewaschuk *et al.*, 2008). Although the ability of each individual strain to improve TEER was determined, the authors did not compare *B. infantis* CM with either live VSL#3 or its CM. A future study comparing these effects would determine whether the ability of VSL#3 to improve intestinal permeability was due to a combination of strains, or to *B. infantis* alone.

The effects of four independent probiotic strains (*Bifidobacterium lactis* 420, *Bifidobacterium lactis* HN109, *Lactobacillus acidophilus* NCFM and *Lactobacillus salivarius* Ls-33) on tight junction integrity have also been investigated (Putala *et al.*, 2008). Differentiated Caco-2 cells were treated with cell-free SNs of each strain. *B. lactis* 420 CM significantly increased TEER suggesting that live bacteria are not always required to exert beneficial effects. These results are supported by earlier

findings which indicated that soluble proteins produced by *Lactobacillus rhamnosus* GG (LGG) were able to protect against hydrogen peroxide-induced epithelial damage (Seth *et al.*, 2008). A separate study using LGG in alcohol-induced gut leakiness provided further evidence of the ability of the strain to improve gut permeability (Forsyth *et al.*, 2009). In this study, rats treated with alcohol and live LGG had significantly reduced gut leakiness compared to alcohol-treated controls. Future studies should compare the effect of live LGG to its cell-free SN in order to determine whether the effects are mediated entirely by secreted factors. The effectiveness of LGG to reduce the severity of multiple forms of gut damage suggests potential for its use in the treatment of chemotherapy-induced intestinal damage.

In addition to improving barrier function in models of intestinal damage, probiotic administration has also been shown to strengthen the already healthy or developing gut. Karczewski *et al.* (2010) administered *L.plantarum* WCFS1 to healthy subjects and reported a subsequent increase in ZO-1. Follow up studies on Caco-2 cells demonstrated that, not only were ZO-1 levels increased again, but Toll-like Receptor-2 signaling was also activated following probiotic administration. Probiotic strains which are able to strengthen an already healthy gut may effectively be employed as pre-treatment strategies prior to the commencement of chemotherapy in cancer sufferers. Furthermore, Patel *et al.* (2012) demonstrated the ability of both heat-inactivated and live LGG to improve intestinal barrier function through the induction of claudin-3 expression in the murine neo-natal gut. Such a strain may assist in the restoration of barrier function post-chemotherapy.

1.3.3 Elimination of pathogenic bacteria

The role of pathogenic bacteria in intestinal mucositis is not yet completely defined; however, recent findings suggest that pathogenic bacteria play a key role in the development of the disorder (Stringer *et al.*, 2007a; Stringer *et al.*, 2009a; Stringer *et al.*, 2009b). Chemotherapeutic drugs have been shown to have a direct toxic effect on commensal bacteria (Stringer *et al.*, 2009b). These changes contribute to the improved survival of pathogenic bacteria which, when combined with increased intestinal permeability and impaired immunity, render mucositis sufferers increasingly susceptible to intestinal infection (Stringer *et al.*, 2009b). Probiotics have demonstrated the capacity to inhibit survival of pathogens in the gastrointestinal tract, suggesting a further mechanism by which they could reduce the severity of mucositis.

By binding to both epithelial cells and the mucus layer throughout the gastrointestinal tract, probiotics can prevent pathogen colonisation by competitive exclusion (Candela *et al.*, 2008). In a condition such as mucositis, where large scale changes to the bacterial population occur (Stringer *et al.*, 2009b), probiotics which are able to competitively-inhibit multiple pathogenic strains are optimal. An example of such a strain is *L. plantarum* 423, which inhibited adhesion of pathogenic *Clostridium sporogenes* and *Enterococcus faecalis* (Ramiah *et al.*, 2008). Moreover, LGG, *L. rhamnosus* LC705, *B. breve* 99 and *Propionibacterium freudenreichii* ssp. *Shermanii* JS represent further examples of probiotic strains capable of inhibiting multiple pathogens (Collado *et al.*, 2007).

Specific probiotic strains also produce anti-microbial substances which target and eliminate pathogens from the gastrointestinal tract. *Lactobacillus johnsonii* NCC533 was shown to eliminate pathogenic *Salmonella enterica* serovar Typhimurium SL1344 through the production of anti-bacterial hydrogen peroxide (Pridmore *et al.*, 2008). Hydrogen peroxide production only occurred in an aerobic environment and, as such, the anti-microbial activity of this strain may be limited in the anaerobic human microbiota (Pridmore *et al.*, 2008). Bacterial strains with broad-spectrum anti-bacterial effects are commonly reported and warrant further investigation in the setting of intestinal mucositis. Muller and colleagues demonstrated that *L. plantarum* LP31 exhibited a bactericidal effect against *Pseudomonas* sp., *Staphylococcus aureus*, *Bacillus cereus* and *Listeria monocytogenes* (Muller *et al.*, 2009). Similarly, *Lactobacillus salivarius* BGH01 was found to produce multiple bacteriocins which were effective in targeting both gram-positive and gram-negative pathogens (Busarcevic and Dalgarrondo 2012).

1.3.4 Prevention of cell apoptosis

An increase in the apoptosis/proliferation ratio is a common feature of intestinal mucositis and plays a key role in the development of the disorder as it leads to increased permeability of the epithelial wall (Sukhotnik *et al.*, 2008). Probiotic administration has been shown to both inhibit and promote apoptosis in a variety of settings. Treatment with live VSL#3 significantly reduced caspase 3 (a positive marker of apoptosis) activation in the colon of rats with DSS-induced colitis (Mennigen *et al.*, 2009). The ability of the probiotic combination to reduce apoptosis in this chemically-induced model of intestinal damage suggests potential efficacy in similar settings, such as chemotherapy-induced mucositis. With the exception of

irinotecan-induced apoptosis (Bowen *et al.*, 2007), the initiation of caspases in healthy intestinal tissues by chemotherapy drugs remains largely undefined. Future studies should further characterise the role of caspases in chemotherapy-induced apoptosis, allowing for the identification of probiotic strains with the ability to reduce caspase activation. The probiotic cocktail, Ecologic[®] 641 (comprising four *Lactobacillus* and two *Bifidobacterium* strains) reduced cellular apoptosis in rats with glycodeoxycholate-induced acute pancreatitis (Lutgendorff *et al.*, 2009). Neither study compared the anti-apoptotic effects of the probiotic combinations in different areas of the gastrointestinal tract, and as such, the degree of site specificity remains unknown. This characteristic may play a key role in the selection of a candidate probiotic strain for the treatment of mucositis. It is well described what sections of the gut area are damaged by chemotherapy, and so probiotic strains which have been shown to have a protective effect in the same sections could be therapeutically effective.

Individual probiotic strains have also demonstrated anti-apoptotic effects. Pre-treatment with *S. boulardii* significantly inhibited TNF- α -induced apoptosis in human colonic T84 cells infected with pathogenic *E. coli* (Dalmaso *et al.*, 2006). The authors investigated the pathways by which *E. coli* initiated apoptosis and discovered that two different pathways were involved. The first involved death receptors and was identified by the activation of caspase 8, while the second comprised a number of intra- and extracellular death stimuli which led to the activation of caspase 9 (Dalmaso *et al.*, 2006). These two pathways converge to trigger caspase 3. Treatment with *S. boulardii* blocked apoptosis by both pathways, leading to an overall inhibition of caspase 3 activation (Dalmaso *et al.*, 2006).

Reports of therapeutic agents inhibiting these pathways in mucositis are limited; however, a trial examining glucagon-like peptide-2 (GLP-2) demonstrated the importance of caspase 8 activation in irinotecan-induced apoptosis (Boushey *et al.*, 2001). Inhibition of caspase 8 was shown to be a key mechanism via which GLP-2 enhanced the survival of epithelial cells following chemotherapy. The ability of *S. boulardii* to exert similar protective effects should be investigated. LGG has also been shown to exert anti-apoptotic effects in a model of cytokine-induced apoptosis (Yan *et al.*, 2007). Interestingly, Yan and colleagues compared the probiotic in its live form with two proteins (p40 and p75) isolated from the probiotic SN. Co-culture with TNF induced apoptosis in KSRI/ MCE mouse colon cells, but this was inhibited following treatment with live LGG. Furthermore, apoptosis was also inhibited by co-culture with the two LGG-derived proteins, suggesting that these factors present in the SN are involved in the anti-apoptotic process. TNF-stimulated caspase 3 activity was also found to be reduced following co-culture of colonic tissue explants with p40 and p75. Further studies determined that p40 was able to ameliorate cytokine-induced apoptosis through activation of the epidermal growth factor pathway and its downstream factor: Protein Kinase B (Yan and Polk 2012). These findings suggest that the secreted factors of probiotics have the potential to be efficacious in the treatment of intestinal mucositis, and should be further explored.

While anti-apoptotic effects are common amongst probiotics, pro-apoptotic effects have also been reported (Myllyluoma *et al.*, 2008). LGG, *L. rhamnosus* Lc705, *B. breve* Bb99 and *P. freudenreichii* subsp. *shermanii* JS were all tested, individually and in combination, for their ability to inhibit *Helicobacter pylori*-induced apoptosis in differentiated Caco-2 cells. At 24h, *H. pylori* infection significantly increased

caspase 3 activation. Co-culture of cells with *H. pylori* and the probiotic combination reduced caspase 3 activity, as did treatment with LGG and *L. rhamnosus* Lc705 individually. No effect was observed following co-culture of *P. freudenreichii* subsp. *shermanii* JS and *H. pylori*, whilst culture of Caco-2 cells with *P. freudenreichii* subsp. *shermanii* JS alone led to a significant increase in caspase 3 activity. Although a rare occurrence, probiotics should be screened for any pro-apoptotic effects prior to investigation as a potential therapy for intestinal mucositis, to eliminate any risk of exacerbating the condition. Furthermore, the probiotics must not protect neoplastic cells from chemotherapy-induced apoptosis. Although such an effect has yet to be reported in the literature, there is evidence to suggest that probiotics can exert deleterious effects on cancerous cells (De Santis *et al.* 2000). As live bacteria can directly impact cancerous cells, strains should be screened to ensure that the efficacy of chemotherapy is not diminished as a result of probiotic administration.

1.3.5 Maintenance of the mucus barrier

Mucins play a number of vital roles in the gastrointestinal tract, including the protection of the mucosa from bacterial overgrowth, providing attachment sites for intestinal flora and protecting the epithelium from luminal factors (Stringer *et al.*, 2009b). Chemotherapy regimens have been shown to alter mucin dynamics, potentially reducing intestinal barrier function (Stringer *et al.*, 2009b) and contributing to the onset of diarrhoea (Gibson *et al.*, 2003). Ideally, candidate probiotics should be able to reduce the severity of chemotherapy-induced damage by maintaining the production of mucins by intestinal epithelial cells.

VSL#3 was tested both *in vivo* and *in vitro* for its ability to induce mucin secretion (Caballero-Franco *et al.*, 2007). Mucin content, mucin secretion and gene expression were all increased *in vivo* following VSL#3 administration. In contrast, treatment of human colonic LS 174T cells with live VSL#3 had no effect *in vitro*. Interestingly, treating cells with products secreted by VSL#3 also significantly increased mucin expression (Caballero-Franco *et al.*, 2007). Furthermore, when the individual live strains of VSL#3 were compared to one another and in combination, secreted products from *Lactobacillus* species were the most potent mucin stimulators; however, the combination was still the greatest potentiator of mucin secretion. Kim and colleagues demonstrated that *L. acidophilus* A4 was able to increase expression of the mucin polypeptide MUC2 *in vitro*, and in turn inhibit binding of *E. coli* 0157:H7 (Kim *et al.*, 2008). However, the authors also reported an increase in IL-8, IL-1 β and TNF- α as a result of probiotic treatment, and it therefore remains unclear whether the reduced binding was due to mucin production, cytokine expression or both. Similarly, increased MUC2 and MUC3 expression and an inhibition of pathogenic *E. coli* 0157:H7 binding to HT-29 cells was reported following co-incubation with *L. plantarum* 299v (Mack *et al.*, 1999). As co-incubation of the pathogenic strain with the probiotic did not alter viability of *E. coli*, the authors dismissed any anti-bacterial activity of the probiotic. However, earlier findings of Kim *et al.* (2008) suggested that cytokine and/or other immune responses to probiotic treatment should also be investigated. Future studies should also determine the correlation between mucin gene expression and mucin secretion to ensure mRNA levels are an accurate representation.

1.3.6 Prevention of oxidative damage

The release of ROS is hypothesised to play a role in the initial stages of mucositis, leading to the oxidative damage of intestinal tissue (Sonis, 1998; Scully *et al.*, 2003). Inhibiting the release of ROS could therefore reduce the overall severity of mucositis. Probiotics have demonstrated anti-oxidant effects in a number of models of oxidative damage and could be effective in reducing both initial tissue damage, and the subsequent host inflammatory response. A rat model of acute pancreatitis was used to demonstrate the anti-oxidant capabilities of the multispecies probiotic combination, Ecologic® 641 (Lutgendorff *et al.*, 2009). Five days pre-treatment with probiotics prevented the acute pancreatitis-induced reduction in lipid peroxidation and mucosal glutathione levels. Interestingly, probiotic therapy also increased mucosal glutathione levels compared to normal controls by up-regulating glutamate-cysteine-ligase activity (a rate-limiting component of glutathione biosynthesis). The effectiveness of a pre-treatment in preventing oxidative damage is particularly relevant in intestinal mucositis, a disorder which is induced deliberately and the time course of disease progression easily predicted. Peran *et al.* (2007) tested probiotic effects on glutathione levels, employing *Lactobacillus fermentum* CECT5716 in a model of TNBS-induced colitis. Probiotic pre-treatment prevented colonic glutathione depletion, although the mechanism behind this protection was not determined. Furthermore, *L. fermentum* reduced nitric oxide synthase expression, a further anti-oxidative effect.

The anti-oxidant capabilities of VSL#3 have also been reported in rats fed a high fat diet (Esposito *et al.*, 2009). Probiotic treatment reduced the expression of inducible

nitric oxide synthase, protein nitrosylation and malondialdehyde levels, all indicators of oxidative damage. That study, along with that of Lutgendorff *et al.* (2009), suggests that probiotic combinations may have applications in mucositis treatment. Future studies should attempt to determine which strains are primarily responsible for any observed effects, as this may facilitate the development of more targeted therapies.

1.4 Risks associated with the use of probiotic based therapies

Although probiotics are considered as harmless bacteria which convey beneficial effects to the host, there is some evidence suggesting that certain probiotics can confer deleterious effects.

Potentially serious side-effects of probiotic therapies include: the development of sepsis (Zyrek *et al.*, 2007), initiation of an extreme inflammatory response (Hirata and Horie, 2003), growth of foreign bacterial colonies which inhibit normal colonisation of other microbiota (Anderson *et al.*, 2010), presence of virulence factors within strains of probiotic bacteria (Anderson *et al.*, 2010), translocation of live bacteria into local tissues (Hirata and Horie, 2003) and the transfer of resistance genes throughout bacterial populations as a result of anti-microbial factors released by the bacteria (Honeycutt *et al.*, 2007). As intestinal mucositis is commonly associated with reduced immune capacity and gut function, patients may be at an increased risk of side-effects such as the development of bacteraemia, alteration of the gut microbiota and uncontrolled inflammation. These concerns prompt consideration of alternative, probiotic based products such as inactivated/dead bacteria (Donato *et al.*, 2010) or the factors secreted by the live bacteria

(Prisciandaro *et al.*, 2009). These alternate forms do not contain live bacteria, and as such there is a reduced risk of bacteraemia and sepsis.

1.5 Probiotics in intestinal mucositis

1.5.1 Animal models of intestinal mucositis

Numerous established animal models of intestinal mucositis can be used to screen potential treatments such as probiotics (Gibson *et al.*, 2007, Tooley *et al.*, 2006, Yeoh *et al.*, 2007). A small number of studies have recently emerged which demonstrate the protective and therapeutic potential of probiotics. von Bultzingslowen and colleagues reported that *L. plantarum* 299v treatment via drinking water increased feed intake and body weight of 5-FU-treated rats, and reduced the 5-FU-induced increase in the total number of facultative anaerobes in the intestine (von Bultzingslowen *et al.*, 2003). In contrast, the incidence of diarrhoea and bacterial translocation to lymph nodes were not altered by probiotic treatment. Probiotics have also demonstrated efficacy in ameliorating methotrexate (MTX)-induced mucositis. Southcott and colleagues tested cow's milk yoghurt fermented with *L. johnsonii* and sheep milk fermented with both *L. bulgaricus* and *S. thermophilus* (Southcott *et al.*, 2008). Both treatments protected the duodenum from MTX-induced damage at the histological level, but this protection was not observed in other sections of the small intestine. However, a decreased lactulose/mannitol ratio for probiotic-treated animals indicated improved small intestinal barrier function. These authors did not test the efficacy of yoghurts which had not been fermented with live bacteria, and thus the active component of the treatment was not determined. *Streptococcus thermophilus* TH-4 (TH-4) has been examined in the MTX and 5-FU models of intestinal mucositis (Tooley *et al.*, 2006; Whitford *et al.*,

2009). Tooley and colleagues tested live *S. thermophilus* TH-4 at two doses, 10^8 and 10^9 colony forming units (cfu)/ml, delivered daily between 48h prior to, and 96h post-MTX treatment. A dose-dependent response was observed, with rats given MTX + 10^9 *S. thermophilus* TH-4 displaying similar jejunum sucrase levels to non-MTX treated controls, indicating a normalisation of intestinal function. Furthermore, the higher dose of *S. thermophilus* TH-4 significantly reduced myeloperoxidase (MPO, an indicator of tissue damage) activity when compared to MTX-treated control animals. These findings contrast the report of Whitford and colleagues who examined live *S. thermophilus* TH-4 and its SN in 5-FU-induced mucositis (Whitford *et al.*, 2009). In this study, live *S. thermophilus* TH-4 was only able to reduce disease severity scores (an objective measurement based on body condition, weight loss, stool consistency and rectal bleeding), while the previously reported increase in sucrase and decrease in MPO activities were not observed. These findings highlight that probiotic effects may vary based on the chemotherapeutic agent and the mechanism of gut damage.

In the 5-FU-induced rat model of mucositis, Mauger and colleagues reported that administration of either *L. fermentum* BR11 (BR11), LGG or *B. lactis* Bb12 at 10^6 cfu/ml had no effect on MPO and sucrase activity, or on histological damage scores (Mauger *et al.*, 2007). The authors suggested that the absence of a probiotic effect may have been due to the low dosage administered; a notion supported by the findings of Smith *et al.* (2008) who reported that the of 10^9 cfu/ml BR11 reduced 5-FU-induced inflammation in the jejunum. The probiotic combination VSL#3 was investigated in rats treated with irinotecan (Bowen *et al.*, 2007). Rats received VSL#3 either pre-irinotecan treatment, post-irinotecan treatment or both. Only the

latter treatment was able to confer protection against chemotherapy-induced symptoms. Pre- and post-chemotherapy VSL#3 administration increased epithelial cell proliferation, reduced epithelial cell apoptosis and prevented water and electrolyte imbalance, subsequently preventing diarrhoea. These observations were attributed to the ability of VSL#3 to prevent the irinotecan-induced increases in goblet cell number and mucin secretion (Bowen *et al.*, 2007), providing further evidence of the benefits that can be achieved using a probiotic combination. To this end, a mechanism to determine the most active strains may facilitate the development of more efficacious probiotic therapies for intestinal mucositis.

1.5.2 *Clinical trials*

There is currently a shortage of well-conducted, large, randomised, double-blind, placebo-controlled trials which investigate the efficacy of candidate probiotic species in intestinal mucositis. This is most likely a result of conflicting data obtained from animal trials and the absence of detailed *in vitro* studies. Delia and colleagues investigated the use of VSL#3 as a preventative treatment for radiation-induced diarrhoea (Delia *et al.*, 2007). The study involved 490 patients who underwent adjuvant postoperative radiation therapy following surgery for cervical, rectal or sigmoid cancer. Subjects received one sachet of VSL#3 (45×10^9 billion live bacteria/g) daily for the duration of radiation therapy. Severe cases of diarrhoea were reported in 55.4% of patients receiving the placebo compared to 1.4% of patients receiving the probiotic. Furthermore the number of bowel movements per day was reduced (5.1 ± 3) when compared to placebo (14.7 ± 6). This study demonstrated that probiotic bacteria can act as a simple, safe and effective method of protecting cancer patients from radiation-induced diarrhoea. The results of this trial, combined

with the earlier results of Bowen *et al.* (2007) and other clinical trials using this combination in intestinal disorders (Miele *et al.*, 2009, Kim *et al.*, 2005), suggest that VSL#3 is an ideal candidate for clinical trials in the setting of chemotherapy-induced intestinal mucositis.

1.6 Summary

Despite continued research, there remains no definitive treatment for chemotherapy-induced mucositis. Recent findings which demonstrate an involvement of the intestinal microbiota in the condition and the ability to manipulate this environment with probiotic bacteria (Sartor, 2004) present a viable option for the development of either a probiotic-based therapy or prophylactic-treatment.

Appropriate probiotic administration has the potential to decrease the severity of intestinal mucositis. A number of potential mechanisms of action have been identified, including reduction of pro-inflammatory cytokine secretion and gene expression, release of anti-inflammatory cytokines, inhibition of inflammatory pathways, improvement of barrier function, maintenance of mucin secretion, prevention of epithelial cell apoptosis and oxidative damage, and the elimination of pathogenic bacteria. However, these mechanisms have been observed predominantly in other disease settings and few studies have investigated the efficacy of probiotics in mucositis. Currently, the identification of the most suitable probiotic strains should be the target for research in this field. While many probiotics have multiple beneficial effects, it remains unlikely that a single strain will be sufficient to counteract such a multi-faceted disorder. The microbial composition of the host may also affect probiotic efficacy. This review proposes the promising efficacy of

probiotic combinations, and the author hypothesises that a strategically-selected combination of strains may be most efficacious in this disorder.

There are a number of possible directions for future research. Primarily, the capacity for probiotics to exert their beneficial effects in the setting of chemotherapy-induced mucositis must be determined. Although there is evidence for efficacy, there remain only a few confirmed studies which have been performed in mucositis models. Future studies should isolate the strain-specific mechanisms by which probiotics are able to ameliorate damage, and elucidate the effects of chemotherapeutic drugs on probiotic cell viability. The effects of chemotherapeutic drugs on the microbiota suggest that their administration may inhibit the survival and thus the effectiveness of live probiotic-based therapies. Furthermore, the use of probiotic-based secreted factors, rather than live bacteria, remains an area of future promise. The absence of live cells reduces the need to maintain cell viability and could be particularly beneficial for chemotherapy patients at increased risk of infection due to impaired intestinal barrier function. Probiotics could potentially be employed as prophylactic treatments which inhibit the development of mucositis or as a post-treatment to facilitate the recovery process.

The overall aim of this thesis was to investigate the efficacy of probiotic-derived SNs in the treatment of 5-FU induced intestinal mucositis. The first step was to identify a number of candidate strains based on proven mechanisms of protective action in other models of damage. These strains were then tested *in vitro* to determine their capacity to protect against 5-FU induced damage. Strains determined to improve indicators of intestinal damage were then tested in an

established *in vivo* model of 5-FU induced intestinal mucositis. Furthermore, this thesis sought to compare the efficacy of probiotic derived-SNs with their live bacteria, as well as to determine if the previously described mechanisms of action are repeatable in models of 5-FU induced damage.

1.7 Table

Probiotic effect relevant to intestinal mucositis	Species/Strain/Combination	Reference
Inhibition of inflammation	<i>F. prausnitzii</i> <i>L. plantarum</i> <i>B. bifidum</i> Yakult <i>B. breve</i> Yakult <i>S. boulardi</i>	Sokol <i>et al.</i> 2008 Petrof <i>et al.</i> 2004 Imaoka <i>et al.</i> 2008 Imaoka <i>et al.</i> 2008 Martins <i>et al.</i> 2009
Maintenance of intestinal permeability	VSL#3 <i>B. infantis</i> <i>L. rhamnosus</i> GG	Mennigen <i>et al.</i> 2009 Ewaschuk <i>et al.</i> 2008 Seth <i>et al.</i> 2008 Forsyth <i>et al.</i> 2009
Elimination of pathogenic bacteria	<i>L. plantarum</i> LP31 <i>L. plantarum</i> 423 <i>L. rhamnosus</i> GG <i>L. johnsonii</i> NCC533	Muller <i>et al.</i> 2009 Ramiah <i>et al.</i> 2008 Collado <i>et al.</i> 2007 Pridmore <i>et al.</i> 2008
Inhibition of cell apoptosis	VSL#3 Ecologic®641 <i>S. boulardii</i> <i>L. rhamnosus</i> GG	Mennigen <i>et al.</i> 2009 Lutgendorff <i>et al.</i> 2009 Dalmaso <i>et al.</i> 2006 Yan <i>et al.</i> 2007
Prevention of oxidative damage	Ecologic ®641 <i>L. fermentum</i> CECT5716 VSL#3	Lutgendorff <i>et al.</i> 2009 Peran <i>et al.</i> 2007 Esposito <i>et al.</i> 2009
Maintenance of mucus barrier	<i>L. acidophilus</i> <i>L. plantarum</i> 299v	Kim <i>et al.</i> 2008 Mack <i>et al.</i> 1999

Table 1. Probiotic species, strains or combination which have previously been shown to exert effects which suggest potential efficacy in either the treatment or prevention of chemotherapy-induced intestinal mucositis.

Chapter 2. Probiotic factors partially protect intestinal cells from chemotherapy-induced cytotoxicity and improve parameters of 5-fluorouracil-induced intestinal mucositis in rats

2.1 Introduction

The anti-metabolite 5-FU is one of the most commonly prescribed chemotherapy drugs in clinical cancer treatment (Fata *et al.*, 1999). However, over 50% of patients receiving 5-FU develop either oral or gastrointestinal mucositis (Decker-Baumann *et al.*, 1999). Symptoms of 5-FU-induced intestinal mucositis include: nausea, vomiting, dyspepsia, dysphasia and diarrhoea (Soares *et al.*, 2008). These occur as a result of reduced enterocyte proliferation and migration, and increased cell apoptosis which combine to disrupt normal intestinal barrier function (Daniele *et al.*, 2001). Damage to the intestine has serious consequences to the patient as it leads to reduced food and fluid intake, impaired ability to absorb nutrients and increased vulnerability to infection (Duncan and Grant, 2003). In addition to the physical damage evident during intestinal mucositis, changes in the composition of the gut microbiota (Haenel, 1970) and gut pH (Gosselink *et al.*, 2004) are also evident and can further impede normal intestinal function (Gall, 1970).

5-FU has been successfully employed in both rats (Mauger *et al.*, 2007; Soares *et al.*, 2008; Xian *et al.*, 2004) and mice (Carneiro-Filho *et al.*, 2004) to create an *in vivo* model of intestinal mucositis. A single 5-FU dose (150mg/kg body weight delivered via intraperitoneal injection) is sufficient to induce the disorder in rats. The drug can also be used to damage cells *in vitro*; as Braga-Neto *et al.* (2008) and Hirata and Horie (2003) added 5-FU to IEC-6 cells to assess potential therapeutics for the prevention of chemotherapy-induced damage.

Despite the prevalence of intestinal mucositis, there remains no definitive treatment for the disorder (Logan *et al.*, 2007). Therefore, potential therapies such as probiotics

are under investigation. Probiotics have been investigated as therapies in a number of gastrointestinal disorders, but limited research has been carried out in the area of intestinal mucositis. Due to impaired immune and barrier function that accompanies the condition (Duncan and Grant, 2003), live bacteria may not be suitable as a therapy. Recent studies have identified certain probiotic SNs as being capable of carrying out the similar functions as the live bacteria from which they are derived, with a reduced risk of infection (Seth *et al.*, 2008; Sokol *et al.*, 2008). Four SN strains were selected for the current study: BR11, EcN, TH-4 and *L. acidophilus* 5 (LA5). Live BR11 had previously demonstrated efficacy in the setting of DSS-colitis (Geier *et al.*, 2007) and was hypothesised to have anti-oxidant properties which may be effective at counteracting the damage caused by the 5-FU-induced release of ROS (Scully *et al.*, 2003; Sonis, 1998). EcN had been investigated previously in a number of different models, and have been shown to promote the release of anti-inflammatory cytokines (Arribas *et al.*, 2009) and strengthen tight junctions (Ukena *et al.*, 2007; Zyrek *et al.*, 2007). In a model of MTX-induced mucositis, TH-4 administration maintained markers of intestinal barrier function and neutrophil aggregation (Tooley *et al.*, 2006); however, its efficacy in 5-FU-induced mucositis had not been properly defined (Whitford *et al.*, 2009). LA5 has yet to be investigated in a model of intestinal damage, but has been shown to possess anti-bacterial properties (Wang *et al.*, 2004) which could help to counteract the changes in the gut microbiota that occur following 5-FU administration (Stringer *et al.*, 2009b). Although these strains have been investigated in their live forms, only EcN SN has been tested previously (Schlee *et al.*, 2007). Therefore, prior to determining the ability of SNs to protect against 5-FU-induced damage, SNs must be shown to have no deleterious effects on cell viability.

The aim of the current study was to screen a range of probiotic SNs for their ability to aid the recovery of intestinal cells following 5-FU-induced damage. SNs were originally screened in an *in vitro* setting prior to selecting the most efficacious for examination *in vivo*.

2.2 Materials and methods

2.2.1 Supernatant preparation

BR11 was a kind gift from Dr Philip Giffard, Dr Mark Turner and Raquel Lo, Queensland University of Technology (Queensland, Australia). EcN was purchased from Ardeypharm (Herdecke, Germany). All SNs were prepared in Dulbecco's Modified Eagle Medium (DMEM; Gibco Invitrogen, California, USA). EcN, provided in lyophilised form, was added directly to medium. BR11 was grown on de Man Rogosa and Sharpe agar (MRS; Oxoid, South Australia, Australia) at 37°C for 48h and then added to medium. All bacterial strains were incubated at 37°C for 48h, before being centrifuged at 1500g for 10 mins. SNs were then collected and buffered with Tris/HCl to a pH of 7.2. SNs were passed through a 0.20-µm filter, and stored at -20°C until use. Prior to administration, SNs were diluted with 50% DMEM.

2.2.1 Cell culture

IEC-6 cell line was obtained from the American Type Culture Collection (Virginia, USA). Cells (passage 40-44) were cultured in medium comprising DMEM, 10% foetal bovine serum (GIBCO[®], Victoria, Australia) and 1% penicillin/streptomycin (GIBCO[®]). IEC-6 cells were seeded into 24-well plates (10⁵ cells/well) and left

overnight to reach confluency prior to addition of the treatments. Plates were stored at 37°C in 95% air, 5% CO₂ for the duration of the study.

2.2.2 *Effect of supernatants on cell viability*

There were six treatment groups: 100% DMEM, 50% Dulbecco's Phosphate Buffered Saline (DPBS) + 50% DMEM, BR11 SN + DMEM, EcN SN + DMEM, TH-4 + DMEM and LA5 + DMEM. Two control groups (100% DMEM and 50% DPBS + 50% DMEM) were included due to the unknown effect of growing bacteria in DMEM. As the growth of the bacteria may have reduced nutrient levels in the DMEM, comparison to DMEM and DPBS + DMEM control group was necessary to reflect a scenario whereby 50% of the DMEM was completely utilised (DPBS + DMEM) or not utilised (100 % DMEM).

At day 0, IEC-6 cells (10^5 cells/well) were seeded in 24-well plates and incubated overnight at 37°C in 95% air, 5% CO₂. At day 1, basal medium was removed from each well and replaced with 1ml of the respective treatment. Following a second overnight incubation, SN mixture was aspirated and replaced by 1ml of basal media. Medium was then changed daily from days 2 to 6.

Cell viability was determined at four time points (days 3, 4, 5 and 6) using a trypan blue assay as described by Zahri *et al.* (2012). Medium was removed from cells in 24-well cell culture plates and collected in Eppendorf tubes. Cells were washed once with Dulbecco's Phosphate Buffered Saline (DPBS; GIBCO®) and once in Trypsin-EDTA (100µl). Trypsin-EDTA (100µl) was then added to each well for a second time and the cells incubated for 15 mins to detach cells from the culture plate. The

trypsin reaction was then terminated by reintroduction of previously removed medium to the corresponding wells. The previously removed medium was re-added to wells to ensure that cells which had detached from the monolayer were included in the assay. Medium (100µl) was then added to 100µl of trypan blue (GIBCO®), which is able to penetrate the membrane of non-viable cells. Viable and non-viable cells were then counted by haemocytometer, and the percentage of viable/non-viable cells and total cell number determined.

2.2.3 Assessment of protective effects of supernatants against 5-fluorouracil-induced damage

On day 0, IEC-6 cells (10^5 per well) were seeded in 24-well plates and incubated overnight at 37°C in 95% air, 5% CO₂. Medium was then removed from each well and replaced with 0.5ml of fresh media. Cell death was induced on day 1 by adding 1mM of 5-FU suspended in DPBS was added to each well for 1h as described previously (Hirata and Horie, 2003). Media and 5-FU were removed from the cells which were then washed in fresh medium. Medium was then aspirated, and 1ml of the previously listed treatments was added to each well, and the plates were incubated overnight. SN mixture was then aspirated and replaced by 1ml of basal growth medium. Cell viability and number was recorded daily on days 2, 3, 4 and 5 and determined via trypan blue assay.

2.2.4 Animal trial protocol

The study was conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (2004) and was approved by the

Animal Care and Ethics Committees of the Children, Youth and Women's Health Service and The University of Adelaide.

Female Dark Agouti rats were individually housed in metabolism cages (Tecniplast, Pennsylvania, USA) in a temperature-controlled room (22°C) with a 12h light-dark cycle. Rats were given *ad libitum* access to fresh water and an 18% casein-based diet (Tomas *et al.*, 1991), and were randomly allocated to one of four treatment groups (n = 8/treatment): Water + Saline (Saline Control), DMEM + 5-FU (5-FU Control), BR11 SN + 5-FU, and EcN SN + 5-FU. Treatments (1ml) were administered daily via oro-gastric gavage from days 0 to 8. On day 5, animals were injected intraperitoneally with 5-FU (150mg/kg DBL[®], Mayne Pharma Pty Ltd, Victoria, Australia) or saline. Food and water intake, body weight and faecal and urine output were recorded daily. On day 9, all rats were killed via carbon dioxide overdose and cervical dislocation. The gastrointestinal tract was removed and the lengths of the duodenum, small intestine and colon were measured. All gastrointestinal organs were then emptied of contents and weighed. Separate 4cm sections of small intestine and colon were collected in either 10% buffered formalin or snap frozen in liquid nitrogen and stored at -80°C. The weights of visceral organs were also recorded.

2.2.5 Histological analysis

Paraffin embedded specimens of jejunum, jejunum-ileum junction (JI) and ileum were sectioned (6µm) and stained with haematoxylin and eosin. Mean villus height and crypt depth measurements were obtained for each region by evaluating 40 villi and crypts per section per rat (Cool *et al.*, 2005). A qualitative histological assessment of damage was performed as described previously (Howarth *et al.*, 1996)

and analysis of the jejunum and ileum were conducted using seven parameters each individually-scored from 0 (normal) to 3 (maximal damage), to achieve an overall damage severity score of up to 21. The median scores for each group were then compared.

2.2.6 Myeloperoxidase activity

Neutrophil infiltration in the jejunum, JI and ileum was determined by MPO assay (Krawisz *et al.*, 1984). Segments (4cm) of tissue were thawed and homogenised in 1ml of saline. Aliquots (200µl) were then centrifuged (1300g, 10 mins) and the pellet re-suspended in hexadecyltrimethylammonium bromide buffer and vortexed for 2 mins to release MPO from the tissue. Samples were then centrifuged (800g, 2 mins) and 50µl of SN added, in duplicate, to a 96-well plate. Hydrogen peroxide and O-dianisidine reaction mixture were then added to each well and absorbance of the reaction mixture measured at 450nm (Sunrise Microplate Absorbance Reader, Tecan Austria GmbH, Grödig, Austria) at 1 min intervals for 15 mins.

2.2.7 Sucrase activity

Small intestinal sucrase activity (jejunum, JI and ileum) was measured using the methods described previously (Cool *et al.*, 2005). Briefly, tissue was homogenised in 1ml saline and diluted (1/100, 1/80 for saline-treated tissue, 1/50, 1/30 for 5-FU-treated tissue) in 50mM phosphate buffer containing Triton-X (Sigma, Missouri, USA) and 50µl of each dilution added to a 96-well plate. Sucrose (0.2mM) was added to each well, and incubated for 30 mins. Tris-glucose oxidase was then added to each well. Glucose production was measured colorimetrically by measuring the optical density of each well at 490nm.

2.2.8 Mucin staining

As individual goblet cells can potentially produce different types of mucin concurrently, the determination of mucin type required two different staining techniques (Forder *et al.*, 2007). For neutral mucins, sections were subjected to mild acid hydrolysis to eliminate the contribution of sialic acid residues before periodic acid-Schiff (PAS) staining. After rinsing with both tap and distilled water, sections were immersed in periodic acid solution (Sigma, Missouri, USA) for 20 mins, washed, and immersed in Schiff's Reagent (Sigma) for a further 20 mins before being rinsed in tap water and dehydrated. Staining of acidic mucins was performed using high iron diamine-alcian blue (HID-AB) pH 2.5 staining. Sections were treated in HID solution for 16h at room temperature, before being rinsed in tap water and stained in alcian blue (pH 2.5). Cells were counted under a microscope at 20x magnification and expressed as number of cells/villi.

2.2.9 Statistical analysis

Statistical analyses were conducted using SPSS 15.0.1 for Windows (SPSS Inc., Illinois, USA). Cell viabilities and cell numbers were analysed using a one-way analysis of variance (ANOVA). Pair wise comparisons were made using a Bonferroni test. Daily metabolic data were analysed using a repeated measures ANOVA with a Holme's *post-hoc* test ($p < 0.05$ significance) to compare differences both amongst groups and within groups across the duration of the trial. Overall histological damage severity was compared by a Kruskal Wallis test with a Bonferroni *post-hoc* test, and expressed as median (range). All other data were compared by one-way ANOVA with a Tukey *post-hoc* test and were expressed as mean \pm SEM. For all data, $p < 0.05$ was considered significant.

2.3 Results

2.3.1 Effects of supernatant preparations on cell viability and number

At all time points, the SN preparations had no significant effect on cell viability compared to DMEM and DPBS controls (Table 1). Cell numbers were significantly reduced in DPBS control, TH-4 and LA5 SN groups at both days 3 and 6 when compared to 100% DMEM ($p < 0.05$, Table 1).

2.3.2 Effect of 5-fluorouracil on IEC-6 cell viability and number and the protective effects of supernatant preparations.

All 5-FU-treated groups had significantly reduced cell numbers compared to untreated controls ($p < 0.05$, Table 2). However, on day 3, the DPBS + 5-FU and DMEM + 5-FU-treated cells had significantly lower viability than the untreated controls ($p < 0.05$, Table 2) whilst viability was not significantly reduced in SN-treated cells. On day 4, viability was significantly lower in all of the 5-FU-treated groups compared to the untreated controls. On day 5, only DMEM + 5-FU, DPBS + 5-FU and LA5 SN + 5-FU-treated cells had reduced viabilities compared to the untreated controls ($p < 0.05$). Finally, by day 6, only DPBS + 5-FU decreased viability compared to the untreated controls.

2.3.3 Metabolism parameters and organ data

Metabolism data, including water and feed consumption, and urine and faecal output were recorded from days 6 to 9. No differences were observed among groups for both water consumption and urine output (Table 3). Healthy control animals consumed up to 60% more feed and had higher faecal outputs than 5-FU-treated

groups ($p < 0.05$). Metabolism data were also analysed over the entire trial, although no significant differences between groups were observed (data not shown). Similarly, no significant differences were observed among treatment groups between individual days (data not shown).

Treatment had no effect on either weights or lengths of gastrointestinal organs (Tables 4 and 5, $p > 0.05$). Similarly, no differences were observed in the weight/length ratio of each section of the intestinal tract (data not shown).

2.3.4 Myeloperoxidase activity

Previous studies have reported significant increases in MPO levels following administration of 5-FU (Mauger *et al.*, 2007). MPO activity in the jejunum was significantly increased in the 5-FU-treated controls and BR11 SN + 5-FU-treated rats compared to the untreated controls ($p < 0.05$, Figure 1); however, no significant increase was observed in EcN SN + 5-FU-treated animals. The most marked increase was observed in the 5-FU-treated controls which exhibited over ten times the MPO activity of the healthy animals. No significant differences were observed among the 5-FU-treated groups (Figure 1). MPO activity did not differ among any of the treatments in both the JI and ileum.

2.3.5 Sucrase activity

Sucrase activity throughout the small intestinal is typically reduced following 5-FU administration (Mauger *et al.*, 2007). As predicted, in the current study sucrase activity was significantly reduced in the jejunum and JI of 5-FU-treated animals compared to controls ($p < 0.05$), however, no significant differences were observed

in the ileum (Figure 2). In the jejunum, the 5-FU-treated controls had the lowest sucrase activity, an 84% reduction compared to healthy animals. Sucrase activity levels of BR11 SN + 5-FU and EcN SN + 5-FU-treated animals were reduced by 63% and 67% respectively. In the JI, the reduction in sucrase activity was not as marked. Rats receiving BR11 SN + 5-FU had the lowest activity, 69% lower than that observed in the healthy control animals. Sucrase activity was also reduced in the 5-FU-treated controls (60%) and EcN + 5-FU-treated rats (63%) compared to the Water + Saline group.

2.3.6 Histological severity scoring and analysis

5-FU administration significantly increased histological severity score in both the jejunum and the ileum for all treatment groups compared to healthy controls (Figure 3). In the jejunum, both the BR11 SN and EcN SN treated groups had significantly lower severity scores compared to 5-FU-treated control animals ($p < 0.05$).

Both villus height and crypt depth in the jejunum were significantly reduced in the 5-FU control animals ($p < 0.05$), however, this reduction was not observed in rats which received BR11 SN and EcN SN; indicating a protective effect (Figure 4).

2.3.7 Mucin staining

5-FU administration significantly decreased the number of goblet cells containing neutral and acidic mucins in the jejunum ($p < 0.05$, Table 6). SN administration partially prevented the decrease in acidic mucin producing goblet cells ($p < 0.05$). The numbers of goblet cells secreting acidic mucins in the ileum were significantly

lower in all 5-FU-treated rats, however, a reduction in neutral mucin secreting cells was only observed in the DMEM + 5-FU and BR11 SN + 5-FU-treated groups.

2.4 Discussion

The current study assessed the potential for probiotic SN to ameliorate 5-FU-induced intestinal damage both *in vitro* and *in vivo*. Previous studies have shown that the soluble compounds secreted by probiotics have, in some cases, been able to exhibit beneficial effects (Borthakur *et al.*, 2008; Roselli *et al.*, 2006; Schlee *et al.*, 2007). None of the SNs assessed in the current study have been previously investigated in the setting of 5-FU-induced damage, whilst only the EcN SN had been tested in any *in vitro* model (Schlee *et al.*, 2007). The present study indicated that although unable to prevent the reduction in cell number, BR11 SN, EcN SN and TH-4 SN were all able to partially maintain cell viability following 5-FU administration. Furthermore, EcN SN and BR11 SN (selected following their beneficial effects *in vitro*) also demonstrated protective effects *in vivo* as indicated by reduced histological damage and neutrophil aggregation. These findings suggest a potential role for the probiotic SNs in either protection or treatment of 5-FU-induced intestinal mucositis; however, no SN was able to significantly improve all measured indicators of 5-FU-induced damage.

Although the *in vivo* model of 5-FU-induced intestinal mucositis is commonly employed, minimal studies have used an *in vitro* model of 5-FU-induced damage (Braga-Neto *et al.*, 2008, Hirata and Horie, 2003). Hirata and Horie (2003) treated IEC-6 cells with 5-FU to examine the effects of a prostaglandin E₁ analogue on epithelial restitution, while Braga-Neto *et al.* (2008) reported an enhanced 5-FU

effect following supplementation with alanyl-glutamine and glutamine. Both studies employed an identical dosage of 1mM 5-FU for 1h, and in both cases this was sufficient to induce epithelial cell damage as indicated by increases in necrosis and apoptosis; and a reduction in cell proliferation. This supports the findings of the current study, in which 5-FU administration significantly reduced both cell viability and cell number at each time point. However, the degree of damage differed in a time dependent manner. Although recordings were taken at different time points (6, 12 and 24h post-5-FU) Braga-Neto *et al.* (2003) also reported a time-dependency. However, their findings contrast with the current study as they reported an increase in cell number at 24h compared to the reduction observed here. Furthermore, levels of necrosis and apoptosis peaked at 12h. Cell counts in the current study revealed that 5-FU significantly reduced cell number in a time-dependent manner. Considering that there was a 95% reduction in cell number by day 6, this 5-FU dose is not likely to be suitable for studies of this duration. Future studies which aim to investigate long-term effects of 5-FU should use a lower dosage in order to closely replicate the changes observed *in vivo*.

In the current study, SN administration following 5-FU incubation had contrasting effects on cell number and viability. It was hypothesised that post-5-FU treatment with SNs may promote the recovery of intestinal cells. None of the SNs were able to improve the reduction in cell number induced by 5-FU, nor promote rapid cell proliferation during the recovery phase. One of the key features of 5-FU was that it causes single and double stranded breaks in DNA during S-phase, which specifically inhibits cell proliferation (De Angelis *et al.*, 2006) and it was clear that none of the SNs were able to counteract this effect. This may be important, however, as 5-FU

was still carrying out a critical function (inducing apoptosis in neoplastic cells) and so reducing its efficacy is undesirable. Future studies should confirm that neither post nor pre-treatment with SN inhibits the ability of 5-FU to induce apoptosis of transformed cells. The DPBS control was the only treatment group to reveal significantly lower cell viability at all time points compared to DMEM controls. This could be attributed to the treatment having the lowest volume of growth medium. As the live bacteria were grown in DMEM (the same medium as the IEC-6 cells) it was possible that compounds which stimulated cell growth were present in the SN mixtures. By comparing viabilities in the DPBS and DMEM control groups, we learnt that at days 3 and 6, increasing the growth media availability for cells had a beneficial effect. Some of the SNs (EcN, BR11 and TH-4) were able to maintain viability when both DMEM and DPBS controls displayed a reduction, indicating that they contained compounds able to exert a beneficial effect beyond any possible DMEM effects. Those effects may have been more pronounced had SN been administered as both and pre- and post-treatment (as they were *in vivo*).

van Vliet and colleagues described numerous pathways by which disruption of the microbiota can influence mucositis, including reducing the capacity for the microbiota to suppress inflammatory responses and oxidative stress, increasing intestinal permeability through increased villus atrophy and diminished mucin production, inhibition of epithelial repair and altering the production and release of effector molecules essential for the regulation of gut homeostasis (van Vliet *et al.*, 2010). Probiotics and their secreted factors have been shown to improve intestinal health via mechanisms which are particularly relevant to mucositis, and could potentially reduce the impact of the changing microbiota. Multiple anti-inflammatory

characteristics of bacterial factors have been described. EcN SN stimulated secretion of the anti-inflammatory cytokines IL-10 and IL-6 in peritoneal cells of rats (Zidek *et al.*, 2010), while factors released by *Lactobacillus casei* Shirota inhibited indomethacin-induced NF- κ B activation and increases in TNF- α mRNA expression (Watanabe *et al.*, 2009). The anti-inflammatory properties were attributed to the bacteria-derived lipopolysaccharide (LPS) and lactic acid production for EcN SN and *L. casei* Shirota, respectively. Similarly to EcN SN, *Bacillus coagulans* secreted factors not only promoted IL-10 expression, but also inhibited oxidative stress-induced formation of ROS (Jensen *et al.*, 2010).

TH-4 was the only strain which had previously been tested in the 5-FU model of intestinal mucositis (Whitford *et al.*, 2009). In the current study, the *in vitro* analysis revealed contrasting findings, as although the SNs were able to normalise cell viability at particular time points following 5-FU, it failed to maintain cell number following 5-FU and even inhibited cell proliferation in untreated cells on days 3 and 6. These negative results supports the findings of Whitford and colleagues which suggested that, despite therapeutic potential in MTX-induced mucositis *in vivo* (Tooley *et al.*, 2006), both live TH-4 and its SN were unable to ameliorate the harmful effects of 5-FU in rats (Whitford *et al.*, 2009). In addition to the different models employed, a potentially important difference between the current study and that of Whitford *et al.* (2009) was the medium in which the TH-4 was grown. Whitford and colleagues cultured TH-4 in MRS broth, while the current study used TH-4 grown in DMEM. The use of different growth medium between the two studies is a significant factor, as it has been shown to directly impact both the rate of growth of the bacteria and the composition of the bacterial SN (Tomas *et al.*, 2003,

Derzelle *et al.*, 2005, Kimoto-Nira *et al.*, 2008). When grown in MRS broth, TH-4 has been shown to synthesise high levels of folate (Sybesma *et al.*, 2003), and it is hypothesised that the high folate production may be responsible for the efficacy of TH-4 in the setting of MTX-induced mucositis (Tooley *et al.*, 2006). Furthermore, folate negatively impacts upon 5-FU efficacy on tumours (Tucker *et al.*, 2002). Should TH-4 administration increase systemic folate levels, it may inhibit the ability of the drug to target neoplastic cells. However, early investigations suggest this is unlikely (Tooley *et al.* 2011). Further research into the composition of the TH-4 SN grown in both DMEM and MRS is required, as well as investigation into the efficacy of 5-FU following treatment with folate-producing TH-4. These findings will be important as any potential treatment for mucositis cannot impede the anti-neoplastic effect of the chemotherapeutic agent.

LA5 had not previously been tested in any model of 5-FU-induced damage, and the current study did not indicate any efficacy in this setting. While EcN SN, BR11 SN and TH-4 SN maintained cell viability *in vitro* compared to 5-FU controls by day 5, the effect of LA5 SN on cell viability was comparable to that of the basal media. Surprisingly, when added to untreated IEC-6 cells, LA5 SN administration appeared to inhibit cell proliferation; reducing cell number at days 3 and 6 compared to untreated controls. This was also observed for TH-4 SN. Inhibiting proliferation could lead to slower repair and rejuvenation of the intestinal cells, suggesting that LA5 SN may not be an effective treatment for the physical damage associated with intestinal mucositis.

Live BR11 has previously demonstrated indications of efficacy in the treatment of DSS-induced colitis (Geier *et al.*, 2007). However, it has yet to be investigated in its SN form in any disease model. In contrast to the findings of Mauger *et al.* (2007) who reported no beneficial effects of live BR11 treatment in 5-FU-treated animals, BR11 SN administration maintained villus height and crypt depth compared to 5-FU-treated control rats in the current study. A number of differences exist between these two studies which could be responsible for the contrasting findings, most significantly the use of the bacterial SNs rather than live bacteria. The differences may also have been the result of a dose-dependent effect of BR11 as a relatively low dose (10^6 cfu/ml) was administered by Mauger and colleagues. The SNs in the current study were obtained from a 10^9 cfu/ml solution, and a greater concentration of bioactive compounds may therefore have reached the target region of the intestine. BR11 was selected for the current setting on the basis of its potential antioxidant effects (Hung *et al.*, 2003; Hung *et al.*, 2005). The release of ROS is hypothesised to play a role in the initial stages of mucositis, leading to the oxidative damage of intestinal tissue (Sonis, 1998). Inhibiting the release of ROS could therefore impede the onset of mucositis and reduce its overall severity. In order to further investigate these mechanisms, studies should be performed to determine the effect of BR11 on the generation of ROS.

The effects of live EcN in models of gastrointestinal inflammation have been studied previously (Arribas *et al.*, 2009; Barth *et al.*, 2009; Guzy *et al.*, 2008; Schlee *et al.*, 2007; Ukena *et al.*, 2007; Zyrek *et al.*, 2007), and a number of molecular mechanisms have been identified which may explain its beneficial effects in the current study. The reduction in MPO levels in the jejunum in the current study may

have been due to the ability of the SN to reduce TNF- α levels, as reported previously in septic mice (Arribas *et al.*, 2009). Furthermore, the reduction in histological severity score in rats receiving EcN SN may have been a consequence of its ability to improve tight junction integrity (Ukena *et al.*, 2007, Zyrek *et al.*, 2007) and induce the expression of cyclooxygenase-2 an enzyme which has a role in intestinal wound healing (Otte *et al.*, 2009). This may have accelerated the rate of repair and rejuvenation of the epithelial cells. Schlee *et al.* (2007) recently determined that EcN induces the anti-microbial peptide human- β defensin-2 which could counteract the increased levels of pathogenic bacteria which arise following 5-FU administration and is believed to play a role in the development of mucositis (Stringer *et al.*, 2009a). Importantly, we must consider that the current study is the first in which EcN SN has been grown in DMEM. Previous studies (Schlee *et al.*, 2007; Ukena *et al.*, 2007; Zyrek *et al.*, 2007) have incubated the live bacteria in tryptic soy broth (TSB); a medium designed specifically to grow *E. coli* strains. Future studies should attempt to elucidate mechanisms by which EcN SN is able to reduce histological damage, and to compare both the bacterial growth rate and composition of SN produced following incubation with DMEM and TSB.

MPO is commonly used as a biochemical marker for neutrophil infiltration into tissues, and as such can be used as an indicator of damage to the gastrointestinal tract (Soares *et al.*, 2008). In the current study, MPO activity was analysed at different sections along the small intestine, although a 5-FU-induced elevation in MPO levels was only observed in the jejunum. These findings partially contradict those of Soares *et al.* (2008) who reported significant increases in MPO activity in both the jejunum and ileum of Wistar rats at day 3 post 5-FU administration. Interestingly, in the

current study, MPO was only increased in the jejunum of rats from the 5-FU control and BR11 SN + 5-FU treatment groups. The MPO levels of EcN SN + 5-FU-treated rats were not statistically different to healthy or 5-FU control animals. However, although no statistical difference was observed, there was a trend toward a decrease in MPO levels in rats treated with EcN SN + 5-FU which warrants further investigation.

5-FU has been shown to negatively impact on mucin dynamics, which may impede intestinal barrier function (Stringer *et al.*, 2009b). Decreases in goblet cell number following 5-FU administration suggest that the protective capabilities of the mucosal barrier may have been diminished following the depletion of stored mucins (Stringer *et al.*, 2009b). However, our findings suggest for the first time in a model of intestinal mucositis that probiotic-based therapies may be able to counter these deleterious effects. While BR11 SN only altered acidic mucin production in the jejunum, EcN SN partially maintained acidic-mucin producing goblet cells in the jejunum and neutral mucin producing goblet cells in the ileum. This finding is supported by other studies in which probiotics have been shown to increase mucin gene expression (Caballero-Franco *et al.*, 2007, Khailova *et al.*, 2010). Future studies should examine probiotic SNs for their capacity to alter mucin gene expression and actual mucin production.

In summary, the probiotic SNs assessed in the current study displayed variable efficacy in the treatment of 5-FU-induced mucositis. The *in vitro* model system used in this study can be further developed to become a valuable tool for screening novel compounds. The *in vivo* results suggest that BR11 and EcN SN may be able to

partially protect the intestine in the setting of 5-FU-induced mucositis as they partially improved specific indicators of gut damage. Future studies involving these strains should aim to characterise the components of the SN, as well as determining mechanisms by which they exert their protective effects.

2.5 Figure Legends and Figures

Figure 1. MPO activity of intestinal segments from rats at day 9 (or 96h post-5-FU or saline administration), receiving Water, DMEM, BR11 SN or EcN SN treatment. Segments from jejunum or jejunum-ileum (JI) junction. Data are expressed as mean (MPO u/g) \pm SEM. * denotes significant differences compared to Saline + Water control group ($p < 0.05$).

Figure 2: Sucrase activity of intestinal segments from rats at day 9 (or 96h post-5-FU or saline administration), receiving Water, DMEM, BR11 SN or EcN SN treatment. Segments from jejunum or jejunum-ileum (JI) junction. Sucrase activity expressed as nmol glucose/well/min/cm tissue, mean \pm SEM. * denotes a significant difference compared to Water + Saline control group ($p < 0.05$).

Figure 3: Histological severity score of (a) jejunum and (b) ileum of rats at day 9 (or 96h post-5-FU or saline administration), receiving Water, DMEM, BR11 or EcN SN treatment. * denotes a significant difference compared to Water + Saline control group ($p < 0.05$) and γ denotes a significant difference compared to DMEM + 5-FU group ($p < 0.05$).

Figure 4. Jejunum villus height and crypt depth of rats at day 9 (or 96h post-5-FU or saline administration), for rats receiving Water, DMEM, BR11 or EcN SN treatment. Data represented as mean \pm SEM. * indicates significant difference from Water + Saline control group ($p < 0.05$).

Figure 1.

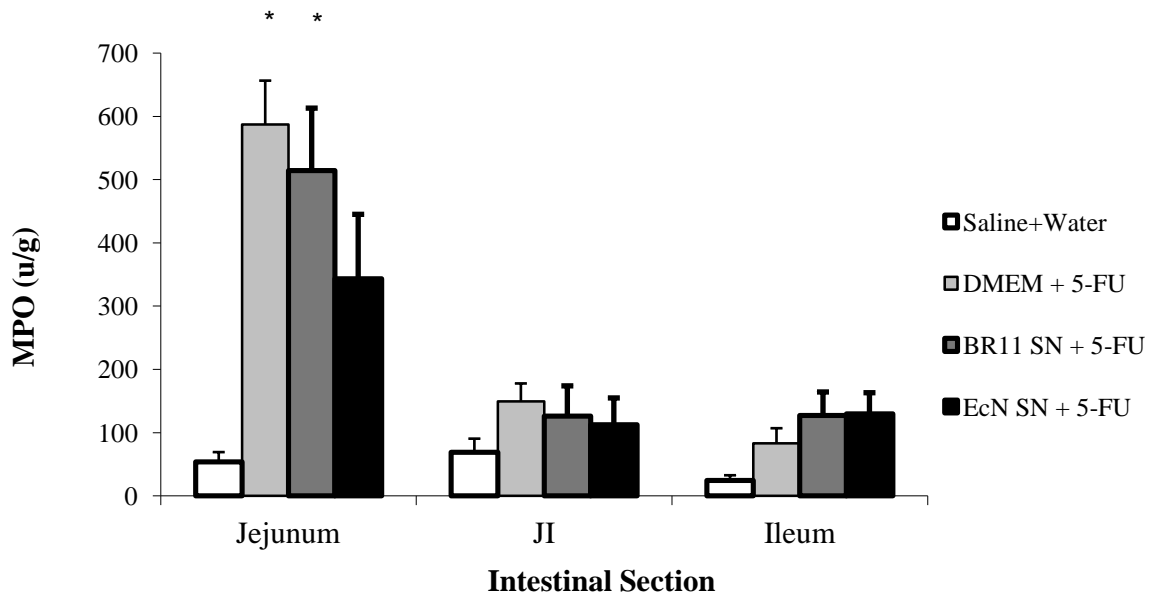


Figure 2.

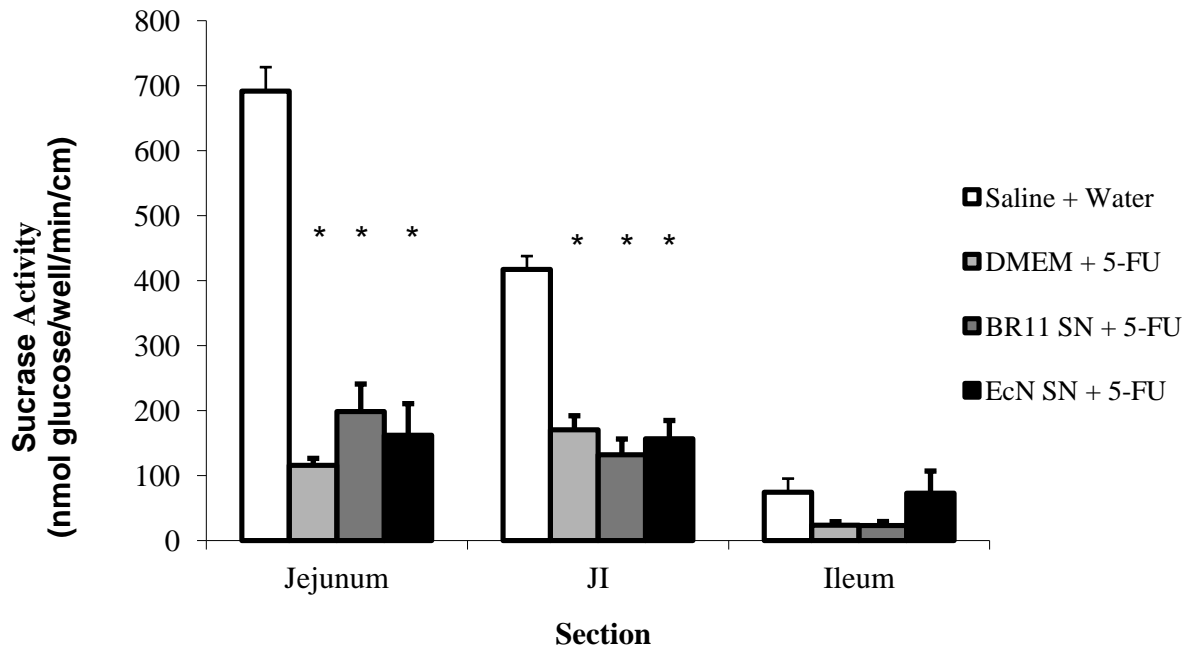


Figure 3.

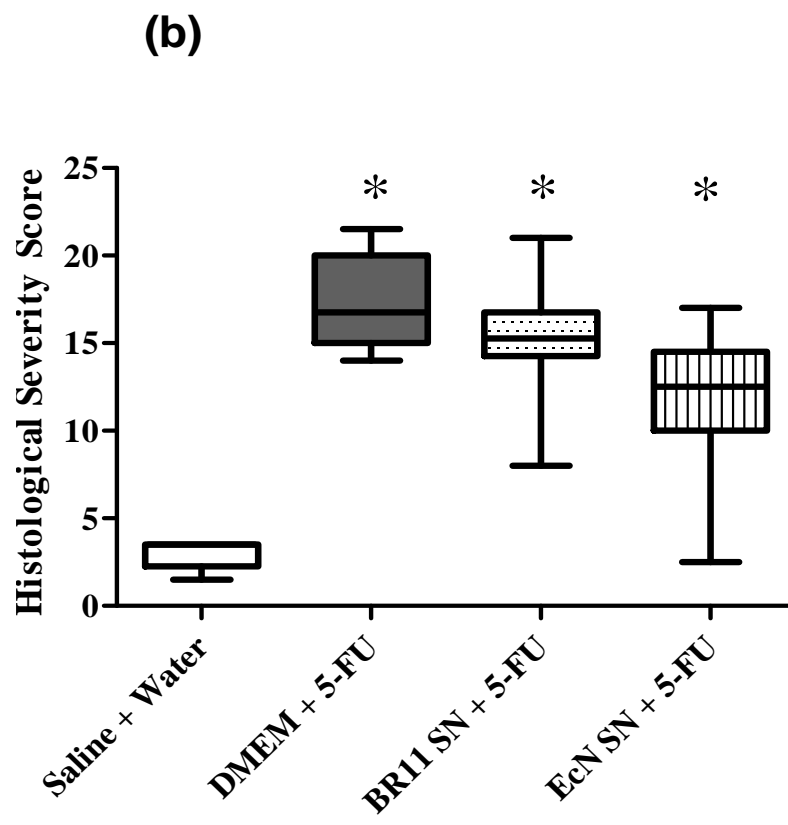
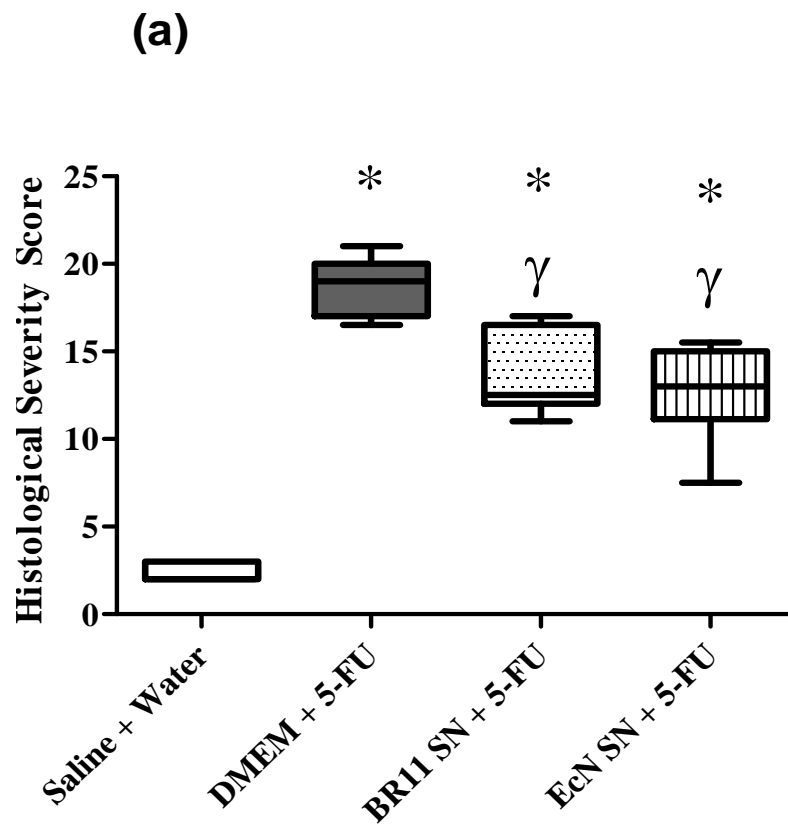
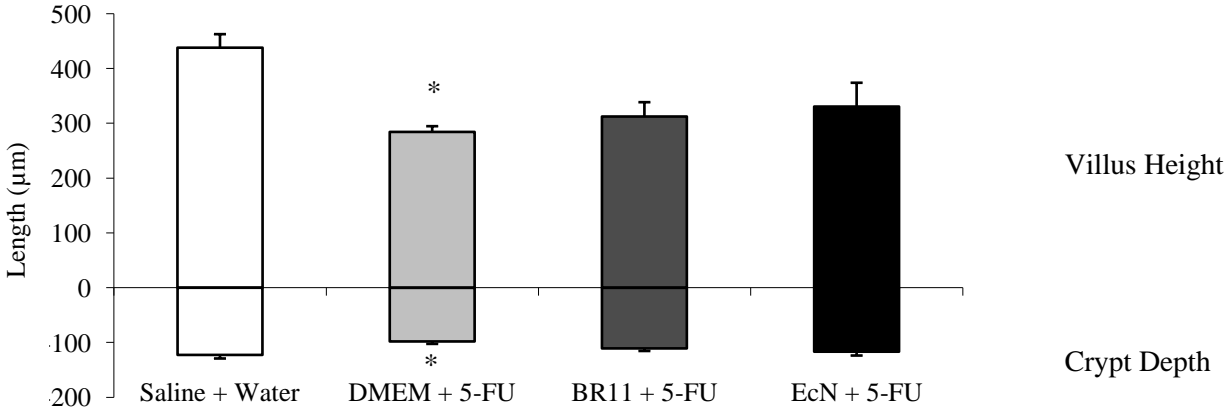


Figure 4.



2.6 Tables

Table 1. Cell numbers (10^5) and viabilities (%) of IEC-6 cells treated with SN preparations at days 3, 4, 5 and 6

Day	Measurement	Treatment					
		100% DMEM	DPBS + DMEM	BR11 SN + DMEM	EcN SN + DMEM	TH4 SN + DMEM	LA5 SN + DMEM
3	Cell Viability (%)	95 ± 1.5	94 ± 1.9	95 ± 1.9	89 ± 4.1	89 ± 5.6	89 ± 2.9
	Cell Number (10^4)	106 ± 12.3	61 ± 10.9*	77 ± 12.2	63 ± 9.2	46 ± 7.2*	46 ± 5.7*
4	Cell Viability (%)	95 ± 1.4	95 ± 0.8	95 ± 1.7	92 ± 3.6	94 ± 1.1	94 ± 0.6
	Cell Number (10^4)	79 ± 23.6	56 ± 21.9	75 ± 30.2	52 ± 24.8	27 ± 4.7	27 ± 7.4
5	Cell Viability (%)	96 ± 0.75	96 ± 2.1	96 ± 1.2	96 ± 0.54	94 ± 1.1	94 ± 0.6
	Cell Number (10^4)	133 ± 9.1	75 ± 29.9	84 ± 4.8	97 ± 13.1	85 ± 12.5	73 ± 7.8
6	Cell Viability (%)	95 ± 1.1	94 ± 1.3	93 ± 1.7	79 ± 11.9	73 ± 12.6	87 ± 2.4
	Cell Number (10^4)	156 ± 26.1	65 ± 24.7*	123 ± 22.9	81 ± 6.3	57 ± 12.4*	64 ± 6.3*

Statistical comparisons were made using a one-way ANOVA with a Bonferroni *post-hoc* test. Groups which are significantly different from 100% DMEM indicated by * ($p < 0.05$ considered significant). Data are expressed as mean ± SEM

Table 2. Cell numbers (10^5) and viabilities (%) of IEC-6 cells treated with SN preparations at 24, 48, 72 and 96h following 5-FU administration.

Day	Measurement	Treatment						
		100% DMEM	DMEM + 5-FU	DPBS + 5-FU	BR11 SN + 5-FU	EcN SN + 5-FU	TH4 SN + 5-FU	LA5 SN + 5-FU
3	Cell Viability (%)	95 ± 1.5	77 ± 4.8*	66 ± 8.2*	76 ± 11.1	85 ± 4.8	81 ± 4.1	89 ± 5.6
	Cell Number (10^4)	53 ± 9.4	10 ± 3.2*	10 ± 2.7*	6 ± 1.8*	9 ± 2.9*	7 ± 1.5*	6 ± 1.2*
4	Cell Viability (%)	94 ± 1.3	65 ± 7.4*	55 ± 8.5*	52 ± 7.6*	44 ± 5.2*	50 ± 9.9*	56 ± 6.9*
	Cell Number (10^4)	79 ± 23.6	23 ± 8.2*	17 ± 2.7*	17 ± 3.8*	18 ± 4.2*	12 ± 2.8*	12 ± 1.9*
5	Cell Viability (%)	96 ± 7.5	55 ± 5.5*	48 ± 6.4*	69 ± 10.8	67 ± 4.6	64 ± 7.3	52 ± 8.9*
	Cell Number (10^4)	134 ± 9.1	18 ± 5.1*	13 ± 4.8*	15 ± 4.1*	18 ± 5.2*	10 ± 3.7*	8 ± 1.5*
6	Cell Viability (%)	94 ± 1.1	87 ± 4.7	68 ± 3.9*	81 ± 8.1	89 ± 5.6	78 ± 4.8	79 ± 7.3
	Cell Number (10^4)	156 ± 26.1	7 ± 1.3*	6 ± 1.1*	5 ± 0.7*	6 ± 2.4*	4 ± 0.7*	4 ± 1.1*

Statistical comparisons were made using a one-way ANOVA with a Bonferroni *post-hoc* test. Groups which are significantly different from 100% DMEM indicated by * ($p < 0.05$ considered significant). Data are expressed as mean ± SEM

Table 3. Metabolism data from days 3 to 6 for rats receiving Water, DMEM, BR11 or EcN SN treatment.

Metabolic data	Group			
	Water + Saline	DMEM + 5-FU	BR11 SN + 5-FU	EcN SN + 5-FU
Water consumption (ml)	78.3 ± 5.9	60.6 ± 11.2	77.5 ± 5.4	84.4 ± 11.2
Urine output (ml)	68.1 ± 5.2	47.4 ± 6.8	59.0 ± 4.2	67.00 ± 7.5
Feed consumption (g)	24.0 ± 0.8	11.8 ± 0.9*	14.6 ± 1.9*	14.1 ± 2.3*
Fecal output (g)	3.6 ± 0.3	1.5 ± 0.2*	1.9 ± 0.2*	1.9 ± 0.2*

Feed consumption and Fecal output of rats from days 3 to 6, receiving either Water, Dulbecco's Modified Eagle Medium, *Lactobacillus fermentum* BR11 SN and *Escherichia coli* Nissle 1917 SN treatment (n = 8/treatment). Data are expressed as mean (g) ± SEM. Water consumption and urine output are expressed as mean (ml) ± SEM * indicates significant difference from rats receiving Water + Saline (p < 0.05).

Table 4. Gastrointestinal organ weights at day 9 for rats receiving Water, DMEM, BR11 or EcN SN treatment.

Organ weights (g) x 10⁻²				
Organ	Water + Saline	DMEM + 5-FU	BR11 SN + 5-FU	EcN SN + 5-FU
Stomach	71 ± 3	70 ± 2	72 ± 2	74 ± 3
Jejunum + Ileum	22 ± 2	25 ± 1	27 ± 2	27 ± 2
Duodenum	22 ± 2	25 ± 1	27 ± 2	27 ± 2
Caecum	42 ± 1	48 ± 2	52 ± 3	48 ± 2
Colon	73 ± 3	76 ± 5	62 ± 2	64 ± 4

Organ weights of rats at day 9, receiving either Water, Dulbecco's Modified Eagle Medium, *Lactobacillus fermentum* BR11 SN and *Escherichia coli* Nissle 1917 SN treatment (n = 8/treatment).

Data are expressed as mean (g) x 10⁻² ± SEM.

Table 5. Gastrointestinal organ lengths from rats at day 9 receiving Water, DMEM, BR11 or EcN SN treatment.

Organ lengths (cm)				
Organ	Water + Saline	DMEM + 5-FU	BR11 SN + 5-FU	EcN SN + 5-FU
Duodenum	5.4 ± 1.4	5.6 ± 0.1	5.5 ± 0.2	5.5 ± 0.2
Colon	12.1 ± 0.5	11.1 ± 0.4	11.1 ± 0.4	11.2 ± 0.4
Jejunum + Ileum	68.7 ± 0.9	65.6 ± 1.1	67.3 ± 0.8	66.6 ± 1.1

Organ lengths of rats at day 9, receiving either Water, Dulbecco's Modified Eagle Medium, *Lactobacillus fermentum* BR11 SN and *Escherichia coli* Nissle 1917 SN treatment (n = 8/treatment). Data are expressed as mean (cm) ± SEM.

Table 6. Differences in acidic and neutral goblet cell mucin composition from rats at day 9, receiving Water, DMEM, BR11 or EcN SN treatment.

Section	Mucin	Treatment			
		Water + Saline	DMEM + 5-FU	BR11 SN + 5-FU	EcN SN + 5-FU
Jejunum	Neutral	24.17 ± 1.3	17.33 ± 1.7*	17.72 ± 1.7*	18.57 ± 1.4*
	Acidic	26.44 ± 2.7	14.94 ± 1.4*	18.83 ± 1.9	19.18 ± 1.0
Ileum	Neutral	26.67 ± 2.4	13.43 ± 2.4*	11.96 ± 2.1*	19.39 ± 4.7
	Acidic	22.48 ± 2.8	13.57 ± 1.4*	11.66 ± 1.2*	14.56 ± 2.1 *

Acidic and neutral goblet cell numbers from crypts of rats at day 9, receiving either Water, Dulbecco's Modified Eagle Medium, *Lactobacillus fermentum* BR11 SN and *Escherichia coli* Nissle 1917 SN treatment (n = 8/treatment). Data are expressed as mean ± SEM. * denotes significance compared to Water + Saline treatment.

**Chapter 3. Probiotic factors partially prevent 5-fluorouracil-
induced changes to caspase 3 and 7 activation and
transepithelial electrical resistance *in vitro***

3.1 Introduction

The high degree of strain specificity associated with mechanisms of probiotic action can be problematic when selecting an appropriate strain for a specific disorder. Consistent with the involvement of apoptosis and barrier disruption in the development of intestinal mucositis, two probiotic strains which have previously been shown to promote cell survival and barrier function, LGG (Donato *et al.*, 2010; Lin *et al.*, 2008a) and EcN (Ukena *et al.*, 2007) were selected for the current study.

Treatment with live EcN has been shown to up-regulate the tight junction molecule, ZO-1 at both the mRNA and protein levels, and reduce intestinal permeability in mice with DSS-induced colitis (Ukena *et al.*, 2007). EcN administration led to an overall increase in intestinal barrier function, reduced body weight loss and leukocyte infiltration. However, the study was limited to analysis of the large intestine and it remains unexplored if EcN would also impact on small intestinal enterocytes. LGG has also been shown to exert beneficial effects on intestinal cells, preventing TEER reduction and maintaining ZO-2 levels in Caco-2 cells treated with pro-inflammatory interferon- δ (Donato *et al.*, 2010). These changes were associated with the inhibition of pro-inflammatory TNF- α expression: a cytokine which is also involved in the development of intestinal mucositis (Logan *et al.*, 2007). LGG has also demonstrated anti-apoptotic effects in a model of *ex vivo* staurosporine-induced apoptosis (Lin *et al.*, 2008a), in which a single dose of LGG was sufficient to inhibit caspase 3 expression and reduce the overall percentage of cells undergoing apoptosis.

EcN and LGG are yet to be thoroughly investigated in the setting of 5-FU-induced damage. Therefore, the primary aim of this study was to test the capacity for probiotic SNs derived from LGG and EcN, to protect epithelial cells from caspase activation and reduction of epithelial barrier function induced by 5-FU.

3.2 Materials and methods

3.2.1 Supernatant preparation

LGG was kindly donated by Valio Ltd. (Helsinki, Finland). EcN was purchased from Ardeypharm (Herdecke, Germany) and was grown in TSB (Oxoid, South Australia, Australia). LGG was grown on MRS agar (Oxoid) at 37°C for overnight and then MRS broth (Oxoid). All bacterial strains were incubated at 37°C for two days and reached a concentration of 10⁹ cfu/ml. Broths were then centrifuged at 1500g for 10 mins. SNs were then collected and buffered with Tris/HCl to a pH of 7.0. SNs were passed through a 0.20-µm filter, and stored at -20°C until use. Prior to administration, SNs were diluted with 50% DMEM.

3.2.2 Transepithelial electrical resistance

IEC-6 rat intestinal epithelial cells (passage 20-23) were cultured in medium comprising DMEM, 10% foetal bovine serum (GIBCO®, Victoria, Australia) and 1% penicillin/streptomycin (GIBCO®). IEC-6 cells were added to the upper layer of 0.33cm² Transwell clear polyester permeable membranes (Corning Costar, New York, USA) at a density of 8 x 10⁴ cells/well. Fresh DMEM (200µl) was then added to the

lower layer. Cells were left overnight to adhere to the plate prior to the beginning of the experiment. Plates were stored at 37°C in 95% air, 5% CO₂ for the duration of the study. Six treatment groups were used for the study: DPBS + Saline (untreated control), DPBS + 5-FU (5-FU control), MRS Broth + 5-FU, TSB + 5-FU, LGG SN + 5-FU and EcN SN + 5-FU. The MRS and TSB groups were included as vehicle controls to determine the effects of the media in which the SNs were grown. Treatment and DMEM (100µl of each) were added together to the upper layer of each well daily for three days. On day 3, 5µM 5-FU in DPBS (500µl) was added to the DMEM in the lower layer and left overnight to induce a reduction in TEER. Medium was then replaced daily with fresh DMEM, without SN or vehicle. TEER measurements were recorded daily on days 3, 4 and 5. Treatments were performed in duplicate while the entire experiment was carried out in triplicate to give n = 6 for each treatment. The resistance across confluent monolayers was measured using a millicell-ERS volt-ohm meter (Millipore, Massachusetts, USA) with electrodes. Values were expressed as ohms per square centimetre (Ω/cm^2), taking into account the surface area of the filter.

3.2.3 Caspase 3/7 activity

IEC-6 rat intestinal epithelial cells (passage 24-26) were cultured in medium comprising DMEM, 10% foetal bovine serum (GIBCO®) and 1% penicillin/streptomycin (GIBCO®). For apoptosis measurements, cells were seeded into a black 96-well plate (0.32 cm²) at a density of 8 x 10³ cells/well. Cells were left overnight to adhere to the plate prior to the addition of treatments. Plates were stored at 37°C in 95% air, 5% CO₂ for the duration of the study.

The six treatment groups were as described previously. Treatment and DMEM (100µl of each) was added to each well daily for four days. On day 3, 5µM 5-FU in DPBS (200µl) was added to each well for 24h to induce apoptosis. Medium was then replaced daily with fresh DMEM without SN or vehicle. Apoptosis measurements were recorded daily on days 4 and 5. Treatments were performed in duplicate, while the entire experiment was carried out in triplicate to provide n = 6 for each treatment.

Apoptosis was measured by caspase 3/7 assay (Apo-One® Homogenous caspase 3/7 assay kit, Promega, Wisconsin, USA). Medium was removed from the cells and briefly stored in Eppendorf tubes, before 50µl was returned to each well. This was done to ensure cells which had undergone apoptosis and detached from the monolayer were included in the assay mixture, and that the volume of medium was uniform for each well. Caspase reagent (50µl) was then added to each well. The plate was covered with aluminium foil and left on a plate shaker at room temperature for 2h. After 2h, the plate was read on a fluorescent plate reader at 485nm/535nm.

3.2.4 *Statistical analysis*

Statistical analysis was conducted using SPSS 15.0.1 for Windows (SPSS Inc., Illinois, USA). Data were compared by one-way ANOVA with a Tukey *post-hoc* test and were expressed as mean ± SEM. For all data, p < 0.05 was considered significant.

3.3 Results

3.3.1 *Transepithelial electrical resistance*

Cells were co-incubated with 5 μ M 5-FU overnight from days 3 to 4. TEER was recorded on days 3, 4 and 5. On day 3 (i.e. pre-5-FU) LGG SN pre-treatment had significantly increased TEER compared to untreated controls ($p < 0.05$, Figure 1a). 5-FU administration significantly reduced TEER on day 4 compared to untreated controls ($p < 0.05$); however, this reduction was not observed in any other treatments (Figure 1b). MRS + 5-FU, LGG SN + 5-FU and EcN SN + 5-FU-treated groups displayed significantly higher TEER readings than 5-FU controls ($p < 0.05$). Importantly, EcN SN treatment also increased TEER compared to its vehicle control, TSB ($p < 0.05$).

The 5-FU-induced reduction in TEER was also observed on day 5 (Figure 1c, $p < 0.05$). As with the measurements on day 4, all vehicle and SN groups prevented this reduction in TEER. LGG and EcN SN + 5-FU groups exhibited increased TEER compared to untreated controls at day 5 ($p < 0.05$). Furthermore, EcN SN treated cells exhibited higher TEER than the TSB vehicle control ($p < 0.05$).

3.3.2 *Caspase 3/7 activity*

Apoptosis was measured via caspase 3/7 activation on days 4 and 5. On day 4, 5-FU administration significantly increased caspase 3/7 activation compared to untreated controls (Figure 2a, $p < 0.05$). All vehicle controls and SN groups displayed lower caspase 3/7 activation than 5-FU controls ($p < 0.05$), and both LGG SN and EcN SN significantly reduced activation compared to their respective vehicle controls, MRS and

TSB ($p < 0.05$). Caspase activation for 5-FU controls remained elevated on day 5 compared to untreated cells ($p < 0.05$, Figure 2b). Consistent with day 4 results, no increase was observed in any vehicle or SN treatment group ($p > 0.05$). Both MRS and LGG SN treated cells displayed lower caspase 3/7 activation compared to 5-FU controls ($p < 0.05$). EcN SN treated cells exhibited significantly reduced caspase 3/7 activation compared to 5-FU controls ($p < 0.05$), although this reduction was not observed in the TSB vehicle control group.

3.4 Discussion

Pre-treatment with EcN SN prevented 5-FU-induced reductions in TEER following an overnight treatment with 5-FU. EcN has been shown to up-regulate expression of ZO-1 (Ukena *et al.*, 2007) and ZO-2 (Zyrek *et al.*, 2007): two tight junction molecules which have previously been associated with improvements in TEER (Basuroy *et al.*, 2003). Whether these changes are responsible for the increases observed in this current study requires further investigation. However, as the effect of 5-FU administration on the expression of ZOs remains undefined, future studies could also investigate the capacity for EcN to alter the distribution of tight junction molecules, such as claudins. The findings of Zyrek and colleagues are particularly relevant, as an increase in ZO-1 was observed in a model of intestinal damage, whereby the authors added enteropathogenic *E. coli* E2348/69 to T84 cells to induce epithelial disruption (Zyrek *et al.*, 2007). The study revealed that co-incubation of EcN with the pathogen, or the addition of EcN following damage, was able to prevent the onset of epithelial disruption and maintain barrier integrity. Given the effect of EcN SN on caspase 3/7 activity in the current study,

we also proposed that the reduced cell apoptosis may have contributed to an overall increase in epithelial integrity.

Caspases 3 and 7 are executioner caspases which can be used as positive markers for cell apoptosis (Bowen *et al.*, 2006). EcN SN successfully prevented the increase in caspase 3/7 activity induced by 5-FU. To our knowledge, this represents the first description of an anti-apoptotic effect of EcN SN. Previously, live EcN has been shown to influence the expression of genes linked to apoptosis regulation (Zyrek *et al.*, 2007) and pro-inflammatory cytokine production (Otte *et al.*, 2009), indicating potential mechanisms underlying the observed results.

In the current study, LGG SN decreased apoptosis and prevented barrier disruption, a finding consistent with previous studies (Johnson-Henry *et al.*, 2008; Myllyluoma *et al.*, 2008). Acute improvement of epithelial integrity has been reported in T84 cells treated with LGG, where a 3h incubation significantly increased TEER (Johnson-Henry *et al.*, 2008). LGG administration was also able to prevent an *E. coli* 0157:H7-induced reduction in TEER, whilst maintaining epithelial permeability and re-distribution of tight junction molecules. Seth *et al.* (2008) isolated two proteins (p40 and p70) from the SN of LGG that prevented hydrogen peroxide-induced damage to barrier function in Caco-2 cells. Further analysis of the LGG SN revealed a number of proteins released by LGG, including LytR and CpsA (Sanchez *et al.*, 2009). These two proteins are components of the LytR/CpsA/Psr protein family which plays a role in cell wall structural maintenance. To determine if these proteins are able to protect against 5-FU-

induced damage, future studies should investigate the ability of these proteins to maintain barrier function, both independently and in combination.

The capacity for LGG to reduce enterocyte apoptosis has been described previously in other models of intestinal damage (Lin *et al.*, 2008b; Yan *et al.*, 2007). Yan and colleagues isolated the secreted proteins p40 and p75 from LGG and found that both proteins were able to inhibit TNF-induced apoptosis, whilst also increasing cell proliferation (Yan *et al.* 2007). Further studies are required to determine the mechanism by which these proteins were able to inhibit apoptosis. The components of the SN responsible for the beneficial results in the current study have not yet been identified.

The current study further suggests that the growth media from which the SN were obtained may be contributing to the observed results. MRS broth improved TEER following incubation with 5-FU, while both MRS and TSB prevented increases in caspase activity post 5-FU treatment. The primary component of TSB is pancreatic digest of casein: a mix of amino acids, of which glutamine is the most abundant (Oxoid, 2010a). Glutamine is an important amino acid for cell proliferation (Tuhacek *et al.*, 2004) and has been associated with maintenance of epithelial cell integrity (Li and Neu, 2009; Potsic *et al.*, 2002). Indeed, early research into the use of glutamine as a treatment for chemotherapy-induced mucositis yielded promising results (Sukhotnik *et al.*, 2009). In a model of MTX-induced mucositis, glutamine supplementation following chemotherapy reduced apoptosis and promoted cell proliferation. It was hypothesised that the abnormally high availability of glutamine, the preferred fuel source of the small

intestine (Windmueller and Spaeth, 1974), may have stimulated mucosal hyperplasia, as well as the release of enteric hormones which in turn had trophic effects on the intestinal mucosa.

Glucose is the major component of MRS broth (Oxoid, 2010b). High glucose containing media have been shown to prevent LPS-induced changes in the induction of apoptosis and epithelial barrier function (Yu *et al.*, 2005), with high glucose containing media preventing LPS-induced reduction of Bcl-2 protein expression and increasing anti-apoptotic Bcl-X_L. These proteins have previously been associated with the development of intestinal mucositis (Bowen *et al.*, 2006) and similar modulation in this current study may have been responsible for the protective effects of MRS. The protective effect of the growth media in the current study suggests that the changes to caspase activity and TEER observed in the SN treated groups may not have been entirely due to the secreted factors. Future studies should attempt to separate the secreted compounds from the growth media using techniques such as super-centrifugation and then compare the two individually.

Secreted factors from LGG and EcN were partially effective at preventing 5-FU-induced alterations in epithelial barrier function and apoptosis, with the growth media also contributing to the observed protection. Future studies should focus on determining the importance of strain specificity and comparisons between the probiotic SNs and their respective live bacteria. Furthermore, investigation into the effects of the probiotic-derived compounds on tight junction expression and intestinal permeability should be

conducted to better understand the mechanisms underlying maintenance of intestinal barrier function.

3.5 Figure legends and figures

Figure 1. Transepithelial electrical resistance readings at (a) day 3, (b) day 4 and (c) day 5. Cells were treated from days 0 to 3 with either phosphate buffered saline (Control and 5-FU), de Man Rogosa Sharpe (MRS) broth, *Lactobacillus rhamnosus* GG supernatant (LGG SN), Tryptic Soy Broth (TSB) or *Escherichia coli* Nissle 1917 supernatant (EcN SN). All treatments were added in a 1:1 dilution with Dulbecco's Modified Eagle Medium. Cells were treated with 5 μ M 5-fluorouracil overnight on day 3. Values are expressed as mean \pm SEM, n = 6. * denotes a significant difference compared to control treatment (p < 0.05), α denotes a significant difference compared to 5-FU treatment (p < 0.05) and β denotes a significant difference compared to TSB + 5-FU (p < 0.05).

Figure 2. Caspase 3/7 activation on days 4 (a) and 5 (b). Cells were treated from days 0 to 3 with either phosphate buffered saline (Control and 5-FU), de Man Rogosa Sharpe (MRS) broth, *Lactobacillus rhamnosus* GG supernatant (LGG SN), Tryptic Soy Broth (TSB) or *Escherichia coli* Nissle 1917 supernatant (EcN SN). Cells were treated with 5 μ M 5-fluorouracil overnight on day 3. All treatments were added in a 1:1 dilution with Dulbecco's Modified Eagle Medium. Values expressed as mean \pm SEM, n = 6. * denotes a significant difference compared to control treatment (p < 0.05), α denotes a significant difference compared to 5-FU (p < 0.05), γ denotes a significant difference compared to MRS + 5-FU (p < 0.05) and β denotes a significant difference compared to TSB + 5-FU (p < 0.05).

Figure 1.

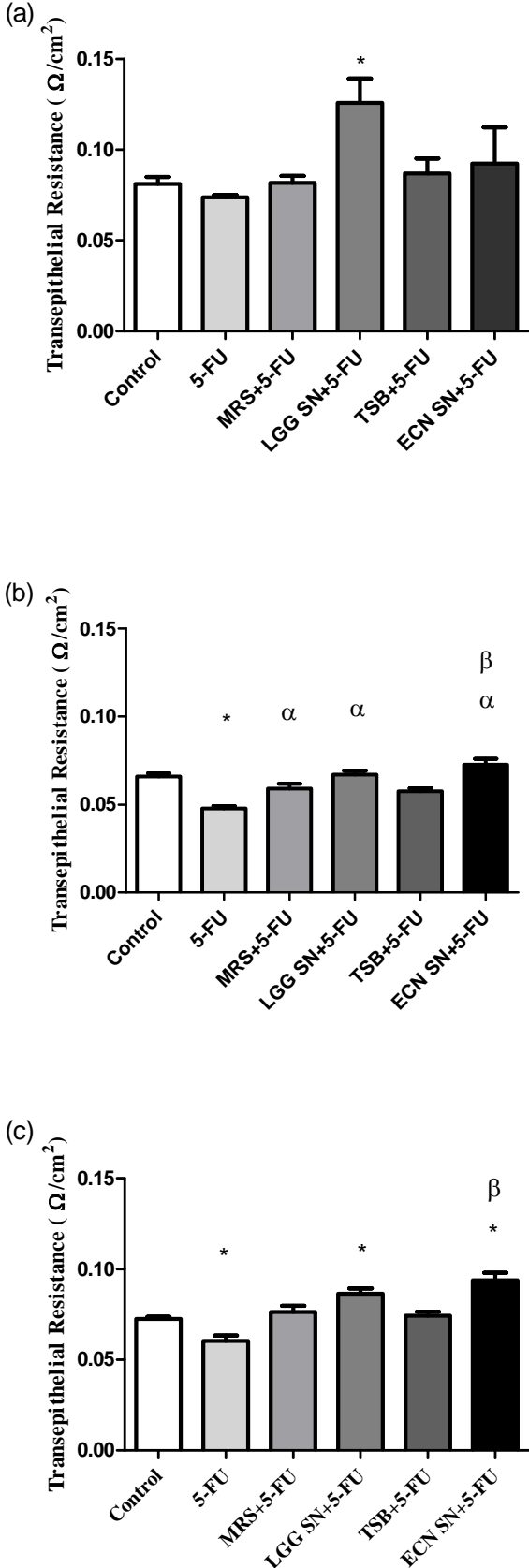
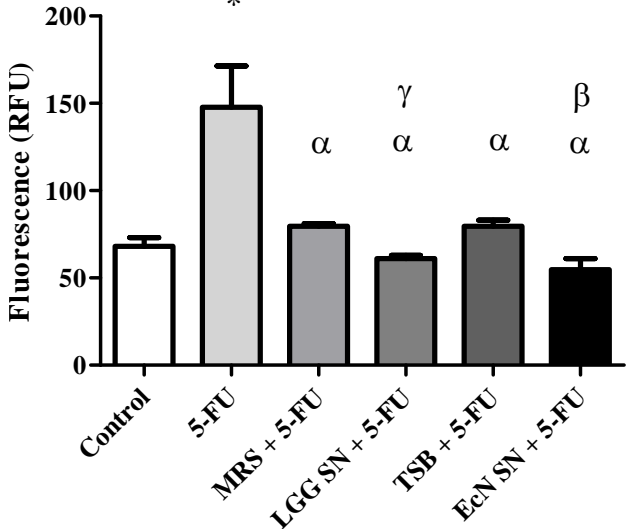
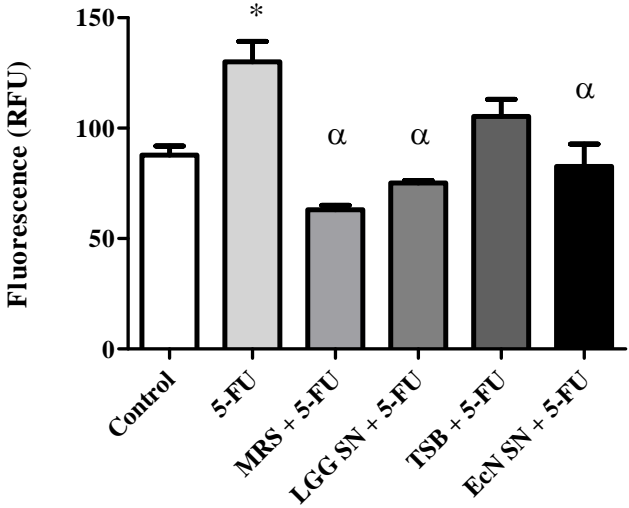


Figure 2.

(a)



(b)



**Chapter 4. Effects of probiotic factor pre-treatment on
5-fluorouracil-induced intestinal mucositis in rats**

4.1 Introduction

Selecting the optimum probiotic strains and treatment regimens is a difficulty associated with the use of probiotic-based therapies. In Chapter 2, bacterial SNs delivered pre-and post-5-FU administration partially reduced the severity of 5-FU-induced intestinal mucositis in rats. In Chapter 3, a four day pre-treatment of intestinal epithelial cells with LGG SN and EcN SN *in vitro* was sufficient to inhibit the 5-FU-induced increase in caspase activity and reduction in TEER. Given the success of pre-treatment in Chapter 3, the current study investigated the potential for a probiotic-based pre-treatment to prevent the onset of 5-FU-induced intestinal mucositis in rats.

While post-treatment regimes aim to enhance healing and restoration of the intestine, pre-treatment is aimed at preparing the host for the oncoming challenge. In the case of intestinal mucositis, this may be achieved by strengthening the intestinal barrier or stimulating the release of anti-inflammatory cytokines. Pre-treatment regimes have been assessed previously to prevent intestinal mucositis using a number of different compounds. CR3294, a benzamidine derivative, administered to rats at 20mg/kg for 3 days prior to 5-FU treatment, effectively prevented 5-FU-induced reduction of crypt cell proliferation, and reduced the incidence of diarrhoea (Letari *et al.*, 2010). In addition, Shiota *et al.* (2010) reported that five-day pre-treatment with spinach extract prevented 5-FU-induced villus atrophy and pro-inflammatory cytokine production in the jejunum of rats. In both of these studies, the anti-oxidative properties of the compounds were believed to be responsible for the observed protection.

Despite not currently being used for the prevention of mucositis, probiotics have been investigated as potential preventative strategies in other conditions (Anderson *et al.*, 2010; Lutgendorff *et al.*, 2009; Nanda Kumar *et al.*, 2008). Five-day pre-treatment with Ecologic 641 (a probiotic combination consisting of *L. acidophilus*, *L. casei*, *L. salivarius*, *Lactococcus lactis*, *B. lactis* and *B. bifidum*) significantly reduced cell apoptosis, passage of pathogenic *Escherichia coli* across the epithelial barrier and prevented the disruption of tight junction proteins in rats with acute pancreatitis (Lutgendorff *et al.*, 2009). It was hypothesised that this may have been due to an up-regulation of mucosal glutathione synthesis following probiotic administration. A probiotic combination was also investigated by Nanda Kumar *et al.* (2008) in the prevention of DSS-colitis in mice. Mice that received Ecologic 641 for one week prior to the induction of colitis displayed lower histological damage scores and decreased mRNA expression of pro-inflammatory cytokines compared to those treated with DSS only. Furthermore, DSS administration altered faecal bacteroides, bifidobacterium and lactobacillus levels, although these changes were not observed in DSS-treated mice administered probiotics.

As described in Chapter 3, pre-treatment with probiotics has demonstrated efficacy *in vitro*. Caco-2 cells pre-treated with *L. plantarum* MB452 for 10h displayed significantly higher levels of tight junction proteins ZO-1, ZO-2 and cingulin (Anderson *et al.*, 2010). *L. plantarum* administration also up-regulated the expression of 19 different tight junction related genes, suggesting an overall improvement to intestinal barrier function. Although Anderson and colleagues did not investigate the protective effects of *L.*

plantarum MB452, it was hypothesised that these changes would render the monolayer less susceptible to chemical damage.

In addition to testing the efficacy of a pre-treatment regime, this current chapter compares the effectiveness of live bacteria to its bacterial SN. This comparison is important as it is possible for SNs to exert different effects to the live bacteria from which it was derived. For example, *Faecalibacterium prausnitzii* SN has been shown to prevent IL-1 β -induced NF- κ B activity in Caco-2 cells, while the live bacteria had no effect (Sokol *et al.*, 2008). These differing results may have been due to different concentrations of active components in the bacterial cells, or due to an interaction between the live bacteria and the active component. However, the same study reported that both the SN and live bacteria had similar protective effects in an *in vivo* model of TNBS-induced colitis (Sokol *et al.*, 2008). Indeed, comparable efficacies between the live bacteria and its SNs appear more common. Borthakur *et al.* (2008) reported that both live *L. acidophilus* and its SN stimulated luminal chloride/hydroxyl exchange activity in Caco-2 cells, in contrast to the heat inactivated form of the bacteria which had no effect. Similarly, EcN and its SN stimulated nitric oxide production and anti-inflammatory cytokine secretion in rat peritoneal cells (Zidek *et al.*, 2010). Further investigation revealed that these changes were due to the production of LPS by the live cells during the growth phase.

Live EcN and LGG are yet to be thoroughly investigated in the setting of 5-FU-induced damage *in vivo*. Therefore, the primary aim of this study was to investigate the capacity

for live LGG and EcN and their SNs to prevent the onset of intestinal mucositis induced by 5-FU injection in rats.

4.2 Materials and methods

4.2.1 Live bacteria and supernatant preparation

Live bacteria were sourced and grown following the methods described in Chapter 3. In order to obtain bacterial SNs, broths were centrifuged at 1500g for 10 mins. SNs were then collected and buffered with Tris/HCl to a pH of 7.0. SNs were passed through a 0.20µm filter, and stored at -20°C until use.

4.2.2 Animal trial protocol

The study was conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (2004) and was approved by the Animal Care and Ethics Committees of the Children, Youth and Women's Health Service and The University of Adelaide.

Female Dark Agouti rats were individually housed in metabolism cages (Tecniplast, Pennsylvania, USA) in a temperature-controlled room (22°C) with a 12h light-dark cycle. Rats were given *ad libitum* access to fresh water and an 18% casein-based diet (Tomas *et al.*, 1991) and were randomly allocated to one of eight treatment groups (n = 8/treatment): Water + Saline (saline control), Water + 5-FU (5-FU Control), MRS + 5-FU broth (vehicle control), Live LGG + 5-FU, LGG SN + 5-FU, TSB + 5-FU (vehicle control), Live EcN + 5-FU and EcN SN + 5-FU. Treatments (1ml) were administered

daily via oro-gastric gavage from days 0 to 9. On day 8, animals were injected intraperitoneally with 5-FU (150mg/kg DBL[®], Mayne Pharma Pty Ltd, VIC, Australia) or saline. Food and water intake, body weight and faecal and urine output were recorded daily. At day 9, all rats were killed via carbon dioxide overdose and cervical dislocation. The gastrointestinal tract was removed and the lengths of the duodenum, small intestine and colon were measured. All gastrointestinal organs were then emptied of contents and weighed. Separate 4cm sections of small intestine and colon were collected in either 10% buffered formalin or snap frozen in liquid nitrogen and stored at -80°C. The weights of visceral organs were also recorded.

4.2.3 Histological analysis and mucin staining

As described in Chapter 2, specimens from the jejunum and ileum were sectioned (6µm) and stained with haematoxylin and eosin, before being analysed for villus height, crypt depth and qualitative histological damage (Howarth *et al.*, 1996). Mucin production was determined by two staining techniques: PAS, to identify neutral mucins, and HID-AB, for acidic mucins. The staining procedures were carried out following the protocol described in Chapter 2 (Forder *et al.*, 2007).

4.2.4 Myeloperoxidase and sucrase activity

As described in Chapter 2, neutrophil infiltration in the jejunum, JI and ileum was determined by MPO assay, following a previously described technique (Krawisz *et al.*, 1984). Small intestinal sucrase activity (jejunum and ileum) was measured by sucrase assay, following a previously described technique (Cool *et al.*, 2005).

4.2.5 Proliferating cell nuclear antigen staining

Sections (5µm) of jejunum were analysed for proliferating epithelial cells using the Proliferating Cell Nuclear Antigen (PCNA) staining technique (Invitrogen, NSW, Australia). Slides were de-paraffinised in two changes of xylene for five mins each, before being rehydrated through a series of graded ethanol solutions. Slides were then heated (60°C) for 25 mins to aid antigen retrieval. Blocking solution (100µl) was then added to the tissue and incubated for 10 mins. This solution was then blotted and replaced by 100µl of biotinylated mouse anti-PCNA primary antibody for 60 mins. Slides were then washed twice in DPBS before 100µl of streptavidin was added to each section. Slides were incubated for 10 mins before being washed twice in DPBS. DAB chromogen (100µl) was then added to each section for 5 mins, followed by 100µl of haematoxylin for 2 mins. Slides were then washed in tap water and then DPBS, before being dehydrated and cleared with xylene. Proliferating cells, as indicated by dark brown stain, were counted using an Olympus WH B10X/20 microscope (Olympus, Tokyo, Japan) at 20x magnification and expressed as the number of cells/mm².

4.2.6 Detection of apoptotic cells

Sections (5µm) of jejunum were analysed for apoptotic cells using a TACS 2 TdT *In Situ* Apoptosis kit (Trevigen, Maryland, USA). Slides were de-paraffinied in two changes of xylene for 5 mins each, before being rehydrated through a series of graded ethanol solutions. After washing with 1x DPBS, samples were covered with 50µl of Proteinase K solution for 30 mins. Slides were then washed in de-ionised water and

immersed in a 10:1 dilution of methanol and 30% hydrogen peroxide for 5 mins. DPBS was again used to wash slides before samples were immersed in 1x TdT labelling buffer for 5 mins. Samples were then incubated with 50µl of Labelling Reaction Mix at 37°C for 60 mins in a humidity chamber. The reaction was stopped by immersing the slides in 1x TdT Stop Buffer for 5 mins. De-ionised water was then used to wash the slides before they were incubated at 37°C for 10 mins in a humidity chamber. Slides were again washed in 1x DPBS and then immersed in DAB solution for 5 mins, before being added to 1% Methyl Green solution for 2 mins. Butanol and xylene were used to wash the slides before they were mounted. Apoptotic cells in the crypts, as indicated by dark brown nuclear staining, were counted using an Olympus WH B10X/20 microscope at 40x magnification and expressed as a percentage of total cells.

4.2.7 Statistical analysis

Statistical analyses were conducted using SPSS 15.0.1 for Windows (SPSS Inc., Illinois, USA). Daily metabolic data were analysed using a repeated measures ANOVA with a Holme's *post-hoc* test ($p < 0.05$ significance) to compare the differences both amongst groups and within groups across the duration of the trial. Overall histological damage severity was compared by a Kruskal Wallis test with a Bonferroni *post-hoc* test, and expressed as median (range). All other data were compared by one-way ANOVA with a Tukey *post-hoc* test and were expressed as mean \pm SEM. For all data, $p < 0.05$ was considered significant.

4.3 Results

4.3.1 Metabolism parameters and organ data

Metabolism data, including water and feed consumption, and urine and faecal output were recorded between days 0 to 9 and were analysed both for the duration of the trial, and for the period following 5-FU administration (day 8 to 9). No changes were observed in any of these parameters over the course of the entire trial (day 0 to 9). However, post-5-FU administration (day 8 to 9), both EcN SN + 5-FU and LGG SN + 5-FU-treated rats had increased urine output compared to healthy controls ($p < 0.05$, Table 1). 5-FU + either Water, MRS or Live EcN administration decreased post-5-FU faecal output compared to healthy controls ($p < 0.05$). No changes were observed in feed consumption in the post-5-FU period ($p > 0.05$); however, water intake was increased in all 5-FU-treated groups ($p < 0.05$).

No significant differences were detected in either the weight (Table 2) or length (data not shown) of the small intestine. Examination of visceral organs revealed that 5-FU + either Water, Live LGG or Live EcN significantly reduced thymus weight compared to healthy controls ($p < 0.05$, Table 2), while Live EcN + 5-FU administration also reduced liver weight compared to 5-FU controls ($p < 0.05$, Table 1). In addition, rats treated with 5-FU + either MRS, TSB, Live LGG or EcN SN, had a significantly lower spleen weight than the healthy controls ($p < 0.05$, Table 2).

4.3.2 Histological analysis and mucin staining

In the jejunum (Figure 1a) and the ileum (Figure 1b), 5-FU treatment resulted in significantly greater histological severity scores compared to healthy controls ($p < 0.05$). Interestingly, 5-FU + either TSB or Live EcN also significantly increased histological damage in both the jejunum and ileum compared to healthy controls ($p < 0.05$). Although offering no protective effects in the ileum, rats pre-treated with MRS broth, Live LGG, LGG SN and EcN SN had severity scores in the jejunum that were not significantly increased compared to untreated controls ($p > 0.05$).

5-FU administration did not significantly alter the number of goblet cells containing either neutral or acidic mucins in the jejunum (Table 3). Furthermore, none of the pre-treatments had any effect on goblet cell number ($p > 0.05$, data not shown).

4.3.3 Myeloperoxidase activity

Only Water + 5-FU administration significantly increased MPO activity in the jejunum ($p < 0.05$, Figure 2a), with these rats producing more than four times the MPO of saline-injected controls. Rats which received either LGG SN or EcN SN + 5-FU had significantly lower MPO activity than 5-FU controls ($p < 0.05$). In the ileum, no significant changes were observed among any groups ($p > 0.05$, Figure 2b).

4.3.4 Sucrase activity

5-FU administration significantly reduced sucrase activity in the jejunum compared to healthy controls ($p < 0.05$, Figure 3a). This decrease was also observed in rats treated

with TSB + 5-FU ($p < 0.05$). Rats treated with Live LGG + 5-FU exhibited significantly higher sucrase activity than rats in the 5-FU control group ($p < 0.05$). No changes in sucrase activity were observed in the ileum in any of the treatment groups (Figure 3b).

4.3.5 PCNA and apoptosis staining

The number of proliferating cell in the jejunum was significantly reduced by 5-FU treatment when compared to untreated controls ($p < 0.05$, Figure 4). This reduction was also observed in the MRS + 5-FU treated group. No significant differences were observed in any of the SN or live bacteria groups compared to 5-FU or untreated controls ($p > 0.05$).

The apoptotic index (i.e. the number of cells which stained positively for apoptosis) was significantly increased in all 5-FU-treated groups when compared to the untreated controls ($p < 0.05$, Figure 5). There were no significant differences observed among any of the live bacteria, SN or vehicle treated groups.

4.4 Discussion

The current study was designed with two primary aims: firstly to assess the potential for a probiotic-based pre-treatment regime to protect against the development of intestinal mucositis, and secondly to compare the efficacy of live probiotic bacteria with the secreted compounds from the same strain. Although Chapter 3 indicated that pre-treatment with probiotic SN was able to prevent changes to specific indicators of 5-FU-induced damage *in vitro*, this protective effect did not appear to translate to the *in vivo*

model under the current experimental protocol. Furthermore, whilst the live bacteria and its corresponding SN generally appeared to have a similar influence on the gut, there were instances where one appeared to be more effective than the other. Overall, the findings presented in this chapter indicated that a pre-treatment regime based on SNs derived from LGG and EcN may not have been an effective strategy to reduce the severity of intestinal mucositis induced by 5-FU

Although indicators of protective effects from either live bacteria or SNs were observed in MPO, sucrase and histological damage analysis; the results of the current study were less promising than those reported in Chapter 3 where pre-treatment of intestinal epithelial cells with probiotic SNs was able to partially prevent 5-FU-induced damage. There are many potential sources of difference between *in vivo* and *in vitro* models, and as such it may be difficult to define the factors responsible for the apparent inconsistency. There are, however, two probable contributing factors of note. The first difference between the two systems was the pre-treatment regimen, specifically the dosage and duration of treatment. Rats were gavaged with 1ml of treatment each day, compared to the cells which were treated with 0.5ml. Furthermore, the duration of the pre-treatment differed between the two studies (four days *in vitro* vs. seven days *in vivo*). As the total number of epithelial cells in the entire rat small intestine is far greater than the 8×10^4 found in each well, the dosage received *in vivo* was much lower than in the *in vitro* study. Further to this, while *in vitro* the SN was diluted into a controlled amount of growth medium, the dilution *in vivo* was subject to some variability due to water and feed consumption and endogenous secretions by the rat. This is particularly

significant as previous studies involving LGG (Zhang *et al.*, 2005) and EcN (Lammers *et al.*, 2002) have shown dose-dependent probiotic effects. EcN has been shown to protect against the damage caused by the non-steroidal anti-inflammatory drug indomethacin when administered at a dose of 10^{10} cfu/ml (as opposed to the 10^9 cfu/ml employed in the current study); such a dose may have been effective in the prevention of 5-FU-induced mucositis (Bures *et al.*, 2011). Twice-daily administration (Geier *et al.*, 2007), or *ad libitum* (Maldonado Galdeano *et al.*, 2011) access to probiotic treatments are also potential methods of increasing the overall dosage. However, in order to determine whether the protective effect observed *in vitro* is dose dependent, a repeat of the experiment performed in Chapter 3 with SNs derived from decreasing concentrations of bacteria should deliver more definitive results.

A second factor contributing to the contrast in results between Chapter 3 and the current study may have been the state of the SN or live bacteria once it reached the small intestine. While *in vitro* experimentation allowed for the treatment to be administered directly to the epithelial cells, oral gavage requires that the treatment pass through the acidic conditions of the gut prior to reaching the intestine. Although viable EcN (Schultz *et al.*, 2005) and LGG (Femia *et al.*, 2002) have been shown to reach the intestine of rats, there has been no investigation into the effect of gut transit on the SN components. Should the acidic or proteinase-rich conditions alter the nature of the secreted products, their ability to protect the intestine may be impaired. Further investigations are required to ascertain the effects of gut transit on the SN. Future studies could also compare the efficacy of different administration regimes which may protect the SN, or live cells,

from damage. Procedures such as intra-rectal delivery of probiotics warrant investigation (Segawa *et al.*, 2011), while significant developments are also being made in the development of micro-encapsulation techniques (Sohail *et al.*, 2011).

In Chapter 3, probiotic SNs were able to significantly inhibit caspase 3 activation following the administration of 5-FU *in vitro*. As caspase 3 is an effector caspase which is up-regulated following 5-FU administration (Bowen *et al.*, 2006), it was hypothesised that inhibition of caspase 3 would translate to an overall inhibition of apoptosis. The results of the current study do not support this hypothesis. This may be a result of the limiting factors listed above. Furthermore, the contrasting results may have been due to the different markers of apoptosis measured in the current study (cells undergoing apoptosis) and Chapter 3 (caspase 3 activation), or a lack of sufficient SN delivery. Caspase 3 is not the only apoptotic protein which is up-regulated during intestinal mucositis. Therefore, while the SNs may have been able to inhibit caspase 3 activation, apoptosis may still have been initiated by other signals triggered by 5-FU. Further *in vitro* investigations are required to determine if the inhibition of caspase 3 by SNs is able to translate to an overall reduction in apoptosis, or *in vivo* to establish if the inhibition of caspase 3 is observed in an animal model. Furthermore, future studies which measure caspase activation would be better served to target initiator caspases (8, 9 and 10) as they may have a greater influence on apoptosis overall.

Although no probiotic-induced changes were observed on cell proliferation or apoptosis in the current study, improvements in MPO and sucrase levels were apparent. Both EcN

and LGG SNs were able to partially reduce MPO activity compared to 5-FU controls, while Live LGG maintained sucrase activity compared to 5-FU controls. The MPO results are particularly interesting as it is difficult to understand why only the secreted components of bacteria significantly reduced MPO activity compared to 5-FU controls. Similar results were observed by Sokol *et al.* (2008) who found that a SN from *F. prausnitzii* prevented IL-1 β -induced NF- κ B activity in Caco-2 cells, while the live bacteria had no effect. In this case, the authors suggested it was due to changes in the growth media as a result of bacterial growth. This theory may have explained the results observed in the current study, as the media in which the live cells were grown was replaced with fresh broth prior to freezing. Another possible reason for this response is a change to the normal gut microflora as a result of treatment. Each rat received a 1ml dose of their respective treatment which, for groups receiving live bacteria, could have significantly increased the concentration of bacterial cells within the gastrointestinal tract and initiated an inflammatory response. The delivery of SNs would not have increased the bacterial concentration. In Chapter 3, administration of growth medium directly to epithelial cells was able to prevent 5-FU-induced increases in caspase 3/7 activation. As there was no significant difference between the treatment groups and their respective vehicle controls, it was possible that the protective effects observed were due in part to components of the growth media. The effect of live LGG on sucrase activity, in the absence of a SN effect, may suggest a role of the bacterial DNA or cell wall components. As no changes to cell-cycling were observed, further studies are required to determine the mechanism of action of the live cells. Donato *et al.* (2010) determined that live LGG could inhibit NF- κ B signalling, which in turn could alter the secretion of pro-

inflammatory cytokines and chemokines. Future studies should investigate whether or not LGG is able to exert these anti-inflammatory effects in the setting of mucositis.

Interestingly, rats treated with Live EcN + 5-FU had significantly lower liver weight than 5-FU controls. The fact that the same reduction was not observed in rats treated with the EcN SN suggests that the mechanism responsible is associated with the presence of live bacteria in the treatment. Although live EcN has been studied extensively, this is the first time such an effect has been reported in the literature, and so further investigations will be required to determine if it is repeatable.

Chapter 2 reported a reduction in goblet cell number following 5-FU administration; but, this was not observed in the current study. The current findings are similar to those of Stringer *et al.* (2009b) who found that a reduction in goblet cell numbers within the jejunum was not observed until two days post-5-FU administration, suggesting that changes to goblet cell production are not as rapid when compared to other 5-FU effects. However, Stringer and colleagues did investigate the effects of 5-FU on goblet cell composition and reported that goblet cells amassed at the base of the villi almost immediately following 5-FU administration, and that by 24h goblet cells became “enlarged and dilated”. The current study, however, did not investigate goblet cell morphology. The current findings suggest that maintenance of mucin dynamics is not solely able to prevent histological damage in the small intestine. Furthermore, as goblet cell counts are typically unchanged at this time point, studies to investigate mucin production at 24h post-5-FU should employ methods such as mucin gene expression.

These investigations may also explain why the changes to goblet cell production were not observed until 48h.

In Chapter 3, LGG and EcN SN were able to prevent 5-FU-induced damage *in vitro* as determined by TEER and caspase activity. The aim of the current study was to determine if LGG, EcN and their SNs could prove protective *in vivo*, however, no clear beneficial effects were observed. A number of differences existed between *in vivo* and *in vitro* models, and as such, further trials using these SNs may prove successful. The importance of dosage and duration of treatment deserves particular attention. Moreover, the results suggest a need for investigations into probiotic-induced changes to the growth media, and its effect on the murine gut.

4.5 Figure legends and figures

Figure 1. Histological severity score of (a) jejunum and (b) ileum of rats at day 9 (or 24h post 5-FU or saline administration), receiving either Water; de Man Rogosa and Sharpe broth (MRS); Live *Lactobacillus rhamnosus* GG (LGG); *Lactobacillus rhamnosus* GG SN; TSB; Live *Escherichia coli* Nissle 1917 (EcN) or *Escherichia coli* Nissle 1917 SN treatment (n = 8/treatment). Data expressed as median and range. * denotes a significant difference compared to Water + Saline control group (p < 0.05).

Figure 2. Myeloperoxidase (MPO) activity in the (a) jejunum and (b) ileum of rats at day 9 (or 24h post 5-FU or saline administration), receiving either Water; de Man Rogosa and Sharpe broth (MRS); Live *Lactobacillus rhamnosus* GG; *Lactobacillus rhamnosus* GG SN; TSB; Live *Escherichia coli* Nissle 1917 or *Escherichia coli* Nissle 1917 SN treatment (n = 8/treatment). Data are expressed as mean (MPO u/mg protein) ± SEM. * denotes a significant difference compared to Water + Saline control group (p < 0.05). α denotes a significant difference compared to Water + 5-FU group (p < 0.05).

Figure 3. Sucrase activity in the (a) jejunum and (b) ileum of rats at day 9 (or 24h post 5-FU or saline administration), receiving either Water; de Man Rogosa and Sharpe broth (MRS); Live *Lactobacillus rhamnosus* GG; *Lactobacillus rhamnosus* GG SN; TSB; Live *Escherichia coli* Nissle 1917 or *Escherichia coli* Nissle 1917 SN treatment (n = 8/treatment). Sucrase activity expressed as mean (nmol glucose/well/min/cm tissue) ± SEM. * denotes a significant difference compared to Water + Saline control group (p <

0.05). α denotes a significant difference compared to Water + 5-FU control group ($p < 0.05$).

Figure 4. Number of proliferating cells in the jejunum of rats at day 9 (or 24h post 5-FU or saline administration), receiving either Water; de Man Rogosa and Sharpe broth (MRS); Live *Lactobacillus rhamnosus* GG; *Lactobacillus rhamnosus* GG SN; TSB; Live *Escherichia coli* Nissle 1917 or *Escherichia coli* Nissle 1917 SN treatment ($n = 8/\text{treatment}$). Data are expressed as mean (cells/mm²) \pm SEM. * denotes a significant difference compared to Water + Saline control group ($p < 0.05$).

Figure 5. The percentage of apoptotic crypt cells in the jejunum of rats at day 9 (or 24h post 5-FU or saline administration), receiving either Water; de Man Rogosa and Sharpe broth (MRS); Live *Lactobacillus rhamnosus* GG; *Lactobacillus rhamnosus* GG SN; Tryptic Soy Broth (TSB); Live *Escherichia coli* Nissle 1917 or *Escherichia coli* Nissle 1917 SN treatment ($n = 8/\text{treatment}$). Data are expressed as mean \pm SEM. * denotes a significant difference compared to Water + Saline control group ($p < 0.05$).

Figure 1.

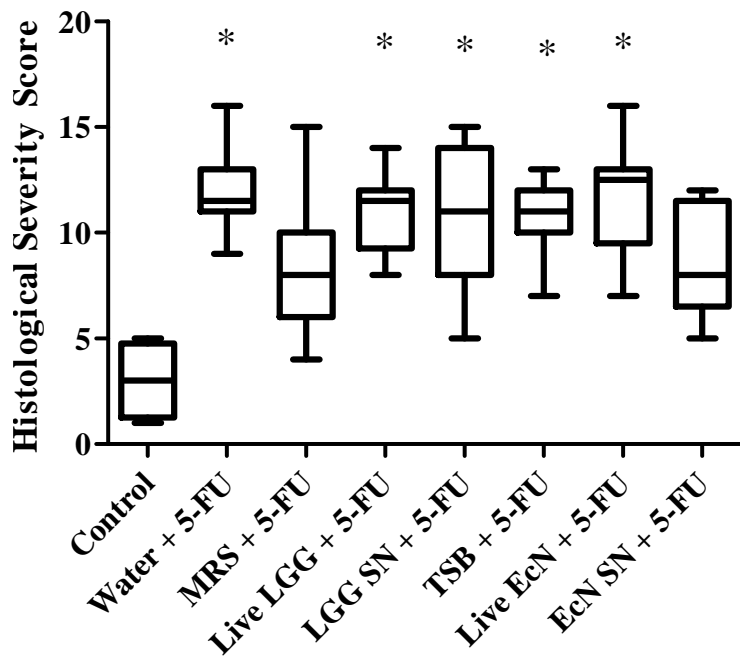
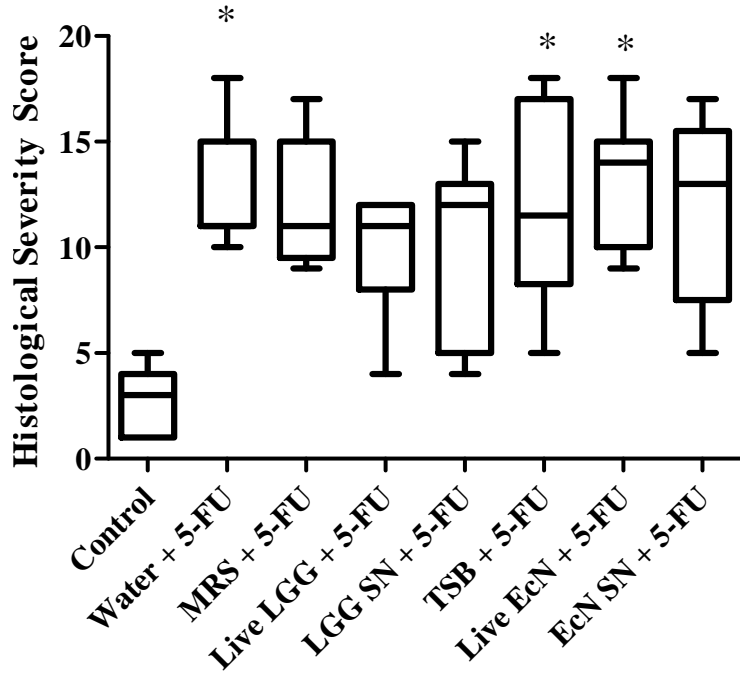
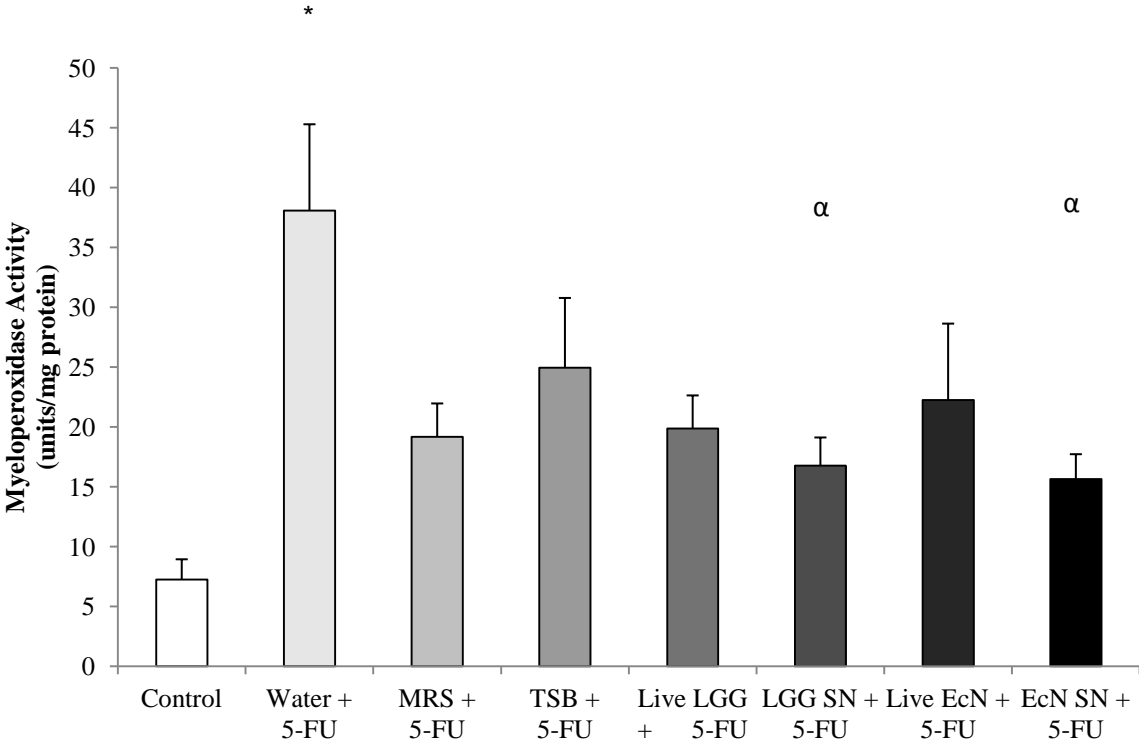


Figure 2.

(a)



(b)

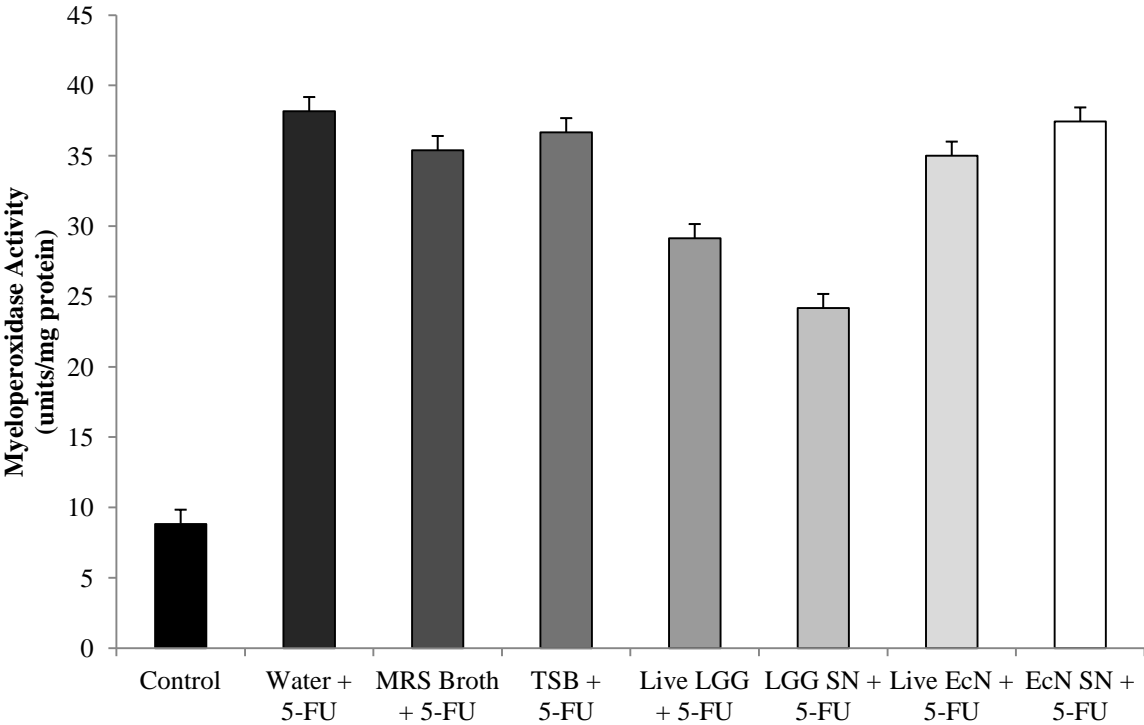


Figure 3.

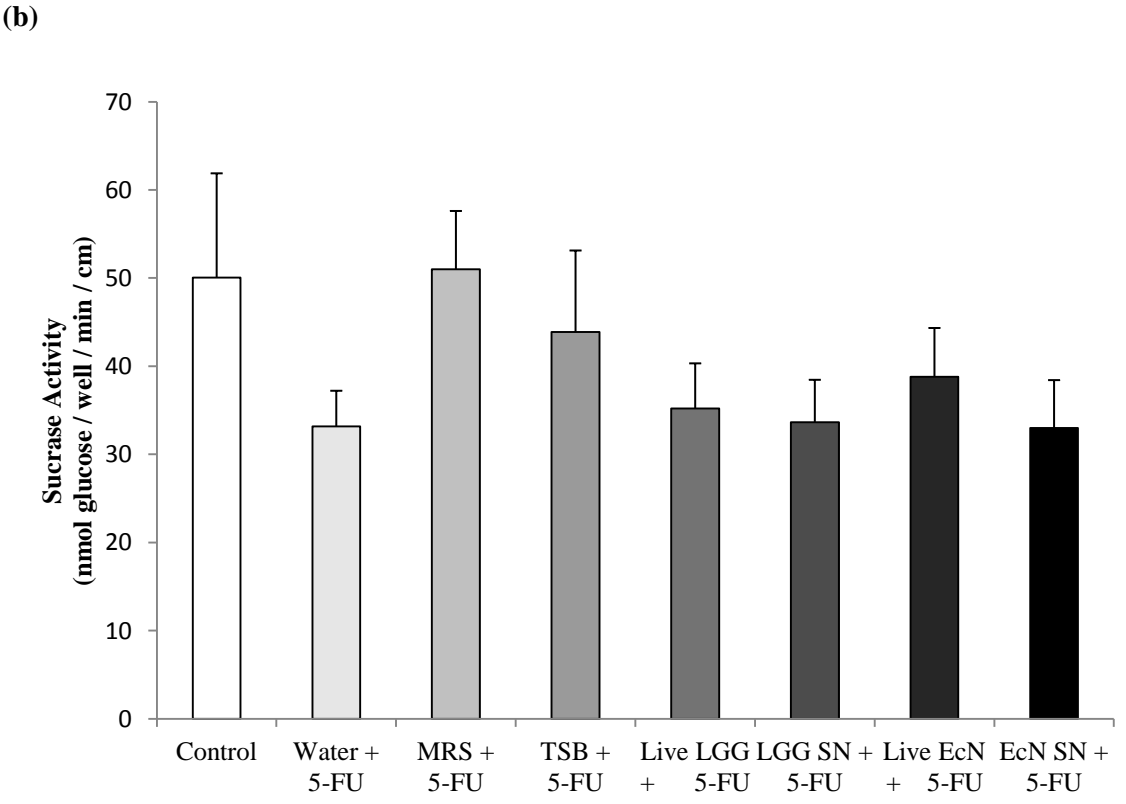
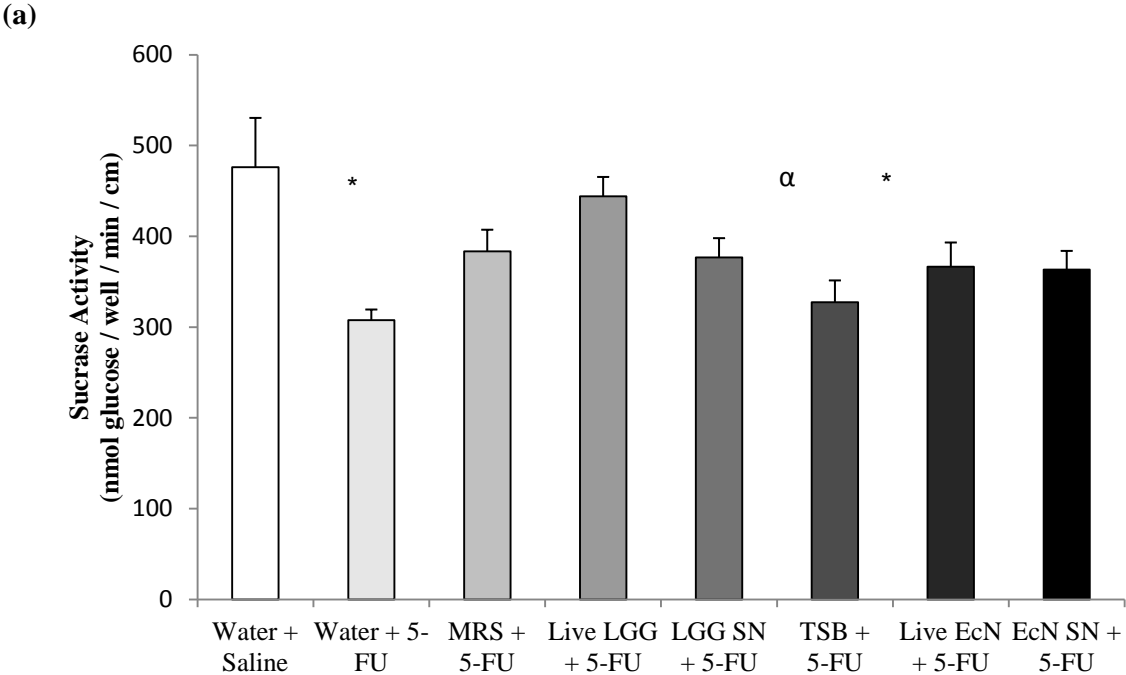


Figure 4.

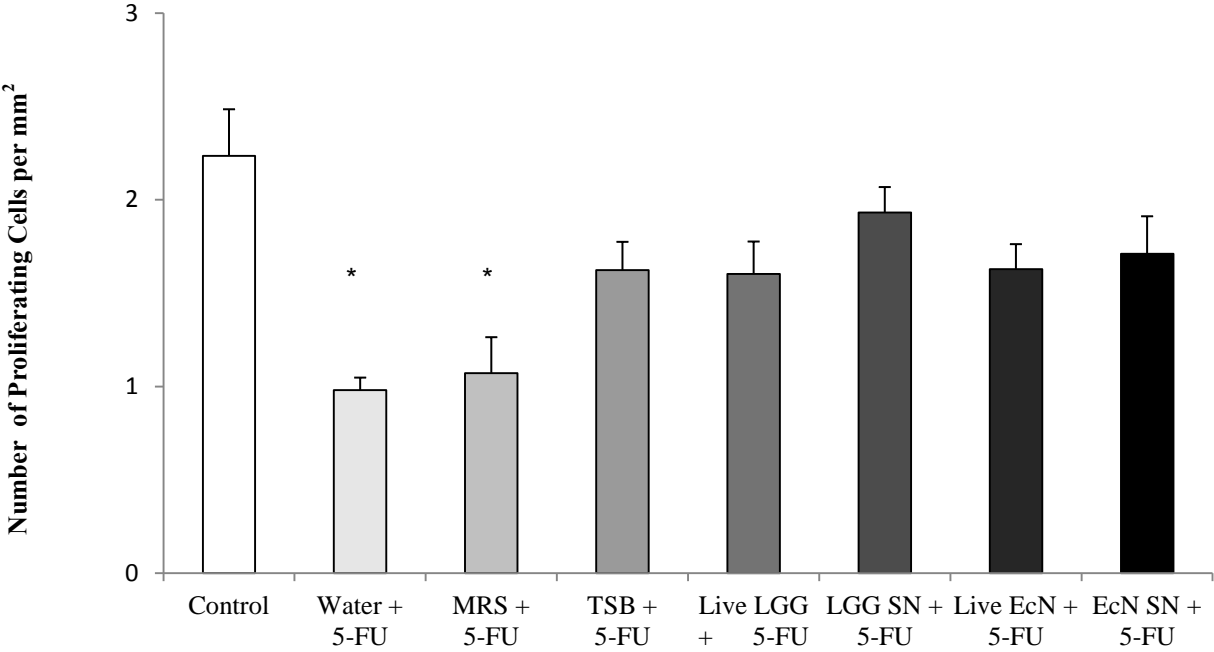
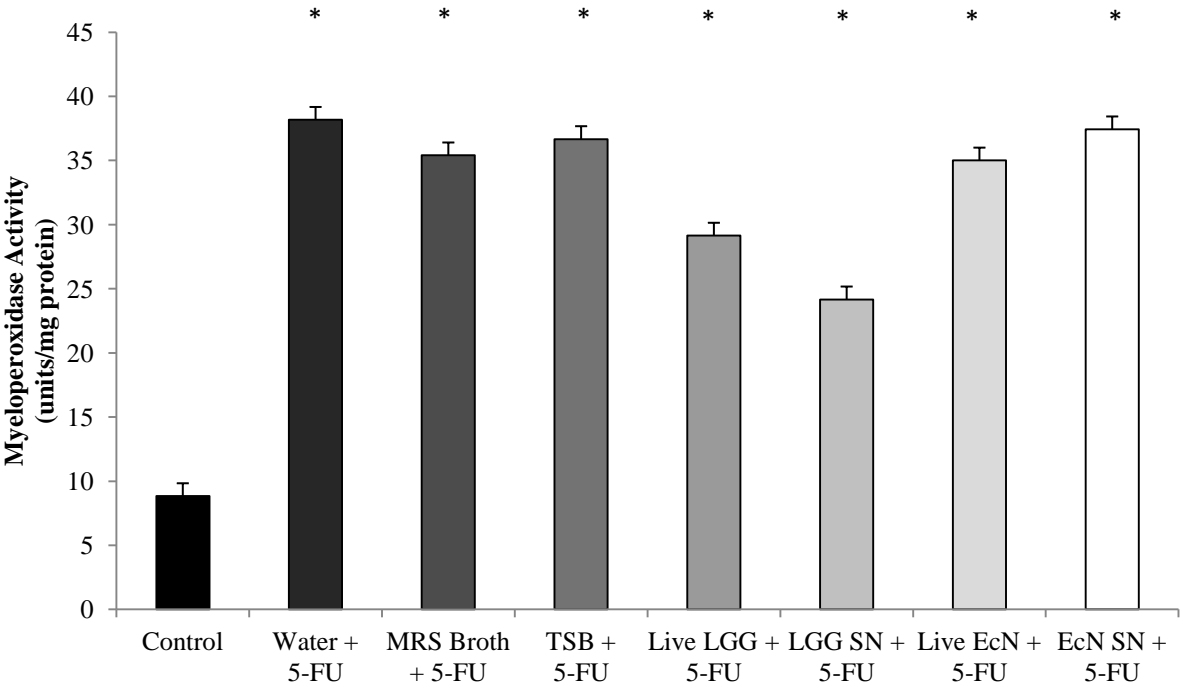


Figure 5.



4.6 Tables

Table 1. Metabolic measurements of rats post 5-FU or saline administration.

	Saline + Water	Water + 5-FU	MRS + 5-FU	Live LGG + 5-FU	LGG SN + 5-FU	TSB + 5-FU	Live EcN + 5-FU	EcN SN + 5-FU
Feed Intake	7.3 ± 1.0	5.3 ± 1.1	5.1 ± 0.8	4.3 ± 0.5	5.1 ± 0.9	5.1 ± 0.6	4.4 ± 0.6	5.8 ± 0.7
Water Intake	22.3 ± 2.2	31.1 ± 3.1*	31.7 ± 2.1*	34.2 ± 2.6*	34.0 ± 5.6*	29.5 ± 3.0*	33.8 ± 4.1*	31.3 ± 4.0*
Faecal Output	1.5 ± 0.1	0.8 ± 0.1*	0.9 ± 0.2*	1.1 ± 0.2	0.9 ± 0.2	0.9 ± 0.1	0.5 ± 0.1*	1.2 ± 0.2
Urine Output	18.1 ± 2.6	27.4 ± 2.7	32.1 ± 1.5	25.2 ± 2.8	35.3 ± 6.3*	32.1 ± 1.9	34.0 ± 4.0*	31.0 ± 3.4

Metabolic measurements of rats at day 9 (24h post 5-FU or saline administration), receiving either Water; de Man, Rogosa and Sharpe (MRS) Broth; Live *Lactobacillus rhamnosus* GG; *Lactobacillus rhamnosus* GG SN; Tryptic Soy Broth (TSB); Live *Escherichia coli* Nissle 1917 or *Escherichia coli* Nissle 1917 SN treatment (n = 8/treatment). * denotes a significant difference compared to Saline + Water controls. Data are expressed as mean (g) ± SEM.

Table 2. Organ weights of rats post 5-FU or saline administration

Organ	Saline + Water	Water + 5-FU	MRS + 5-FU	Live LGG + 5-FU	LGG SN + 5-FU	TSB + 5-FU	Live EcN + 5-FU	EcN SN + 5-FU
Thymus	0.19 ± 0.01	0.13 ± 0.01*	0.15 ± 0.01	0.13 ± 0.01*	0.17 ± 0.01	0.15 ± 0.01	0.14 ± 0.01*	0.15 ± 0.01
Liver	4.04 ± 0.29	3.47 ± 0.12	3.60 ± 0.11	3.43 ± 0.12	3.51 ± 0.07	3.45 ± 0.13	3.37 ± 0.08 ^α	3.60 ± 0.12
Spleen	0.21 ± 0.01	0.19 ± 0.01	0.18 ± 0.01*	0.18 ± 0.01*	0.20 ± 0.01	0.18 ± .01*	0.19 ± 0.01	0.18 ± 0.01*
Stomach	0.84 ± 0.03	0.79 ± 0.03	0.76 ± 0.04	0.85 ± 0.03	0.81 ± 0.01	0.79 ± 0.02	0.71 ± 0.07	0.78 ± 0.02
Duodenum	1.08 ± 0.55	1.02 ± .48	1.41 ± 0.57	1.00 ± 0.51	1.33 ± 0.49	1.05 ± 0.53	1.41 ± 0.57	1.45 ± 0.61
Jejunum-Ileum	2.00 ± 0.11	1.93 ± 0.07	1.81 ± 0.07	1.79 ± 0.12	1.97 ± 0.02	1.90 ± 0.03	1.89 ± 0.05	1.90 ± 0.03
Caecum	0.48 ± 0.02	0.46 ± 0.03	0.60 ± 0.05	0.51 ± 0.04	0.48 ± 0.03	0.51 ± 0.04	0.50 ± 0.02	0.47 ± 0.04

Organ weights of rats at day 9 (24h post 5-FU or saline administration), receiving either Water; de Man, Rogosa and Sharpe (MRS) Broth; Live *Lactobacillus rhamnosus* GG; *Lactobacillus rhamnosus* GG SN; Tryptic Soy Broth (TSB); Live *Escherichia coli* Nissle 1917 or *Escherichia coli* Nissle 1917 SN treatment (n = 8/treatment). * denotes a significant difference compared to Water + Saline controls. ^α denotes a significant difference compared to Water + 5-FU controls. Data are expressed as mean (g) ± SEM.

Table 3. Number of acidic or neutral mucin in the jejunum of rats post-5-FU administration

	Saline + Water	Water + 5-FU	MRS + 5-FU	Live LGG + 5-FU	LGG SN + 5-FU	TSB + 5-FU	Live EcN + 5-FU	EcN SN + 5-FU
Neutral	2.62 ± 0.12	2.90 ± 0.24	2.65 ± 0.13	2.53 ± 0.12	2.76 ± 0.30	2.65 ± 0.15	2.61 ± 0.15	2.60 ± 0.10
Acidic	2.51 ± 0.12	2.60 ± 0.23	2.60 ± 0.14	2.45 ± 0.16	2.75 ± 0.24	2.48 ± 0.11	2.51 ± 0.12	2.77 ± 0.09

Number of acidic or neutral mucins in the jejunum of rats at day 9 (24h post 5-FU or saline administration), receiving either Water; de Man, Rogosa and Sharpe (MRS) Broth; Live *Lactobacillus rhamnosus* GG; *Lactobacillus rhamnosus* GG SN; Tryptic Soy Broth (TSB); Live *Escherichia coli* Nissle 1917 or *Escherichia coli* Nissle 1917 SN treatment (n = 8/treatment). Data are expressed as mean ± SEM (cells/mm²).

Chapter 5. Effects of proteinase or acid-treatment on probiotic factors

5.1 Introduction

Oral delivery may be the simplest method of administering probiotics to the intestinal tract, however, there are significant associated difficulties. Upon entry to the stomach, the environment changes significantly and the organism encounters a number of stress factors, including an acidic pH of 1.5-3.0 (Cotter and Hill, 2003). At this point, an uncontrolled influx of protons leads to the disintegration of the bacterial membrane and disrupts the metabolism and reproductive processes of the bacteria (Chen *et al.*, 2011). To be considered as a probiotic, bacteria must have a proven ability to pass through the gastrointestinal tract and remain viable (Wall *et al.*, 2007). Those strains that survive this environment are able to do so by employing various defence mechanisms, including proton pumps to remove excessive protons from the cytoplasm, the production of general stress proteins, alkalisation of the external environment or through altered cell metabolism and envelope structure (Cotter and Hill, 2003; De Angelis and Gobbetti, 2004). These mechanisms, however, do not protect the SNs which are secreted by the live bacteria. Therefore, all secreted factors must also be resistant to highly acidic conditions. Furthermore, SN which contain active proteins must also be resistant to the high concentration of proteases found in the stomach (Del Piano *et al.*, 2011). Pepsin, trypsin and chymotrypsin are the three most common proteases found in the digestive system, with pepsin being the most prevalent in the stomach (Hussack *et al.*, 2011).

Both LGG and EcN are able to survive passage through the stomach, and subsequently colonise the human gut (Blum-Oehler *et al.*, 2003, Jacobsen *et al.*, 1999). Koponen *et al.* (2012) recently investigated how LGG adapted to the highly acidic conditions of the

stomach. Multiple mechanisms were identified, including modulated pyruvate metabolism, increased protein and glycolytic enzyme phosphorylation, and the production of a specific surface antigen, all of which were initiated simultaneously by the bacteria when it was exposed to a pH of 4.8. As a result of these changes, the growth rate of LGG in an acidic environment was approximately 70% of that observed under ideal growth conditions. EcN has also developed multiple mechanisms to ensure its survival through the gut, such as iron acquisition systems and different adhesive fimbriae which allow adhesion to the mucus layer (Joeres-Nguyen-Xuan *et al.*, 2010).

While this information supports the use of live bacteria-based therapies, little is known of the effect of gut transit on probiotic SNs. Due to its low pH and high concentration of broad-spectrum proteases, the stomach represents an extremely unfavourable environment for the SN to pass through. The current study was performed to determine if the protective capabilities of the SN were inhibited following exposure to either highly acidic conditions or co-incubation with proteases.

5.2 Materials and methods

5.2.1 Supernatant preparation

Live bacteria were sourced and grown following the methods described in Chapter 3. In order to obtain bacterial SNs, broths were centrifuged at 1500g for 10 mins. SNs were then collected and buffered with Tris/HCl to a pH of 7.0. SNs were passed through a 0.20µm filter, and stored at -20°C until use.

5.2.2 Acid and proteinase treatment of supernatants

The SNs were treated with either hydrochloric (HCl) acid or proteinase-K (EMD Chemicals, New Jersey, USA). Acid treatment was performed by adding 4.2ml of 1M HCl to SNs until the pH reached 4.0. SNs were then stored at 37°C for 1h before 3.9ml 1M sodium hydroxide was added to the solution and the pH was restored to 7.0. For protease treatment, 0.25g proteinase-K was added at a concentration of 1 g/L (equating to 150 units per well) at 37°C for 30mins. Post-treatment, SNs were aliquoted and stored at -20°C until use.

5.2.3 Cell density and 5-fluorouracil dose determination

Prior to commencement of the experiment, a pilot study was completed to determine the dosage of 5-FU and IEC-6 cell density required to yield significant reductions in cell proliferation. At day 0, Cells were seeded at three concentrations 5×10^2 , 5×10^3 and 5×10^4 cells per well and left for four days to adhere to the plate. Medium was replaced daily. On day 4, cells were incubated overnight with 0, 2.5, 5, 10 and 20 μ M 5-FU before the proliferation assay was carried out. The following concentrations were examined in the current study: 0, 2.5, 5, 10 and 20 μ M to determine which concentration would yield significant reductions in cell proliferation, without reducing cell number to such a degree that an increase due to treatment may not be observed.

5.2.4 WST-1 cell cytotoxicity assay

IEC-6 rat intestinal epithelial cells (passage 27-30) were cultured in medium comprising DMEM, 10% foetal bovine serum (GIBCO®, Victoria, Australia) and 1% penicillin/streptomycin (GIBCO®). Cells were seeded into a clear 96-well plate (0.32 cm²) at a density of 5×10^2 cells/well. Cells were allowed 24h to adhere to the plate prior to the addition of treatments. Plates were stored at 37°C in 95% air, 5% CO₂ for the duration of the study.

Six treatment groups were used for the study: DPBS + Saline (untreated control), DPBS + 5-FU (5-FU control), MRS Broth + 5-FU, TSB + 5-FU, LGG SN + 5-FU and EcN SN + 5-FU. The MRS and TSB groups were included as vehicle controls to determine the effects of the growth media in which the SNs were grown. To examine the effects of proteinase incubation, MRS Broth and TSB were treated with proteinase-K at the previously described concentration. Similarly, to determine the impact of acid treatment, HCl was added to both MRS Broth and TSB until the pH reached 4.0, then stored for 1h until initial pH was restored. However, the vehicle control and SN groups underwent the aforementioned pre-treatment. Each treatment and DMEM (50µl of each) were added to each well daily for four days. Treatments were performed in duplicate while the entire experiment was carried out in triplicate to provide $n = 6$ for each treatment.

Proliferation was measured by WST-1 Cell Cytotoxicity Assay (G-Biosciences, Missouri, USA). WST assay buffer was added to both WST-1 Tetrazolium Salt and CytoScan Electron Carrier (CEC) solutions, before WST-1 and CEC were combined in

a 1:1 dilution to prepare the assay dye solution. Assay dye solution (10ml) was added to each well and gently mixed for 30sec before the plate was covered and incubated at 37°C for 1h. The plate was then placed in a microplate reader (Sunrise Microplate Absorbance Reader, Tecan Austria GmbH, Grödig, Austria) and shaken for 1min before absorbance was measured at 420nm/535nm.

5.2.5 Statistical analysis

Statistical analysis was conducted using SPSS 15.0.1 for Windows (SPSS Inc., Illinois, USA). Data were compared by one-way ANOVA with a Tukey *post-hoc* test and were expressed as mean \pm SEM. For all data, $p < 0.05$ was considered significant.

5.3 Results

5.3.1 Cell density and 5-fluorouracil dose determination pilot study

The proportion of cells undergoing proliferation was determined following an overnight incubation with 5-FU. In cells seeded at 5×10^2 , all concentrations of 5-FU significantly inhibited cell proliferation ($p < 0.05$, Figure 1a), while cells seeded at densities of 5×10^3 (Figure 1b) or 5×10^4 (Figure 1c) cells per well showed no significant differences when compared to their respective controls. Based on these results, a 5-FU concentration of 2.5 μ M was determined to be the most suitable for the current study as the number of cells undergoing proliferation remained above 30% of the number of proliferating cells in the untreated group.

5.3.2 Effect of *E. coli* Nissle 1917 supernatants on cell proliferation

Cell proliferation was determined following an overnight with 2.5 μ M 5-FU. Cells in the 5-FU control group showed significantly less proliferation when compared to the untreated controls ($p < 0.05$, Figure 2). The TSB + 5-FU vehicle control group, as well as all of the EcN treated cells showed significantly higher proliferation when compared to the 5-FU controls ($p < 0.05$). No differences were observed between either the standard, protease- or acid-treated EcN groups.

5.3.3 Effect of *L. rhamnosus* GG supernatants on cell proliferation

As was observed previously, cells in the 5-FU control group showed significantly lower levels of proliferation when compared to the healthy controls ($p < 0.05$, Figure 3). However, both the protease- and acid-treated LGG SN also displayed significantly less proliferation when compared to the healthy controls ($p < 0.05$). In contrast, proliferation of cells treated with either MRS or LGG SN + 5-FU was similar to the untreated cells. Furthermore, the LGG SN group showed significantly higher proliferative activity when compared to the 5-FU controls, and the protease- and acid-treated LGG SN ($p < 0.05$).

5.4 Discussion

The aim of the study was to determine if probiotic SNs could maintain their efficacy following exposure to a highly acidic or proteinase-rich environment, as would be found in the human digestive tract. This question was raised in earlier chapters when beneficial effects were observed *in vitro* but could not be replicated *in vivo*. In the case of LGG in particular, exposure to the upper gastrointestinal environment did inhibit SNs in their

ability to protect the small intestine from 5-FU-induced changes in cell proliferation. Cells treated with LGG SN which had been exposed to either acidic conditions or proteinase-K were unable to maintain cell proliferation as successfully as normal LGG SN, however, neither treatment inhibited the protective effects of EcN SN. These findings support the hypothesis that transit to the small intestine can be deleterious to SN-based therapies, and suggest that future studies must explore more effective delivery methods.

In Chapter 3, LGG SN was able to protect intestinal epithelial cells from 5-FU-induced apoptosis. The current study demonstrated that LGG SN was also able to maintain IEC-6 cell proliferation following administration of the chemotherapeutic agent. The LGG SN has been studied extensively, and two of its secreted proteins have been isolated and shown to exert protective effects when delivered to the intestinal tract (Yan *et al.*, 2011). Most recently, Yan *et al.* (2011) demonstrated that the protein, p40, was able to prevent DSS-induced colitis by up-regulating the expression of the extracellular protein, epidermal growth factor (EGF). EGF has been shown to increase enterocyte proliferation by inducing the expression of p21 mRNA and protein (Sheng *et al.* 2006), and the same mechanism may be responsible for the protective effect described here. Similar to orally-delivered LGG SN in Chapter 4, neither acid-nor protease-treated LGG SN was able to protect the epithelial cells from 5-FU-induced changes to cell proliferation in the current study. It is possible that beneficial effects of LGG SN observed in Chapter 3 were chiefly due to proteins such as p40, and that exposure to proteases or acidic conditions may have degraded or destroyed these proteins,

preventing them from exerting any beneficial effects on the small intestine. With the aim of delivering p40 directly to the colon, Yan *et al.* (2011) employed pectin/zeal-based hydrogel beads filled with the protein. Pectin, a polysaccharide which is degraded by colonic microbiota, is resistant to both gastric and small intestinal enzymes and, therefore, ensured that p40 reached the colon undamaged. These findings suggest that the future studies using LGG SN, p40, or other candidate proteins in the intestine should all employ targeted delivery methods to ensure that the compounds reach their desired location.

While the efficacy of LGG SN in the current study was diminished by the two pre-treatments, no significant difference was observed in proliferation of cells treated with the three forms of EcN SN. Previous studies into the composition of the EcN SN have identified two compounds which have been shown to exert protective effects: flagellin (Schlee *et al.*, 2007) and LPS (Arribas *et al.*, 2009; Zidek *et al.*, 2010). The flagellin protein was reported by Schlee *et al.* (2007) to activate the human β -Defensin 2 gene when administered to epithelial cells. In earlier chapters, it was hypothesised that this activation may have contributed to the observed protective effects of EcN SN by strengthening the intestinal barrier and eliminating pathogenic bacteria from the microbiota (Schlee *et al.*, 2007). However, the results of the current work suggest that this may not be the case. Treatment with a broad-spectrum proteinase would have potentially degraded any flagellin which was present in the EcN SN. In contrast, LPS may have been unaltered by both exposure to acidic conditions or co-incubation with proteinase-K. The presence of LPS in EcN SN was first reported by Zidek *et al.* (2010).

Investigation into the composition of EcN SN revealed high quantities of LPS, and determined that this compound mediated the stimulatory effects of EcN on cytokine production (including IL-10). Further studies into EcN SN revealed an ability to improve barrier function *in vitro* via LPS-dependent mechanisms. There is currently no mention in the literature of the effects of EcN SN *in vivo* or the effects of gut transit on its efficacy; however, the current study suggests that the SN contains secreted factors which remain viable through transit, LPS being a potentially active compound. Future studies should investigate the efficacy of EcN SN in comparison to LPS in models of 5-FU-induced damage.

As this pilot study only investigates a single end-point, it did not yield conclusive findings on the effects of gut transit on SNs. However, the results suggest that the effects of gut transit are likely to be strain specific and dependent on the composition of the SN. This presents a challenge to future studies as, in almost all cases the complete composition of the SN is not known and may be variable. Therefore, its susceptibility to proteinases or an acidic environment is unknown. Although not yet investigated in SNs, there is significant research into the microencapsulation of live probiotics to improve survival. This process provides a physical barrier between the probiotic cells and the surrounding environment, thus improving the survival of bacteria (Jimenez-Pranteda *et al.*, 2012). There is currently a wide variety of microcapsules which can vary in both composition and size, depending on the target delivery location. These are described in detail in a review by Prakash *et al.* (2011). Polysaccharides are among the most common materials employed for microencapsulation as they can be broken down by intestinal

microbiota which facilitates the targeted delivery to the human intestine (Yan *et al.*, 2011). The composition of the encapsulating material can also be tailored to suit delivery to the small intestine. Dube *et al.* (2011) described the use of chitosan-tripolyphosphate nanoparticles to deliver the anti-oxidant flavonoid epigallocatechin gallate (EGCG) to the jejunum of adult mice. Encapsulation more than doubled the exposure of the flavonoids to the jejunum and significantly increased EGCG levels in the plasma of treated mice. Similar microencapsulation compounds should be investigated for the delivery of probiotic SNs in models of intestinal mucositis.

The findings of the current study suggest that the effects of gut transit on probiotic SNs are dependent on its composition and, hence, the bacterial strain from which it is derived. For strains which appear to derive some of their beneficial effects from active proteins (such as LGG), the use of techniques such as microencapsulation is advised to maintain stability throughout the stomach. Further research is required to determine the method that will facilitate the most efficient delivery of the SN to the small intestine.

5.5 Figure legends and figures

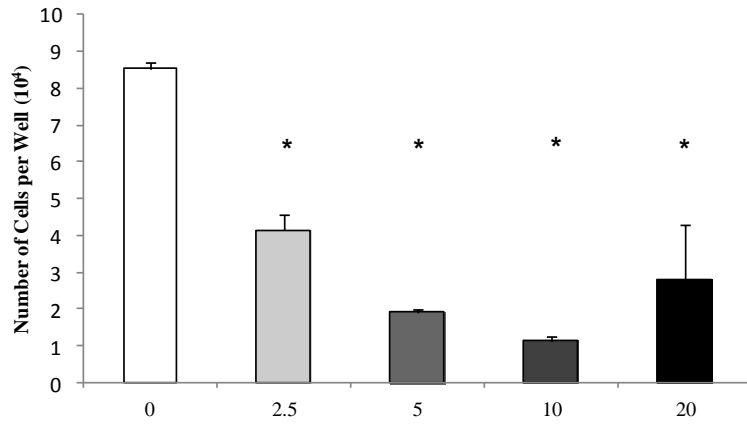
Figure 1. Number of IEC-6 cells per well at day 5 (24h post 5-FU administration). Cells were seeded at (a) 5×10^2 , (b) 5×10^3 or (c) 5×10^4 cells per well and then treated with 0, 2.5, 5, 10 or 20 μ M 5-FU for 24h. Data expressed as mean \pm SEM. * denotes a significant difference compared to the untreated control group ($p < 0.05$).

Figure 2. Number of IEC-6 cells per well at day 5 (24h post 5-FU administration), receiving either DPBS; TSB; EcN SN; Protease-Treated EcN SN (EcN SN-P) and Acid-Treated EcN SN (EcN SN-A); ($n = 6$ /treatment). Data expressed as mean \pm SEM. * denotes a significant difference compared to untreated controls ($p < 0.05$). α denotes a significant difference compared to DPBS + 5-FU control group ($p < 0.05$).

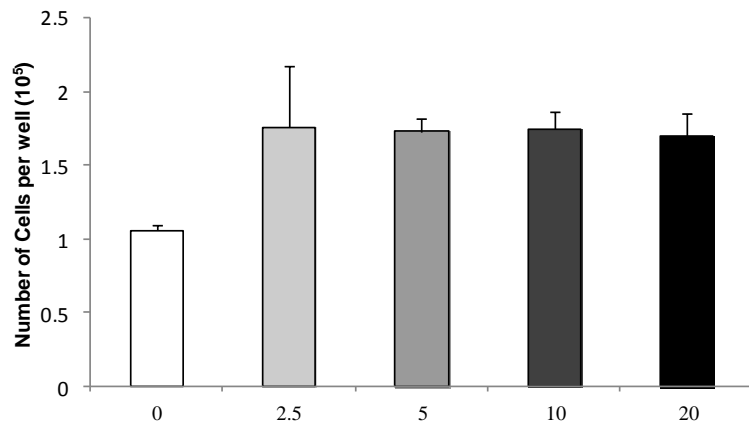
Figure 3. Number of IEC-6 cells per well at day 5 (24h post 5-FU administration), receiving either DPBS; de Man Rogosa and Sharpe broth (MRS); LGG SN; Protease-Treated LGG SN (LGG SN-P) and Acid Treated-LGG SN (LGG SN-A); ($n = 6$ /treatment). Data expressed as mean \pm SEM. * denotes a significant difference compared to untreated controls ($p < 0.05$). β denotes a significant difference compared to LGG SN + 5-FU group ($p < 0.05$).

Figure 1.

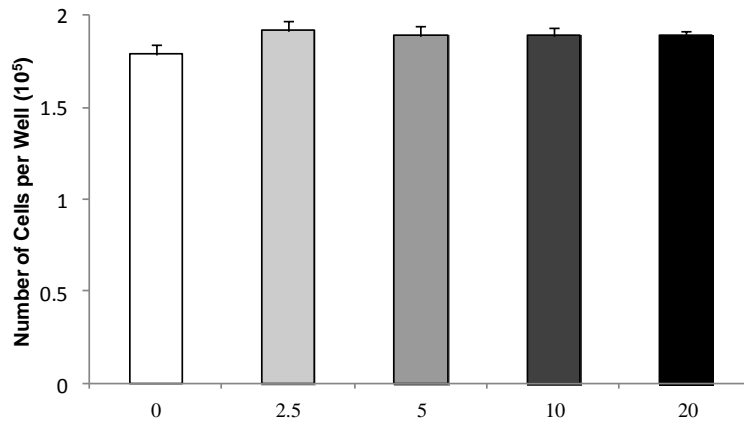
(a)



(b)



(c)



5-FU Concentration (μM)

Figure 2.

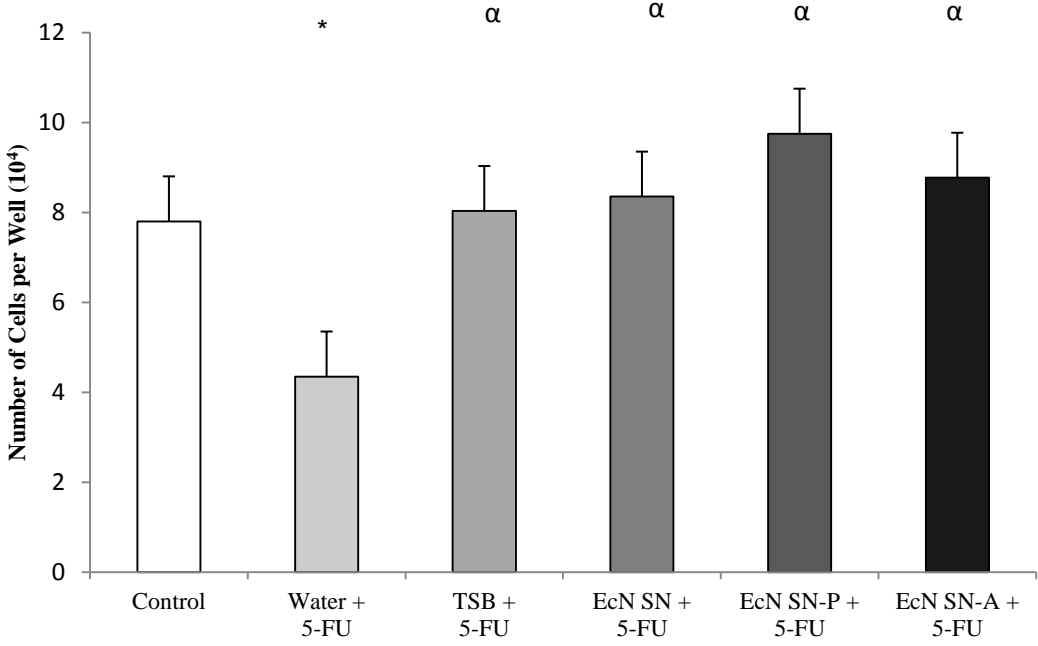
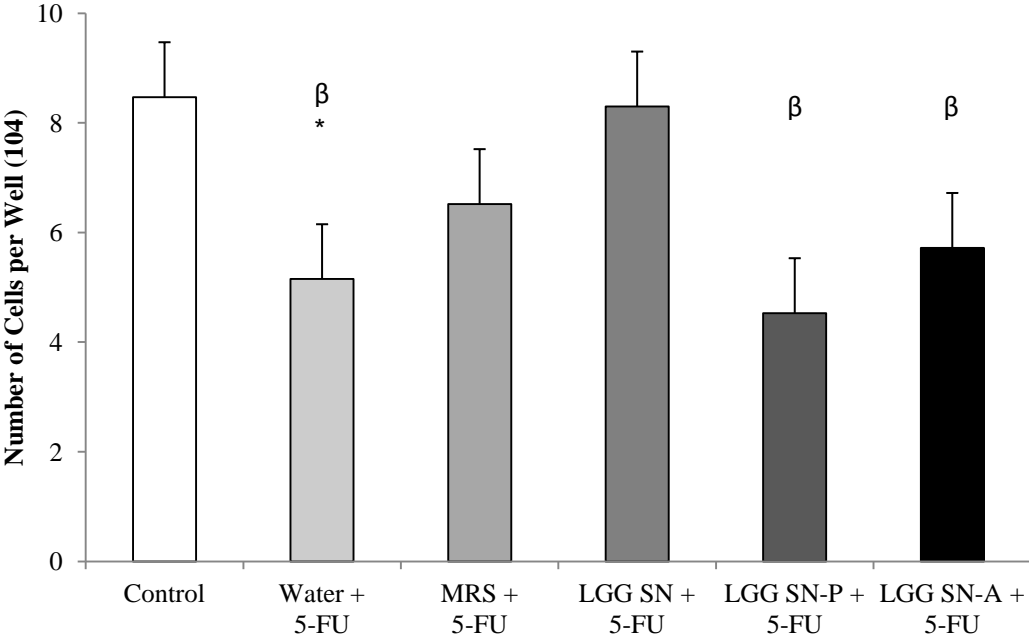


Figure 3.



Chapter 6. Summary and conclusions

6.1 Introduction

The chemotherapeutic drug 5-FU is widely administered in oncologic practice (Chang *et al.*, 2012). It is commonly used to treat a variety of cancers, including breast, colon and neck cancer. A variety of side-effects are associated with 5-FU treatment, including dermatitis and myelosuppression; however, the most common ailment is mucositis, reported in approximately 80% of patients receiving 5-FU. Mucositis can present in two forms: oral or intestinal. Intestinal mucositis is characterised by uncontrolled epithelial cell apoptosis and villus atrophy, leading to a breakdown of the intestinal barrier (Logan *et al.*, 2007, Sonis *et al.*, 2004). Despite its prevalence amongst cancer sufferers, there remains no definitive therapy for mucositis.

The uncertainty surrounding the pathogenesis of intestinal mucositis contributes to the difficulty in developing an effective therapeutic strategy. It is widely accepted that mucositis develops through the overlapping five-stage process described previously by Sonis (2004). This process is not definitive as more recent studies have identified further physiological changes which occur during mucositis development. For example, Stringer *et al.* (2007a) described in detail changes that occurred in the gastrointestinal tract following the delivery of chemotherapeutic drugs. The changes included the reduction in population of beneficial bacteria and number of protective mucin-secreting goblet cells within the gastrointestinal tract; however, the effects were specific to the chemotherapy drug administered. Kaczmarek *et al.* (2012) also identified a role for toll-like receptors 2 and 9 in the development of mucositis, while Yasuda *et al.* (2012)

reported that NADPH oxidase was involved in the activation of caspases 3 and 8 following chemotherapy.

Probiotic-based therapies have been shown to be beneficial in a range of intestinal disorders (Mack, 2011), but are yet to be investigated in great detail for the treatment of intestinal mucositis. There are, however, a wide range of probiotic mechanisms which may be effective in the treatment or prevention of mucositis. These include: broad spectrum anti-inflammatory effects (Yoon and Sun, 2011), maintenance of crypt cell cycling (Khailova *et al.*, 2010; Mennigen and Bruewer, 2009), regulation of the intestinal microbiota (Madsen, 2011) and improvement of intestinal barrier function (Mennigen *et al.*, 2009). A number of studies have investigated the use of probiotics to treat intestinal mucositis, with varying levels of success. TH-4 was reported to protect against methotrexate-induced mucositis (Tooley *et al.*, 2006), but was largely ineffective following 5-FU administration (Whitford *et al.*, 2009). The probiotic combination VSL#3 administered pre- and post-chemotherapy maintained the rate of epithelial cell proliferation and apoptosis, as well as preventing diarrhoea (Bowen *et al.*, 2007). A study of the probiotic strains BR11, LGG and *B. lactis* Bb12 in the setting of 5-FU-induced mucositis reported no protective effects (Mauger *et al.*, 2007). In addition to research with live bacteria, certain studies have also investigated the use of active compounds secreted by the bacterial cells. Although not yet investigated in the setting of intestinal mucositis, these SNs have often been shown to convey similar beneficial effects to the bacteria from which they were derived (Segawa *et al.*, 2011; Yan *et al.*, 2007; Yan *et al.*, 2011). Given that impaired immune function is a common side-effect

of anti-neoplastic therapy, the use of cell-free treatments represents a safer therapeutic option for intestinal mucositis.

There is evidence to suggest that probiotic-derived SNs may be of therapeutic benefit in the treatment of intestinal mucositis. Although preliminary studies have been performed, further research is required to identify which strains may be most effective to treat this disorder, and also, the mechanisms by which they may exert their protective effects. The series of experiments outlined in this thesis describe (i) the screening and identification of bacterial strains with potential for use in the treatment/prevention of 5-FU-induced intestinal mucositis, (ii) preliminary studies to begin to determine mechanisms by which SNs derived from EcN and LGG can protect against 5-FU-induced damage, (iii) a comparison of efficacy between SNs and the live bacteria from which they were derived, and (iv) a pilot study investigating the effect of gut transit on the efficacy of SNs.

6.2 Probiotic factors partially protect intestinal cells from chemotherapy-induced cytotoxicity and improve parameters of 5-fluorouracil-induced intestinal mucositis in rats

Probiotic SNs have been investigated in models of gastrointestinal damage, demonstrating that these SNs were able to confer similar beneficial effects as live bacteria. However, SNs have yet to be considered in models of chemotherapy-induced damage. Therefore, this *in vitro* study performed strain-to-strain comparisons of four candidate probiotic strains, EcN (Arribas *et al.*, 2009; Stetinova *et al.*, 2010), TH-4

(Tooley *et al.*, 2006), *L. acidophilus* LA-5 (Macouzet *et al.*, 2009) and BR11 (Geier *et al.*, 2007). SNs obtained from the four strains were tested for their ability to protect IEC-6 cells from 5-FU-induced reductions in cell number and viability. Although no strains were able to maintain cell number, EcN and BR11 were partially able to maintain the percentage of viable cells and, therefore, were selected for further investigations *in vivo*. In a murine model of 5-FU-induced intestinal mucositis, both EcN and BR11 SN administration improved histological damage scores, while EcN SN was able to prevent 5-FU-induced increases in MPO activity. However, SNs were unable to protect against 5-FU-induced changes to metabolic parameters, goblet cell number or intestinal sucrase activity. Based on the outcome of this study, only EcN was selected for further assessment of protective effects in subsequent chapters. LGG SN was selected to replace BR11 in future studies. The method of SN preparation and timing of administration was also reviewed.

6.3 Probiotic factors partially prevent changes to caspases 3 and 7 activation and transepithelial electrical resistance in a model of 5-fluorouracil-induced epithelial cell damage

Numerous physiological changes are involved in the pathogenesis of intestinal mucositis, but amongst the most important are reduced barrier function and an increase in the percentage of intestinal cells undergoing apoptosis (Han *et al.*, 2011, Sonis *et al.*, 2004). Previous studies have identified two executor caspases, 3 and 7, as two positive markers for cell apoptosis involved in intestinal mucositis (Bowen *et al.*, 2006), while TEER is commonly employed to examine intestinal barrier function. The current study,

therefore, investigated the ability of two probiotic SNs, EcN and LGG, for their ability to protect against 5-FU damage *in vitro*. Due to the absence of an observed protective effect in Chapter 3, BR11 was replaced in the current study by LGG. This strain (and EcN) was selected based on previous studies which described their ability to maintain epithelial integrity and prevent cell apoptosis (Schultz, 2008; Yan and Polk, 2012). This study differed to the *in vitro* trial performed in Chapter 2 in that cells were only treated with SNs prior to receiving chemotherapy, rather than before and after 5-FU. As expected, 5-FU up-regulated caspase 3 and 7 activation and reduced TEER. LGG SN increased TEER prior to 5-FU administration on day 3, while both LGG and EcN SN administration successfully maintained TEER on days 4 and 5 (24 and 48h post-5-FU, respectively). Similarly, caspase 3 and 7 activation was inhibited in cells pre-treated with probiotic SN at days 4 and 5. These findings support previous studies indicating that both strains were effective in maintaining epithelial barrier function; however, this was the first description of an anti-apoptotic effect of EcN. The outcome of this study supported the continued examination of both EcN and LGG SN in subsequent experiments, while also providing an indication that pre-treatment regimes are able to protect against epithelial damage following chemotherapy.

6.4 Effects of pre-treatment with probiotic factors on 5-fluorouracil-induced intestinal mucositis in rats

While *in vitro* studies are effective for screening and mechanistic studies, *in vivo* trials are invaluable for examining whole body effects of a treatment as well as any potential interactions of compounds with the gastrointestinal environment. Therefore, to follow-

up on earlier findings, an *in vivo* study was designed to examine if pre-treatment with EcN and LGG was able to confer similar protective effects in rats treated with 5-FU. The current study also compared efficacy of probiotic SNs with the live bacterial cells from which they were derived to determine if both forms exerted similar effects on the gastrointestinal tract. The results of the current study were surprising and, in some cases, conflicted with earlier work. Both SNs and live bacteria partially maintained cell proliferation following 5-FU; however, the anti-apoptotic effect observed *in vitro* could not be replicated *in vivo*. This result gave the first indication that the delivery method employed throughout this thesis (i.e. oral gavage) may not be ideal for unprotected probiotic-based therapies. Finally, the current findings did not portray any consistent pattern of difference or similarities between probiotic SNs and their live bacteria. However, this may also have been influenced by delivery method of the treatments.

6.5 Exposure to proteases and acidic conditions can reduce efficacy of probiotic SNs in the prevention of 5-fluorouracil-induced damage to epithelial cells: a pilot study

In order to investigate the contrasting results of Chapters 3 and 4, a pilot study was designed to gain an insight into the potential impact of gut transit on SNs derived from LGG and EcN. Prior to reaching the small intestine, orally delivered therapeutics must pass through the highly acidic, proteinase-rich stomach environment (Cotter and Hill, 2003). In order to replicate these conditions, SNs were incubated for 1h at a pH of 4.0 or treated with broad spectrum proteinase-K for 30mins. SNs were then tested for their ability to maintain the rate of cell proliferation in 5-FU-treated IEC-6 cells. SNs derived

from EcN were unaffected by either acid or proteinase exposure, although only untreated EcN SN was able to improve cell proliferation compared to untreated controls. This indicated that the active components of the SN were non-proteinaceous, acid-resistant compounds. The efficacy of the SN derived from LGG was diminished following acid and protease treatments. This supported the theories described in earlier chapters which identified the secreted proteins p40 and p75 as likely active components responsible for the beneficial effects of the LGG SN. The results of this chapter indicated a need for research into the ideal administration method for probiotic-based therapeutics. Current research into techniques such as microencapsulation which protect therapeutics from the harsh conditions of the stomach before being degraded at the target site (Jimenez-Pranteda *et al.*, 2012) represent a promising option to increase effectiveness.

6.6 Overall conclusions

The series of experiments described in this thesis sought to assess the use of probiotics for the treatment and/or prevention of 5-FU-induced intestinal mucositis. Probiotic-based therapies have been shown to be effective in ameliorating indicators of intestinal damage, but have yet to be extensively investigated to treat side-effects of chemotherapy. The studies described in this thesis confirmed that probiotic-based therapies represent a potential treatment, but also highlighted the need for further research before definitive conclusions can be reached.

A major issue facing the field of probiotics is the determination of strains best suited to treat a given disorder, and this is no different in the current research. Three strains were investigated: BR11, EcN and LGG. Each strain was selected based on mechanisms reported previously in different models of intestinal damage. BR11 was selected due to its previously reported anti-oxidative effects. It was hypothesised that administration of this strain would inhibit the release of ROS and, in turn, reduce the severity of intestinal damage. However, Chapter 2 revealed that this probiotic only offered a minimal protective effect. The ability of BR11 to inhibit the release of ROS following 5-FU administration was not determined. Even if BR11 does inhibit ROS production, it may not be the most effective method of reducing/preventing intestinal damage as it does not have a significant role in the inflammatory cascade which occurs during the development of the disorder. Rather, future studies should focus on inhibiting the early stages of mucositis development. Activation of NF- κ B plays a role in the development of intestinal mucositis as it is responsible for the up-regulation of pro-inflammatory cytokines, representing a viable therapeutic target for probiotic manipulation (Chang *et al.* 2012). Indeed, both EcN (Wehkamp *et al.*, 2004) and LGG (Donato *et al.*, 2010) have been shown to augment NF- κ activation previously and this mechanism may have contributed to the protective effects described in Chapters 3 and 4. Strains, such as *Lactobacillus delbrueckii* (Hegazy and El-Bedewy 2010) and *Lactobacillus reuteri* DSM 17938 (Liu *et al.*, 2009), which have been shown to inhibit the NF- κ B pathway, warrant further investigation. Further to NF- κ B signalling, probiotic strains exhibiting broad-spectrum anti-inflammatory actions also should be tested in models of intestinal mucositis. Studies incorporating anti-inflammatory compounds have shown promising

results in the treatment of the disorder (Wu *et al.*, 2010), and strains such as *Lactobacillus casei* (Amdekar *et al.*, 2011) or genetically engineered IL-10 secreting *Lactococcus lactis* (del Carmen *et al.*, 2011). It is also important to consider that individual strains do not need to be administered alone, and probiotic combinations tailored specifically to intestinal mucositis may have a greater efficacy than single strains.

Given the range of physiological changes which occur during intestinal mucositis, future studies may also benefit from the use of specifically-designed probiotic SN combinations. The use of probiotic combinations has been documented extensively, with VSL#3 representing the most commonly utilised combination. The efficacy of VSL#3, a high-concentration probiotic preparation of eight live freeze-dried bacterial species found in the human microbiota, including four strains of lactobacilli (*Lactobacillus casei*, *L. plantarum*, *L. acidophilus*, and *L. delbrueckii subsp. bulgaricus*), three strains of bifidobacteria (*Bifidobacterium longum*, *B. breve*, and *B. infantis*), and *Streptococcus salivarius subsp. thermophilus* (Bowen *et al.*, 2007), has been shown in multiple disease settings, including ulcerative colitis (Sood *et al.*, 2009), irritable bowel syndrome (Michail and Kenche, 2011) and even intestinal mucositis (Bowen *et al.*, 2007). The delivery of a high concentration of multiple strains to the gastrointestinal tract not only aids in restoration of a healthy microbiota, but may also incorporate multiple mechanisms of probiotic action to improve intestinal health. Chapter 1 describes the range of probiotic strains best aligned with the treatment of different aspects of intestinal mucositis pathogenesis. By combining SNs, or even the specific active components

from multiple strains, we may be able to target multiple aspects of mucositis development. This, in turn, may decrease the overall development of the disorder. Furthermore, it may be possible to design a long-term course of probiotic-based therapeutics which can incorporate both a pre-treatment aimed to prepare the patient for the oncoming challenge and a post-treatment which aids in the healing and restoration phase of the disorder.

Determination of the optimal administration technique represents a significant issue for probiotic SN research. SNs are likely to be highly susceptible to the harsh conditions found in the stomach, and therefore steps must be taken to ensure safe passage to the small intestine. This may be the most important aspect of study design, as it is impossible to determine the efficacy of treatment without guaranteeing that the compound has reached its target area. Currently, microencapsulation presents a viable method of minimising probiotic SN degradation during transit. Microcapsules can differ greatly depending on the compound encapsulated and the selected delivery site, factors which have been reviewed extensively by Prakash *et al.* (2011). In order to maximise efficacy, SNs targeted at the treatment or prevention of mucositis should be released in the jejunum, the site of maximal damage with most chemotherapeutic drugs (Sonis, 2004). Targeted delivery to the jejunum has previously been described in the literature, although there are no reports of successful delivery of probiotic-based therapeutics. Dube and colleagues doubled the exposure of flavonoid epigallocatechin gallate (ECGC) to the jejunum by delivering the compound in chitosan-triphosphate nanoparticles (Dube *et al.*, 2011). Patten *et al.* (2009) employed a microcapsule

composed of heated casein, glucose and dried glucose syrup to maximise the delivery of fish oil to the small intestine. Future studies into probiotic-based therapies for intestinal mucositis should utilise such techniques to maximise probiotic availability within the small intestine.

Beneficial effects of probiotic growth media on intestinal epithelial cells were described in Chapters 3 and 4 of this thesis. Comparisons between vehicle controls and SN-treated cells suggested that, in some settings, the growth media may be efficacious. Chapter 3 describes glucose (Yu *et al.*, 2005) and glutamine (Tuhacek *et al.*, 2004, Li and Neu, 2009) as the components of the respective media most likely to be responsible for the observed effect. These results warrant further research into the use of glucose and glutamine in intestinal health, as well as highlighting the importance of appropriate vehicle control groups in probiotic studies.

The 5-FU model of intestinal mucositis is regularly employed to investigate potential therapies. The current studies provide further evidence that 5-FU is able to induce morphological changes within the jejunum. Furthermore, Chapters 2, 3 and 5 demonstrate that 5-FU can also be utilised *in vitro* to damage intestinal epithelial cells in culture. *In vitro* screening of potential therapeutics provides a high throughput system by which high numbers of therapeutics can be tested, while also facilitating simple studies to examine the mechanism responsible for observed effects. However, given the susceptibility to gastric transit, this may not be the most suitable model for the assessment of SNs. Instead animal or simulated gastrointestinal models, such as the

gastrointestinal model TIM (Khalf *et al.*, 2010) may give a more accurate indication of the efficacy of tested SNs.

To conclude, these experiments suggest that certain defined probiotic-derived SNs may be beneficial in both the treatment and prevention of 5-FU-induced mucositis. This thesis represents the first methodical investigation into the efficacy of SNs, and raises a number of questions which require further study. Future mucositis studies should focus on (i) identification of strains which are most effective, (ii) determination of the ideal treatment regime and method of administration, and (iii) identification and isolation of the specific bioactive factors responsible for the observed SN effects.

7. References

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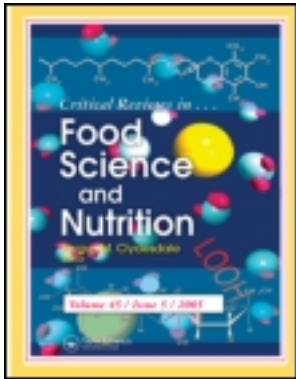
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8. Appendices



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Evidence Supporting the use of Probiotics for the Prevention and Treatment of Chemotherapy-Induced Intestinal Mucositis

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