The Role of Soluble Plant Fibres (Non-Starch Polysaccharides, NSP) in the Maintenance of Intestinal Health and Prevention of Diarrhoeal Disease

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy

By

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February 2016

DECLARATION

I hereby declare that this thesis is a presentation of my original work. Wherever contributions of others are involved, every effort has been made to indicate this clearly, with due reference to the literature.

The work was performed under the joint guidance of Professor Barry J Campbell and Professor Jonathan M Rhodes, both from the Institute of Translational Medicine, at the University of Liverpool.

For Alison

ACKNOWLEDGEMENTS

Firstly, I would like to express my utmost gratitude to my supervisors Professor Barry Campbell and Professor Jonathan Rhodes for their continual support, guidance and encouragement throughout my PhD. It has been an absolute pleasure working with you and I have learnt so much. Thank you to the BBSRC for funding this Industrial Case Studentship.

I would like to extend my thanks to Dr Fabio Miyajima, Dr Paul Roberts, Dr Gillian Douce and Dr Anthony Buckley, all of whom provided me with *C. difficile* toxins and/or clinical isolates. Also, thanks to Dr Niamh O'Kennedy for providing me with the NSP fibres.

I want to say a massive thanks to everyone in the Gastroenterology Unit, past and present, for being such approachable and supportive individuals. In particular, I would like to thank Dr Carol Roberts and Dr Bryony Parsons for training me in all things plantain at the start of the project, as well as their unfailing support throughout my time in the lab. Bryony and Yvette, thank you providing a sympathetic ear when plantain filtering, agar pouring or CFU counting sent me over the edge. Stephanie, thank you for brightening up my days with your witty banter and cat-loving ways. Helen, thanks for the early morning lifts when I was too lazy to get the bus - you have a heart of gold. Sue, thank you for being my substitute mum and for always being there for a good old natter when I needed a break. I also want to say an extremely special thank you to Sophie. Together, we have celebrated the successes and supported each other during the lows. I couldn't imagine my time in Gastro without you by my side, and I know that I have made a friend for life. Thanks for being amazing!

To my Mummy, Daddy and two gorgeous sisters Chloe and Ruth, thanks for supporting me during my studies and for being the best family around. Mummy, I can honestly say that I wouldn't have got to this stage in life without you and your strength. I love you beyond words. I also want to say a huge thanks to Karen and Derek for being there and helping me get through the last four years, it means such a lot and I will never forget it.

My biggest thank you is reserved for Aaron. Thank you for collecting me from the lab, for making my dinner, for calming me down during thesis writing frenzies, for making me laugh on a daily basis, for making an effort to understand what Enterotoxigenic *E. coli* is, and most importantly, for being my best friend. I couldn't have achieved this without you.

ABSTRACT

It has long been proposed that a high intake of dietary fibre promotes good intestinal health. Work performed previously by our research group suggests that soluble dietary fibre might have a particularly beneficial impact on intestinal health via its ability to inhibit potentially harmful interactions between bacterial pathogens and the gut epithelium. The aims of this thesis were to evaluate soluble plantain NSP for its ability to disrupt the epithelial interactions of diarrhoeal pathogens *C. difficile* and Enterotoxigenic *Escherichia coli* (ETEC), as well as other bacterial components implicated in diarrhoeal disease. Work was also performed to characterise the specific inhibitory fraction of plantain NSP, and in addition, to establish the molecular mechanism underlying its inhibitory activity.

A range of soluble dietary fibres were shown to significantly inhibit the *in vitro* epithelial adhesion of *C. difficile* and ETEC, but out of all the fibres tested, soluble plantain NSP exhibited the highest efficacy. Plantain NSP also significantly inhibited the epithelial adhesion of eleven *C. difficile* clinical isolates, irrespective of their toxin expression or ribotype status. Furthermore, plantain NSP blocked the epithelial interactions of five purified *C. difficile* spore preparations. In addition to its anti-adhesive effects, soluble plantain NSP significantly down-regulated the pro-inflammatory, cytotoxicity and apoptotic response induced by *C. difficile* and its toxins. Similar effects were also found with respect to mucosally-associated *E. coli* isolated from ulcerative colitis (UC) patients, as well as bacterial components such as flagellin and LPS.

Results demonstrated that the inhibitory activity of plantain NSP was mediated by its acidic polysaccharide fraction, which is mainly composed of pectic material. In addition, it was

shown that soluble plantain disrupted bacterial-epithelial interactions via an interaction with the intestinal epithelium. Whilst plantain NSP induced increased cellular chloride secretion, this mechanism was not responsible for inhibitory activity. It was also hypothesised that plantain NSP might mimic intestinal MUC2 glycans by interacting with cell-surface galectin-3, with consequent nuclear localisation of β-catenin and down-regulation of inflammatory cytokines. Whilst plantain NSP was shown to induce activation of β-catenin, the knockdown of surface galectin-3 expression had no effect on inhibitory activity. Thus, the specific mechanism underlying the inhibitory activity of plantain NSP requires further investigation.

This work supports the hypothesis that soluble plantain fibre can inhibit harmful interactions between bacteria and the human intestinal epithelium. Indeed, these studies provide convincing evidence to suggest that soluble plantain fibre, acting as a 'contrabiotic', could be developed as a potential prophylaxis or treatment against *C. difficile* and ETEC, which represent the main cause of antibiotic-associated diarrhoea and traveller's diarrhoea, respectively. In addition, dietary supplementation with soluble plantain NSP may also confer a therapeutic benefit in inflammatory bowel disease (IBD).

PUBLICATIONS ARISING FROM THIS THESIS

Papers

Parsons, B. N., Wigley, P., **Simpson, H. L.**, Williams, J. M., Humphrey, S., Salisbury, A., Watson, A. J. M., Fry, S. C., O'Brien, D., Roberts, C. L., O'Kennedy, N., Keita, Å. V., Söderholm, J. D., Rhodes, J. M. and Campbell, B. J. Dietary supplementation with soluble plantain non-starch polysaccharides inhibits intestinal invasion of *Salmonella* Typhimurium in the chicken. Plos One. 2014; 9(2): e87658.

In preparation. **Simpson, H. L.**, Roberts, C. L., Miyajima, F., Roberts, P., O'Kennedy, N., Rhodes, J. M. and Campbell, B. J. Soluble plantain fibre (non-starch polysaccharide) inhibits the *in vitro* epithelial interactions of *Clostridium difficile*.

Reviews

Simpson, H. L. and Campbell, B. J. Soluble dietary non-starch polysaccharides may improve health by inhibiting the adhesion, invasion and translocation of enteric gut pathogens. 2014. In: Benkliba, N. ed. Polysaccharides: Natural Fibres in Food and Nutrition. CRC Press.

Simpson, H. L., Campbell, B. J. and Rhodes, J. M. IBD: Microbiota manipulation through diet and modified bacteria. Digestive Diseases. 2014 Dec; 32(Suppl. 1): 18-25.

Simpson, H. L. and Campbell, B. J. Review article: dietary fibre-microbiota interactions. Alimentary Pharmacology and Therapeutics. 2015 Jul; 42(2): 158-179.

Abstracts

Simpson, H. L., Roberts, C. L., Rhodes, J. M. & Campbell, B. J. Soluble plant fibres, particularly leek and plantain, inhibit adherence of diarrhoea-associated pathogens *C. difficile* and Enterotoxigenic *E. coli* to intestinal epithelial cells. DDF 2012, Liverpool.

Campbell, B. J., **Simpson, H. L.**, Parsons, B, N. & Rhodes, J. M. The acidic polysaccharide component of soluble plantain fibre inhibits the adhesion of diarrhoea-associated pathogens *Salmonella*, *C. difficile* and Enterotoxigenic *E. coli* to intestinal epithelial cells. BSG Annual Meeting 2013, Glasgow

Simpson, H. L., Keita, Å. V., Söderholm, J. D., Rhodes, J. M. & Campbell, B. J. Soluble plantain fibre inhibits the epithelial adhesion of diarrhoeal pathogens *Clostridium difficile*, *Salmonella* and Enterotoxigenic *E. coli* (ETEC) through interaction with the intestinal epithelium. BSG Annual Meeting 2014, Manchester.

Simpson, H. L., Rhodes, J. M. & Campbell, B. J. Soluble plantain (banana) fibre inhibits epithelial cell damage in response to *Clostridium difficile* and its toxins. DDW 2015, Washington DC.

Simpson, H. L., Rhodes, J. M. & Campbell, B. J. Soluble plantain (banana) fibre inhibits the epithelial IL-8 and cytotoxicity response to ulcerative colitis (UC) mucosally-associated *Escherichia coli.* DDW 2015, Washington DC.

Reade, S., **Simpson, H. L.**, Aggio, R. B. M. & Probert, C. S. J. Hypervirulent *Clostridium difficile* has a unique odour profile. DDF 2015, London.

ABBREVIATIONS USED IN THIS THESIS

AAD	Antibiotic-associated diarrhoea
AIEC	Adherent invasive *E. coli*
AK	Adenylate kinase
AMP	Adenosine monophosphate
AUL	Arbitrary units of luminescence
BHI	Brain heart infusion
BSA	Bovine serum albumin
CCNA	Cytotoxicity neutralisation assay
CD	Crohn's disease
CDAD	*C. difficile* associated disease
CDI	*C. difficile* infection
CDRN	*C. difficile* Ribotyping Network
CF	Colonisation factor
CFTR	Cystic fibrosis transmembrane regulator
CFTR-inh-172	Cystic fibrosis transmembrane regulator inhibitor 172
CFU	Colony forming unit
CHO	Chinese hamster ovarian
CLE	Cortex lytic enzymes
CSPG4	Chondroitin sulphate proteoglycan 4
DAPI	4',6-diamidino-2-phenylindole
DC	Dendritic cell
DE	Dextrose equivalent
DEAC	Diffusely adherent *E. coli*
DMEM	Dulbecco's Modified Eagle's Medium

DPA	Dipicolinic acid
DPX	Distrene, Plasticiser, Xylene
DSS	Dextran sulphate sodium
EIA	Enzyme immunoassay
EAEC	Enteroaggregative *E. coli*
ECACC	European Collection of Authenticated Cell Cultures
EPEC	Enteropathogenic *E. coli*
ETEC	Enterotoxigenic *E. coli*
FAE	Follicle-associated epithelium
FCS	Foetal calf serum
FDA	Food and Drug Administration
FMT	Faecal microbiota transplantation
GalA	Galacturonate
GBF	Germinated barley foodstuff
GDH	Glutamate dehydrogenase
GMP	Guanosine monophosphate
GR	Germinant receptor
GRO- α	Growth-related oncogene alpha
HG	Homogalacturonan
HPA	Health Protection Agency
HR	Hazard ratio
HRP	Horseradish peroxidase
H_2	Histamine receptor 2
IBD	Inflammatory bowel disease
IBS	Irritable bowel syndrome
IKK	IκB kinase

IL	Interleukin
Ig	Immunoglobulin
I_{SC}	Transmucosal short circuit current
IVIG	Intravenous immunoglobulin
LacNac	N-acetyllactosamine
LB	Luria Burtani
LPS	Lipopolysaccharide
LT	Heat-labile toxin
MAb	Monoclonal antibodies
M-cell	Microfold cell
MCP-1	Monocyte-chemotactic protein 1
MOI	Multiplicity of infection
NAAT	Nucleic acid amplification test
NeuGc	N-glycolylneuraminic acid
NF-κβ	Nuclear factor kappa β
NICE	National Institute for Health and Care Excellence
NOD	Nucleotide oligomerisation domain
NPPB	5-nitro-2-(-3-phenylpropyl-amino) benzoic acid
NSAID	Non-steroidal anti-inflammatory drug
NSP	Non-starch polysaccharide
OD	Optical density
OMV	Outer membrane vesicle
OPD	*O*-phenylenediamine
OR	Odds ratio
PBS	Phosphate-buffered saline
PC	Phosphatidylcholine

PFA	Paraformaldehyde
PG	Peptidoglycan
PMC	Pseudomembranous colitis
PPI	Proton pump inhibitor
PVRL3	Poliovirus receptor-like 3
RG	Rhamnogalacturonan
RR	Relative risk
SCFA	Short chain fatty acid
SMC	Sorbitol MacConkey
Spp.	Species
ST	Heat-stable toxin
TEER	Transepithelial electrical resistance
TEM	Transmission electron microgram
TGY	Tryptic glucose yeast
TLR	Toll-like receptor
TNBS	2,4,6-trinitrobenzenesulfonic acid
Tslp	Thymic stromal lymphopoietin
UC	Ulcerative colitis
VE	Villous epithelium
VRE	Vancomycin-resistant enterococci
WHO	World Health Organisation

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Chapter 1

Introduction

1.1 DIARRHOEAL DISEASES

1.1.1 Diarrhoea – a definition

Diarrhoea is a perturbation of normal bowel movement, which is characterised by an increase in the water content, volume or frequency of stools (Baldi *et al.* 2009). The World Health Organisation (WHO) defines diarrhoea as three or more loose or watery stools (taking the shape of the container in which it is collected) in a 24-hour period (Abba *et al.* 2009).

1.1.2 Acute, persistent and chronic diarrhoea

From a clinical perspective, diarrhoea can be classified according to certain characteristics, such as episode length. Diarrhoeal episodes lasting less than 2 weeks are defined as acute whilst diarrhoea lasting between 2-4 weeks is termed as persistent. Episodes of diarrhoea lasting longer than 4 weeks are classed as chronic (Abba *et al.* 2009; Thiagarajah *et al.* 2015).

Most cases of acute diarrhoea occur due to an infectious agent or pathogen such as a bacterium, virus or parasite (Baldi *et al.* 2009; Abba *et al.* 2009). Also known as pathogen-related diarrhoea, this diarrhoeal subtype represents a global problem that persistently causes significant mortality and morbidity, resulting in 1.8 million deaths annually (World Health Organisation 2007) . Pathogen-related diarrhoea is of bacterial aetiology in 1.5 – 5.6% of cases (Baldi *et al.* 2009). In developing countries, the most common causes of bacterial mediated diarrhoea include enterotoxin-producing *Vibrio cholerae* and Enterotoxigenic

Escherichia coli (ETEC) (Thiagarajah *et al.* 2015; Baldi *et al.* 2009). In developed countries, main bacterial causes of diarrhoeal disease include enterotoxin-producing *Clostridium difficile*, as well as enteroinvasive *Salmonella* species (spp.) and *Campylobacter* spp. (Thiagarajah *et al.* 2015). Most common causes of chronic diarrhoea include intestinal inflammatory and autoimmune conditions, which include inflammatory bowel disease (IBD), irritable bowel syndrome (IBS) and coeliac disease (Thomas *et al.* 2003).

For the purpose of this thesis, three important causes of diarrhoea will be reviewed that cause significant disease and are somewhat problematic in terms of their treatment. This includes acute infectious diarrhoeal disease caused by *C. difficile*, which represents one of the leading causes of antibiotic-associated diarrhoea in a healthcare setting (Ananthakrishnan 2011). Acute infectious diarrhoea caused by ETEC will also be reviewed, which represents the main cause of Traveller's diarrhoea and a common cause of infantile diarrhoea in developing countries (Qadri *et al.* 2005). Lastly, ulcerative colitis (UC), a major form of IBD (Danese and Fiocchi 2011), will be reviewed as a chronic cause of diarrhoeal disease.

1.2 *C. DIFFICILE* INFECTION – AN ACUTE DIARRHOEAL DISEASE

C. difficile is a Gram-positive, spore-forming, anaerobic bacillus, which belongs to the phylum Firmicutes (*Clostridium* cluster XI) (Cairns *et al.* 2012). The bacterium resides in the environment (soil, water and animal faeces) and in the human gut where it can act as a normal commensal (Bruggemann and Gottschalk 2008). Indeed, some populations are carriers of *C. difficile* but do not develop symptoms of infection. *C. difficile* infection (CDI) refers to patients who exhibit symptoms of gastrointestinal illness (Dawson *et al.* 2009).

C. difficile was first isolated from the stool of a healthy infant by Hall and O'Toole in 1935, and the species name chosen to reflect the difficulty with its isolation and culture (Carroll and Bartlett 2011). Over the next 40 years, there were infrequent reports of *C. difficile* isolation, with limited findings to suggest that the organism could actually contribute towards disease. It was not until 1978 that *C. difficile* was shown to be the primary isolate in patients undergoing clindamycin treatment and the causative agent of pseudomembranous colitis (PMC) (Bartlett *et al.* 1978). Today, *C. difficile* is recognised as the worldwide leading cause of antibiotic-associated diarrhoea (AAD), with CDI attributing 15-25% of all cases (Ananthakrishnan 2011).

1.2.1 Pathogenesis of CDI

Asymptomatic carriage of *C. difficile* is common in both a community and hospital setting, which is illustrated by the small proportion patients (approximately 35-50%) who go on to develop active disease (Goudarzi *et al.* 2014; Donskey *et al.* 2015). This clearly demonstrates that a number of events must first take place to induce symptomatic infection. Indeed, the pathogenesis of CDI can be characterised by four main steps. These include the perturbation of the normal intestinal microbiota (Seekatz and Young 2014), successful *C. difficile* colonisation of the intestinal epithelium and the subsequent release of *C. difficile* toxins (Voth and Ballard 2005). It has also become evident that dormant *C. difficile* spores play an important role in CDI transmission and pathogenesis (Sarker and Paredes-Sabja 2012).

1.2.1.1 Disruption of the normal intestinal microbiota

The intestinal microbiota plays an essential role in CDI pathogenesis (Seekatz and Young 2014). *C. difficile* is considered a poor competitor with the normal intestinal microbiota, and as such, a healthy microbiota provides colonisation resistance against the bacterium (Britton and Young 2014). Conversely, disruption of the intestinal microbiota leads to the creation of an ecological niche in which opportunistic *C. difficile* can flourish and effectively colonise the intestinal epithelium (Britton and Young 2014; Seekatz and Young 2014). Many of the risk factors associated with CDI therefore result in perturbation of the normal intestinal microbiota (as reviewed in *Section 1.2.3*).

1.2.1.2 Colonisation of the intestinal epithelium

C. difficile colonisation of the intestinal epithelium represents an early and essential step in the pathogenesis of CDI. Following the disruption of the colonic microbiota, *C. difficile* penetrates the mucus layer overlying enterocytes, subsequently colonising the intestinal epithelium. Indeed, numerous studies have demonstrated that *C. difficile* can adhere *in vitro* to various cell lines such as Caco2 cells and Vero cells, as well as *in vivo* to the caecal mucus of mice (GomezTrevino *et al.* 1996; Cerquetti *et al.* 2002; Karjalainen *et al.* 1994).

A number of different colonisation factors have been identified which are thought to mediate this process. These include proteolytic enzymes, such as the cysteine protease Cwp84 (Janoir *et al.* 2007), and flagella components flagellin (FliC) and flagellar cap protein (FliD) (Tasteyre *et al.* 2001). Multiple adhesins have also been characterised, which include S-layer P36 and P47 proteins (Calabi *et al.* 2002; Cerquetti *et al.* 2000), a 66-kDa cell-wall protein

Cwp66 (Waligora *et al.* 2001), the GroEL heat-shock protein (Hennequin *et al.* 2001) and a 68-kDa fibronectin-binding protein (Hennequin *et al.* 2003).

It has been demonstrated that *C. difficile* colonisation factors exhibit carbohydrate-binding (lectin) activity (Stevenson *et al.* 2015). These surface proteins recognise and bind to complementary carbohydrates on the surface of host cells, promoting bacteria-epithelial adherence (Sharon 2006). Importantly, close proximity or adhesion of *C. difficile* to the intestinal mucosa is likely to be necessary for the release of its toxins (Voth and Ballard 2005). Therefore, this initial phase of infection is an essential mediator of pathogenesis.

1.2.1.3 Release of toxins

Pathogenic strains of *C. difficile* secrete two potent exotoxins, Toxin A and Toxin B (TcdA and TcdB, respectively), which induce mucosal inflammation and diarrhoea. In addition to these exotoxins, some *C. difficile* strains produce an adenosine diphosphate (ADP)-ribosylating binary toxin (CDT), however, its role in disease is somewhat less understood (Voth and Ballard 2005).

TcdA (308 kDa) and TcdB (270 kDa) belong to the family of large clostridial toxins, and are amongst the largest bacterial toxins reported to date (Voth and Ballard 2005; Just and Gerhard 2004). The precise mechanism of toxin uptake into the cells is unclear, but there is sufficient evidence to suggest that the mechanism can be characterised into certain stages **(Figure 1.1)**. Receptor binding is the first essential step in toxin cell entry, thought to be mediated by carbohydrate structures present on the host cell surface (Voth and Ballard 2005; Just and Gerhard 2004).

Although TcdA and TcdB are homologues, they are thought to have different receptors, based on differences in sensitivities among cell types *in vitro* (Chaves-Olarte *et al.* 1997; Dingle *et al.* 2010). Multiple receptors for TcdA have been identified including the disaccharide Galβ1-4GlcNAc, which is expressed on I, X and Y carbohydrate antigens present on the human intestinal epithelium (Tucker and Wilkins 1991). In line with these findings, treatment of cells or tissue with β-galactosidase reduces binding by TcdA (Clark *et al.* 1987; Smith *et al.* 1997). Other proposed TcdA receptors include rabbit sucrase isomaltase (Pothoulakis *et al.* 1996) and glycoprotein (gp)-96 (Na *et al.* 2008). TcdB receptors are somewhat more elusive, and until recently, they were completely undefined (Voth and Ballard 2005). Yuan and colleagues demonstrated the first identification of a TcdB receptor. In this landmark study, it was shown that chondroitin sulphate proteoglycan 4 (CSPG4) is critical for TcdB cell attachment, inducing cytoskeleton disruption and cell death (Yang *et al.* 1993). Poliovirus receptor-like 3 (PVRL3) has also recently been characterised as an epithelial cell receptor responsible for TcdB-induced cytotoxicity (LaFrance *et al.* 2015).

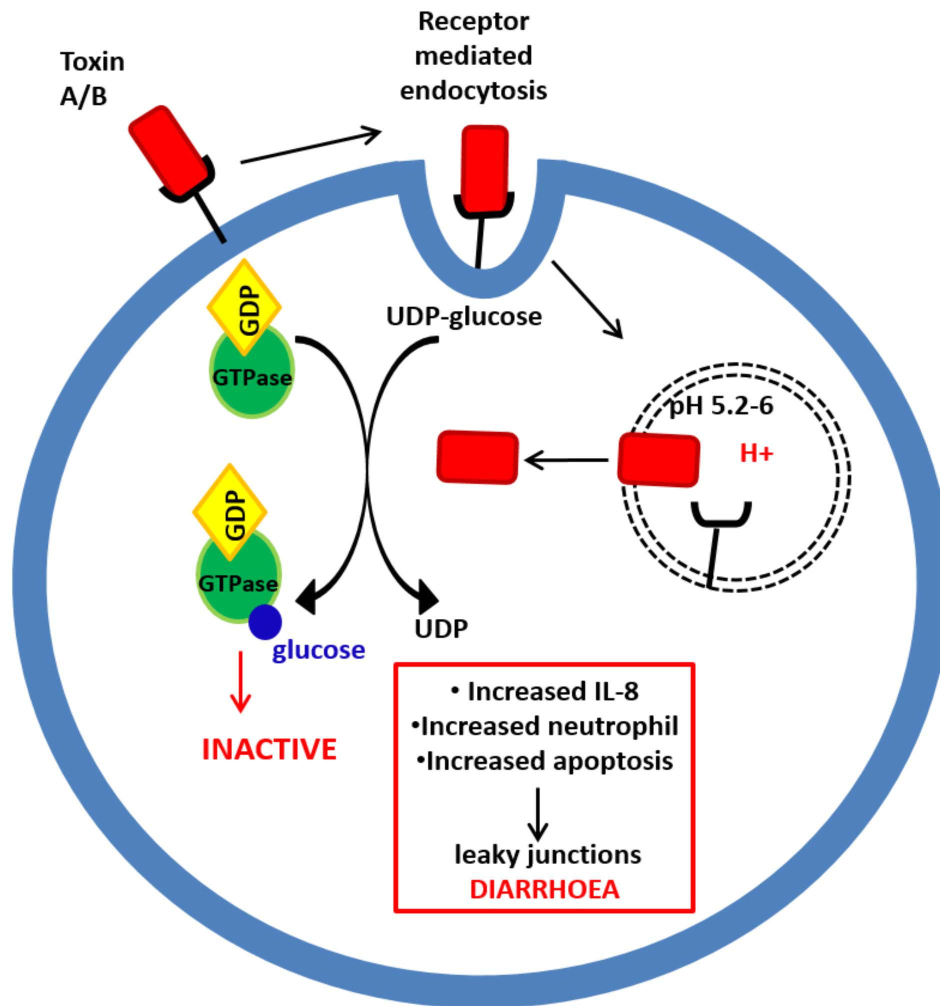


Figure 1.1 Summary of the mechanism of *C. difficile* TcdA and TcdB. Adapted from (Voth and Ballard 2005).

Subsequent to binding carbohydrate membrane structures, toxin internalisation is induced by receptor-mediated endocytosis (Voth and Ballard 2005). Studies have shown that this step requires a low pH, which is likely due to the toxins undergoing important structural changes. An acidic endosomal pH induces toxin refolding which leads to the exposure of the hydrophobic transmembrane domain, and aids insertion into the target endosomal membrane (Qa'Dan *et al.* 2000). It is understood that TcdA and TcdB next undergo autoproteolysis, allowing only the catalytic domain to be translocated into the cytoplasm of the cell, where it finally catalyses the mono-glucosylation of small regulatory Rho-GTPases (Rho, Rac and Cdc42) (Voth and Ballard 2005).

The irreversible modification of Rho proteins prevents their interaction with downstream regulators, resulting in their ensuing inactivation (Sehr *et al.* 1998). This leads to the disruption of vital cell signalling pathways and a number of toxin mediated effects, which include increased cell rounding, loss of actin stress fibres, disruption of intercellular tight junctions and resultant diarrhoea (Voth and Ballard 2005).

Other cellular effects of TcdA and TcdB include the induction of the pro-inflammatory response through the release of cytokine interleukin 8 (IL-8), mediated by the activation of nuclear factor kappa β (NF-κβ) (Kim *et al.* 2006). *C. difficile* TcdA and TcdB also mediate apoptosis through the activation of both the intrinsic and extrinsic apoptotic pathways, and subsequent activator of executioner caspase-3 (Gerhard *et al.* 2008).

The role of CDT in infection is not well characterised, but it is known that its structure consists of two unlinked polypeptides; enzymatic component CdtA and transporter component CdtB. CdtA is a G-actin specific, mono ADP-ribosyl transferase which becomes internalised into the host cell, most likely via a CdtB heptamer on the cell surface. Studies

suggest that in the cytosol, CdtA irreversibly ADP-ribosylates G-actin which inhibits its polymerisation to F-actin thus disrupting the actin cytoskeleton, resulting in cell rounding and cell death (Carman *et al.* 2011; Davies *et al.* 2011). Studies have also suggested that CDT may increase bacterial adhesion to target cells through the formation of microtubule protrusions (Schwan *et al.* 2009).

1.2.1.4 C. difficile spores

When environmental conditions become unfavourable, such as through a lack of nutrients, *C. difficile* has the ability to rapidly produce highly resistant and dormant endospores (Barra-Carrasco and Paredes-Sabja 2014; Paredes-Sabja *et al.* 2014). During infection, *C. difficile* initiates an efficient sporulation cycle whereby patients enter a highly contagious 'super-shedder' state with high levels of spore excretion into the surrounding environment (Barra-Carrasco and Paredes-Sabja 2014). Studies have also shown that compared with non-carriers, asymptomatic patients exhibit high rates of skin (61% versus 19%, P = 0.001) and environmental *C. difficile* spore contamination (59% versus 24%, P = 0.004) (Riggs *et al.* 2007). As such, *C. difficile* spores are considered the key vehicle of CDI transmission (Sarker and Paredes-Sabja 2012; Paredes-Sabja *et al.* 2014).

C. difficile spores are resistant to commonly used disinfectants, therefore, they can survive indefinitely outside of the host and persist on clinical surfaces for long periods of time (Sarker and Paredes-Sabja 2012; Barra-Carrasco and Paredes-Sabja 2014). Once ingested by a susceptible individual, spores return to vegetative growth through germination, which is particularly triggered by primary bile acids (Wilson 1983; Wilson *et al.* 1982). Under optimal conditions, this can result in colonisation, toxin release and subsequent active disease (Burns

et al. 2010). *C. difficile* spores can also persist in the colonic tract of patients, contributing to recurrent infection (Drekonja *et al.* 2011). Consequently, *C. difficile* spores play an important role in disease pathogenesis.

Much of the understanding about *C. difficile* sporulation is rather limited and relies on comparisons with other spore-forming bacteria, such as the model spore-forming Gram positive organism *Bacillus subtilis,* also found in soil and the gastrointestinal tract of mammals (Edwards and McBride 2014). The process of *C. difficile* sporulation is almost identical to that of *B. subtilis*, and is typically characterised into seven morphological stages (as summarised in **Figure 1.2**) (Leggett *et al.* 2012). The molecular mechanisms underlying *C. difficile* sporulation are less clearly understood. In many *Bacillus* and *Clostridium* species, the decision to enter sporulation is regulated by several orphan histidine kinases that can phosphorylate the master regulator of sporulation Spo0A (Edwards and McBride 2014).

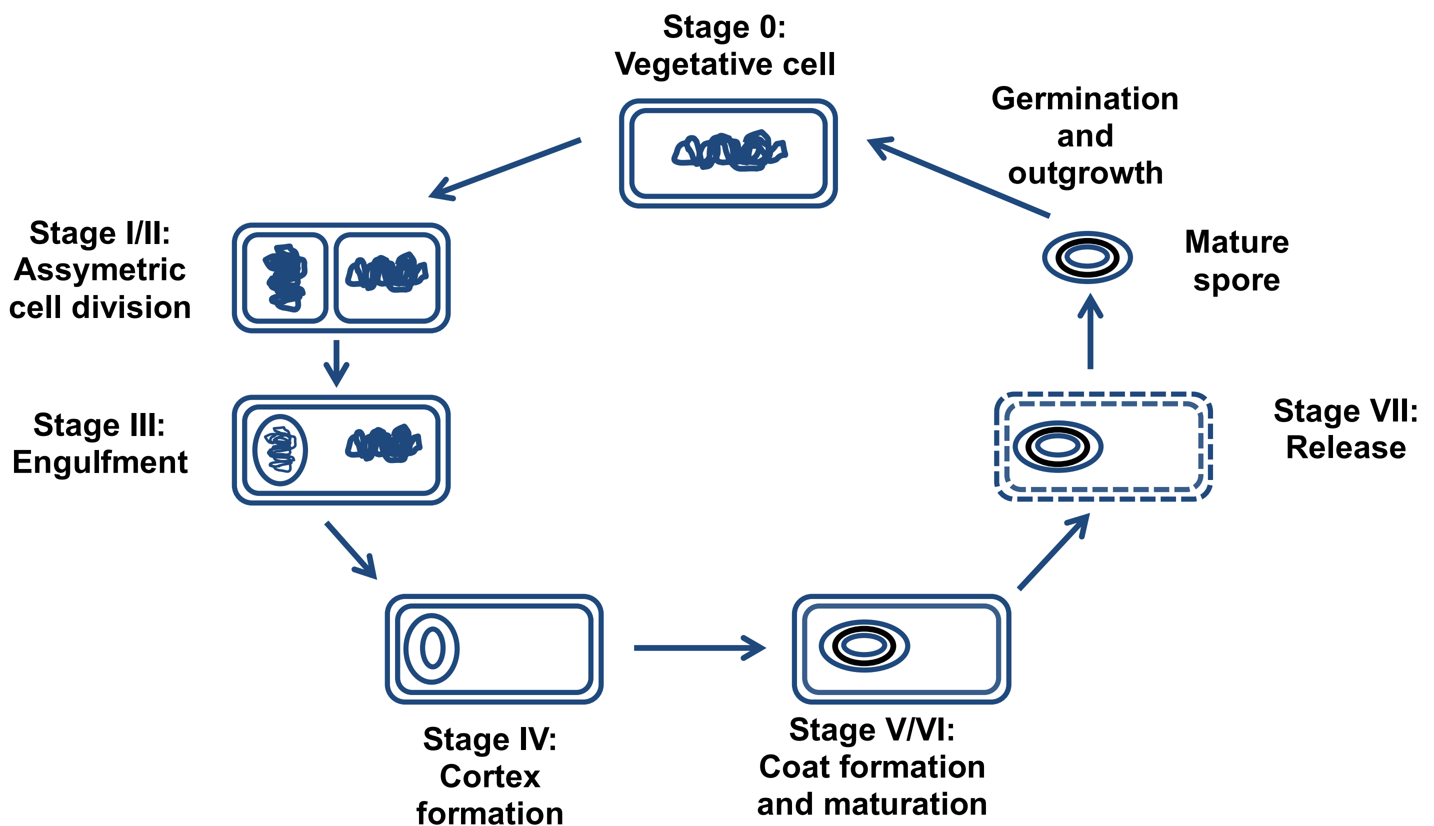


Figure 1.2. Summary of the morphological changes that take place during *C. difficile* sporulation. *Stage 0)* Normal vegetative growth. *Stage I/II)* Vegetative cell undergoes asymmetric cell division, which forms two compartments separated by the septum. The smaller of the two compartments is termed the pre-spore. *Stage III)* Pre-spore engulfed by the mother cell to form a distinct cell termed the fore-spore bound by inner and outer fore-spore membranes. *Stage IV)* Spore cortex formation between inner and outer fore-spore membranes. *Stage V/VI)* Mother cell synthesises spore specific pyridine-2, 6-dicarboxylic acid (dipicolinic acid; DPA), which accumulates in the fore-spore and reduces fore-spore water content. This is accompanied by spore maturation and increased density of spore coat. *Stage VII)* Lysis of the mother cell and release of mature spore. Resistant nature of the mature spore ensures it survives until conditions become more favourable for vegetative cell growth. The dormant spore is then re-activated and undergoes germination and outgrowth. Figure adapted from (Leggett *et al.* 2012).

Following Spo0A phosphorylation, gene expression shifts to support spore formation rather than vegetative growth (Edwards and McBride 2014). Sequencing analyses have demonstrated that *C. difficile* strain 630 genome encodes five orphan histidines (CD1352, CD1492, CD1579, CD1949 and CD2492) (Underwood *et al.* 2009) and it is thought that these may undergo autophosphorylation to directly phosphorylate Spo0A (Paredes-Sabja *et al.* 2014). Studies have shown that knockdown of CD2492 reduces *C. difficile* spore formation by 3.5-fold, whilst mutation of *spo0A* completely abolishes spore formation, indicating an essential role in the process (Underwood *et al.* 2009).

Bacterial spores differ considerably from their vegetative counterparts, both in their structure and chemical composition (Paredes-Sabja *et al.* 2014; Leggett *et al.* 2012) (**Figure 1.3**). Several groups have published transmission electron micrograms (TEMs) of *C. difficile* spores, demonstrating that the basic ultrastructural layers are similar to *B. subtilis* and *B. anthracis* (Barra-Carrasco and Paredes-Sabja 2014). In the centre of the spore lies the dehydrated spore core (77% of the spore core wet weight) that contains the spore's DNA, RNA, ribosomes and most of its enzymes (Barra-Carrasco and Paredes-Sabja 2014). The core also contains high levels of pyridine-2, 6-dicarboxylic acid (dipicolinic acid; DPA), which exists as a 1:1 chelate with divalent cations, most notably Ca^{2+} (Ca-DPA) (Leggett *et al.* 2012). The spore core is surrounded by a remarkably impermeable inner membrane that acts to protect the core from DNA-damaging chemicals (Paredes-Sabja *et al.* 2014; Barra-Carrasco and Paredes-Sabja 2014). Surrounding the inner membrane is the germ cell wall, composed of a peptidoglycan (PG) cortex with a similar structure to that of growing cells (Paredes-Sabja *et al.* 2014). The germ cell wall layer is surrounded by a spore-specific cortex which provides protection against cellular cortex hydrolases through unique glycan residue modifications,

and an outer membrane that plays a key role in spore formation but has little effect on spore resistance (Leggett *et al.* 2012; Barra-Carrasco and Paredes-Sabja 2014). These layers are surrounded by a proteinaceous spore coat, which provides protection against reactive chemicals, as well as lytic and microbial enzymes (Leggett *et al.* 2012; Barra-Carrasco and Paredes-Sabja 2014). The electrodense exosporium layer represents the outermost part of the *C. difficile* spore, the structure of which varies considerably between *C. difficile* strains (Escobar-Cortes *et al.* 2013) (**Figure 1.3**). Recent studies have suggested that the exosporium of *C. difficile* spores may play a key role in adherence to epithelial cells and contribute to spore surface hydrophobicity and spore dormancy (Paredes-Sabja and Sarker 2012; Joshi *et al.* 2012; Paredes-Sabja *et al.* 2012).

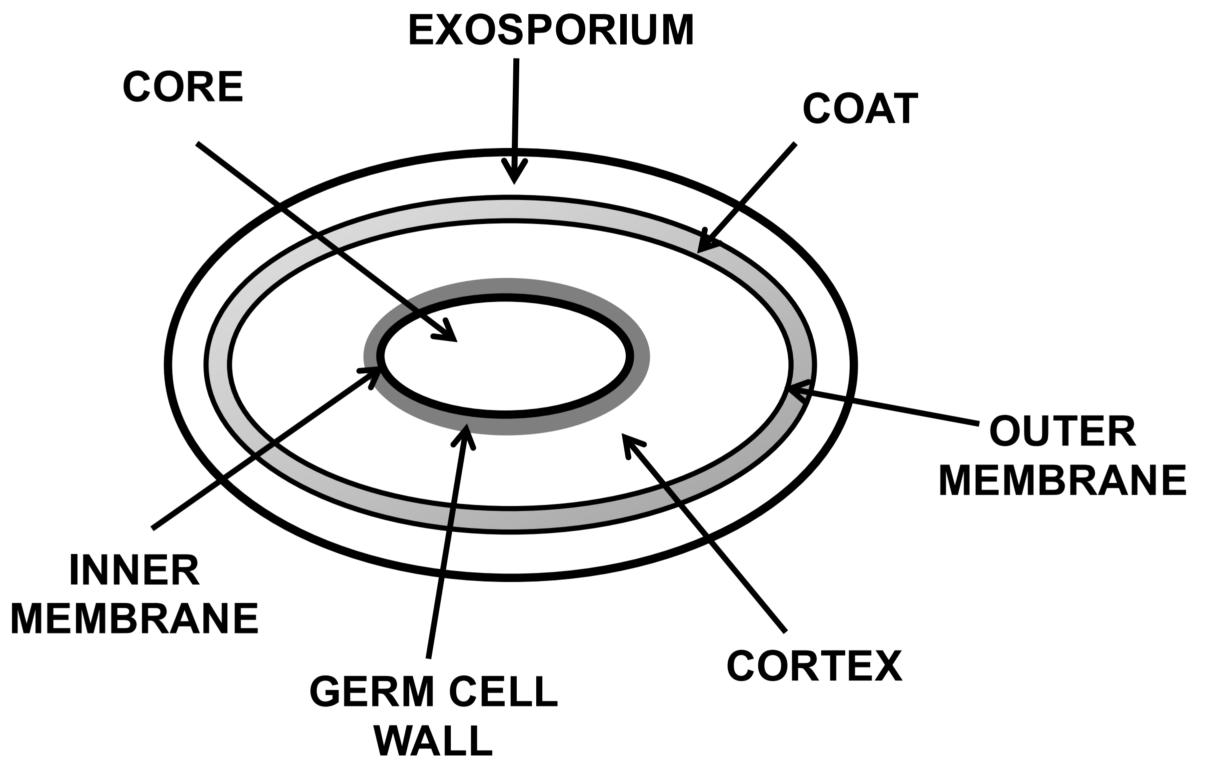


Figure 1.3. Representative diagram of *C. difficile* spore structure. Structure is not drawn to scale. Modified from (Leggett *et al.* 2012).

Spore germination is triggered when cognate germinant receptors (GRs) bind germinant factors. A number of spore germinant factors have been characterised, which include specific primary bile acids cholate, taurocholate and glycocholate (Wilson 1983; Wilson *et al.* 1982). More recently, studies have suggested that glycine, ʟ-phenylalanine, ʟ-arginine (Howerton *et al.* 2011) and histidine can also act as co-germinants with cholate derivatives (Howerton *et al.* 2011; Sorg and Sonenshein 2008; Wheeldon *et al.* 2011), whilst secondary bile acids such as chenodeoxycholate inhibit germination (Sorg and Sonenshein 2010; Sorg and Sonenshein 2009). Germinant factor binding prompts the release of monovalent cations (H^+, Na^+ and K^+) and the spore core's large depot of Ca-DPA. These events initiate hydrolysis of the spore PG cortex, allowing spore core expansion and water uptake. When the water content in the spore core reaches similar levels to that found in vegetative cells, enzyme activity is restored, cell metabolism is initiated and spore outgrowth takes place (Sarker and Paredes-Sabja 2012). The signals that activate cortex hydrolysis in *C. difficile* spores are currently unknown, however, in *Bacillus* species, hydrolysis occurs via direct activation of cortex lytic enzymes (CLEs) (Paredes-Sabja *et al.* 2011).

Interestingly, it has been shown that the disruption of the intestinal microbiota might result in increased *C. difficile* spore germination. In individuals with a healthy intestinal microbiota, co-germinants such as cholate and its derivatives are quickly metabolised, reducing the relative abundance of germinants, thus preventing *C. difficile* spore germination. However, following antibiotic treatment, disruption of the intestinal microbiota may lead to an increase in the availability of germinants, thus promoting *C. difficile* spore germination (Sarker and Paredes-Sabja 2012). A study by Giel and colleagues provides evidence towards this, whereby caecal extracts from antibiotic-treated mice contained elevated levels of

primary bile salts and increased colony formation of *C. difficile* spores in comparison with caecal extracts from untreated mice (Giel *et al.* 2010). Studies have also demonstrated that CDI disease severity is correlated to germination rates of *C. difficile* clinical isolates (Carlson *et al.* 2015)

1.2.2 Epidemiology of CDI

1.2.2.1 Incidence rates

In recent years, there has been a significant change in the epidemiology of CDI. Prior to 2000, the annual CDI rate was relatively stable. However, since the turn of the century, a continuous rise in CDI incidence has been observed across the Western world, including Canada (Pepin *et al.* 2004), the USA (Dallal *et al.* 2002) and Europe (Jones *et al.* 2013; Kuijper *et al.* 2008).

Dramatic increases in CDI rates were reported initially in Quebec, Canada, where incidence increased from 35.6 cases per 100,000 of the general population in 1991 to 156.3 cases per 100,000 of the population in 2003 (Pepin *et al.* 2004). Amongst patients 65 years or more, incidence rates increased 8-fold from 102.0 per 100,000 to 886.5 per 100,000 of the population (Pepin *et al.* 2004). A parallel increase was observed in North America, where there was a 5-fold increase in CDI incidence in the whole population, with a disproportionate 8-fold increase in the elderly population (McDonald *et al.* 2006). A similar pattern has also been observed in Europe. Increased CDI rates were first observed in the UK between 2003-2004, then in the Netherlands and Belgium from 2005, followed by France and other European countries (Kuijper *et al.* 2008; Bassetti *et al.* 2012). Other observed epidemiological

changes include increased disease severity, increased complications, higher mortality and increased risk of recurrence (Pepin *et al.* 2004). This changing pattern of disease has been attributed largely to the emergence of the hypervirulent and epidemic BI/NAP1/027 strain (Freeman *et al.* 2010).

1.2.2.2 A United Kingdom (UK) perspective

In the UK, the first known outbreak caused by the BI/NAP1/027 strain occurred at Stoke Mandeville Hospital (Buckinghamshire Hospitals NHS Trust) in 2003, which involved 174 cases and 19 deaths attributable to CDI (Smith 2005). Over the coming years, disease outbreaks became widespread across England with more than 55,000 CDI cases reported in 2007/08, of which 20% were in younger age groups previously not considered as high risk (Wilcox *et al.* 2012). Due to the increase in disease incidence and severity, the Health Protection Agency (HPA) introduced an enhanced *C. difficile* surveillance programme in 2007, in which all NHS hospitals in England were required to report all CDI cases on a mandatory basis (Wilcox *et al.* 2012). Reduction targets (30% annual decrease in CDI incidence) were also established, alongside the introduction of the *C. difficile* Ribotyping Network (CDRN) (Freeman *et al.* 2010).

Dramatic declines in CDI incidence were observed rapidly following the introduction of enhanced surveillance, and incidence rates have continued to fall on an annual basis. In 2013/14, the total number of reported CDI cases reached the lowest level (13,361 cases) since mandatory surveillance began. However, this annual decline has slowed substantially, with only a 9.1% reduction between 2012/13 and 2013/14, in comparison to a 19.1% reduction between 2011/12 and 2012/13 (Public Health England 2014) . Furthermore, there has been a

slower decline in community-acquired CDI (Public Health England 2014) , with an increase in the proportion of *C. difficile* deaths occurring in care homes (5.3 - 6.7% increase in proportion between 2007-09 and 2010-12), homes (1.3 - 1.9%) and hospices (0.8 - 1.4%) (Cole 2013).

1.2.3 Risk factors of CDI

1.2.3.1 Antibiotic exposure

The main risk factor for CDI is exposure to antibiotics. Broad-spectrum antibiotics, particularly those that target anaerobic bacteria, alter the normal ratio of different intestinal microbial communities (Ananthakrishnan 2011). As described in several studies, the administration of antibiotics causes a decrease in carbohydrate-fermenting and butyrate-producing bacteria members of Bacteroidetes and Firmicutes phyla (Antonopoulos *et al.* 2009; Jernberg *et al.* 2007; Manges *et al.* 2010). It is thought that the resultant intestinal dysbiosis creates an ecological niche that favours the multiplication of *C. difficile*, allowing it to become an established gut pathogen. As such, most CDI patients have been exposed to antimicrobials several weeks to several months before diagnosis (Owens *et al.* 2008). In one retrospective study, more than two-thirds of CDI patients had received antibiotics in the 2 months preceding diagnosis (Pepin *et al.* 2004). In another case-controlled study, it was reported that up to 94% of hospitalised CDI patients had received antibiotics at some point either before or during their stay (Loo *et al.* 2005).

Almost all antibiotics have been associated with the development of CDI, but some carry a higher risk than others (Sullivan *et al.* 2001). Indeed, these associations may parallel temporal changes in the frequency of use of each antibiotic category (Ananthakrishnan 2011).

Initially, antibiotics that held the greatest association with CDI development included clindamycin, cephalosporins and amoxicillin (Ananthakrishnan 2011). More recently, fluoroquinolones have also emerged as a significant risk factor for CDI (Pepin *et al.* 2005). In a study by Pepin and colleagues, the use of fluoroquinolones was associated with a three-fold increase in the risk of CDI among hospitalised patients (hazard ratio [HR] 3.4, 95% confidence interval [CI] 2.7-4.5) (Pepin *et al.* 2004). This is likely due to the widespread use of these drugs to treat a variety of infections, leading to the emergence of fluoroquinolone-resistant *C. difficile* strains (Ananthakrishnan 2011; Bassetti *et al.* 2012). Other microbial CDI risk factors include the use of multiple antibiotics (Gerding *et al.* 1986; Thibault *et al.* 1991; Nelson *et al.* 1994; Chang and Nelson 2000), as well as prolonged duration of antibiotic treatment (Pepin *et al.* 2004; Gaynes *et al.* 2004; Zimmerman 1991)

1.2.3.2 Increased age

Older age is also a well-recognised CDI risk factor (Ananthakrishnan 2011; Bartlett 2010; Carroll and Bartlett 2011; Bartlett and Gerding 2008). Most reports show a sharp increase in CDI incidence and mortality in those above 65 years old (Bassetti *et al.* 2012), with CDI rates up to 20-fold higher in this sub-population when compared to younger individuals (Pepin *et al.* 2005; Loo *et al.* 2005; Aronsson *et al.* 1984; Karlstrom *et al.* 1998). This could be due to a number of contributing factors; the elderly population is more likely to have increased comorbidity, increasing the probability of hospitalisation and antibiotic usage (Ananthakrishnan 2011). Immunosenescence may also play a role in an increased CDI incidence in the elderly population (Carroll and Bartlett 2011; Ananthakrishnan 2011).

1.2.3.3 Healthcare exposure

Recent healthcare exposure also poses a significant patient risk factor for CDI, whether this includes residence at long-term care facilities or short-term stay at acute-care hospitals (Bartlett 2006). *C. difficile* colonisation occurs in 20-40% of hospitalised patients compared to only 2-3% of normal, healthy adults (Heinlen and Ballard 2010; Bartlett 2006), which reflects the widespread contamination of hospital environments. The duration of hospital stay has also been identified as increasing CDI risk. One study reported a 4% increase in CDI risk with each additional day of hospitalisation (Debast *et al.* 2009), with a five-fold increase associated with hospitalisations lasting longer than one week (Pepin *et al.* 2005). Hospitalisation poses a major risk because it brings together multiple major issues, including exposure to antibiotics and a vulnerable, elderly population. Furthermore, the healthcare system provides a setting where highly infectious *C. difficile* spores are encountered (Bartlett 2010). This problem is exacerbated through sub-optimal hand hygiene of healthcare workers, resulting in the rapid transmission of CDI through the hospital environment (Depestel and Aronoff 2013).

1.2.3.4 Anti-toxin antibodies

A patient's ability to mount an adequate immune response against *C. difficile* may also be a significant risk for CDI development and recurrence (Rupnik *et al.* 2009). In adults, the ability to produce anti-toxin antibodies may be a key determinant of asymptomatic disease versus active infection (Ananthakrishnan 2011). Studies have illustrated that individuals who produce lower levels of immunoglobulin G (IgG) directed against *C. difficile* toxin A or B are more likely to develop CDI, and could also be at a higher risk of developing recurrent disease

(Kyne *et al.* 2000; Kyne *et al.* 2001). One such study, by Kyne and colleagues, illustrated that CDI patients with low IgG titres against *C. difficile* toxin A showed a 48-fold increased risk of developing recurrent disease (Kyne *et al.* 2001). In contrast, individuals who produce adequate levels of IgG are more likely to remain asymptomatic carriers of infection (Kyne *et al.* 2000).

1.2.3.5 Other risk factors

Immune compromised patients, including those undergoing chemotherapy or organ transplant, are at increased risk of CDI (Alonso and Marr 2013; Tsapepas *et al.* 2015; Chopra *et al.* 2010). Patients with underlying IBD have also been identified as high risk (Nitzan *et al.* 2013; Reddy and Brandt 2013; Berg *et al.* 2013). These groups of patients are more likely to experience disruption of colonic mucosal barrier function, dysbiosis of intestinal microbiota, immunosuppression owed to the use of drugs, as well as malnutrition, and it is thought that these all contribute to the increased risk of disease (Ananthakrishnan 2011).

Exposure to stomach acid-reducing agents, such as histamine receptor 2 (H_2) antagonists and proton pump inhibitors (PPIs), may also promote CDI. Gastric acid suppression is thought to promote the persistence of ingested *C. difficile* spores, therefore contributing to an increased CDI risk. Using a case-control study design, Diel and colleagues identified 1,672 CDI cases in the UK, and matched each case with 10 healthy controls. Multivariate analysis demonstrated that PPI use was associated with a three-fold increased risk of CDI (Odds ratio [OR] 2.9%, 95% confidence interval [CI] 2.4-3.4), with a lower but still significant increased risk associated with the use of H_2 antagonists (OR 2.0%, 95% CI 1.6-2.7) (Dial *et al.* 2005; Dial *et al.* 2004). There has been some question as to whether these agents

truly contribute towards CDI risk, or if there are confounding factors present due to underlying disease. However, there is accumulating evidence to support that PPIs in particular pose an increased risk of CDI, including data that shows a dose-dependent response (Howell *et al.* 2010; Biswal 2014).

1.2.4 Clinical presentation of CDI

C. difficile causes a broad spectrum of clinical symptoms, ranging from mild diarrhoea to severe and life-threatening illness (Ananthakrishnan 2011; Heinlen and Ballard 2010). Symptomatic patients most commonly present with *C. difficile*-associated diarrhoea (CDAD), which is sometimes accompanied by lower abdominal cramps (Goudarzi *et al.* 2014). Stools are usually watery with a characteristic foul odour, although mucoid or soft stools can also occur (Bartlett 2010; Bartlett and Gerding 2008). An individual with CDAD can experience more than 10 bowel movements per day (Ananthakrishnan 2011). Symptoms usually begin during or shortly after antibiotic therapy, but occasionally these may be delayed for several weeks (Goudarzi *et al.* 2014).

More severe forms of CDI include colitis and PMC. *C. difficile* colitis is characterised by abdominal cramps, fever, leukocytosis and severe high-volume diarrhoea which can have some trace blood. Colonic inflammation can be visualised by endoscopy (Bassetti *et al.* 2012; Bartlett and Gerding 2008). PMC is a systemic illness that has many causes; however, *C. difficile* is responsible for almost all cases. Patients experience abdominal pain and tenderness, fever and severe bloody diarrhoea. Fever and leukocytosis may also be severe in patients, with temperatures occasionally reaching 40°C and white blood cell counts over 20,000 cells/mm^3. Hypoalbuminemia is also common, which is the result of large protein losses attributable to leakage of albumin (< 3.0 g/dL albumin) (Bartlett and Gerding 2008).

PMC is characterised by inflammatory lesions with coalesce within the colon creating a pseudomembrane of immune cells, mucous and necrotic tissue. Endoscopic visualisation shows the presence of classic pseudomembranes, which appear as raised yellow plaques 2-10 mm in diameter scattered over the colorectal mucosa (Heinlen and Ballard 2010).

CDI presents as fulminant colitis in approximately 3% of patients and accounts for the majority of serious complications. These include bowel perforation, prolonged ileus, toxic megacolon and death (Cairns *et al.* 2012; Ananthakrishnan 2011). Fulminant CDI can also necessitate a total colectomy, which carries a high average mortality rate, occurring at 67% (Heinlen and Ballard 2010). Rising serum lactate levels (≥ 5 mmol/L) (Butala and Divino 2010), leukocytosis (≥ 35 x 10^4 white blood cells/µL) (Sailhamer *et al.* 2009; Henrich *et al.* 2009), acute renal failure (Khanna *et al.* 2013) and respiratory distress (Sailhamer *et al.* 2009) are also associated with poor prognosis and high mortality.

1.2.5 Diagnosis of CDI

Laboratory analysis of stool samples is the standard diagnostic test for CDI, and is recommended for adults and children (≥ 1 year old) who present with unexplained diarrhoea associated with recent antibiotic use (Bartlett and Gerding 2008). Several laboratory tests have been developed for the diagnosis of CDI.

The cell cytotoxicity neutralisation assay (CCNA) has historically been considered as the gold standard for CDI diagnosis (Iv *et al.* 2014). In this assay, faecal filtrates are evaluated for their ability to induce toxin-induced cytopathic cell rounding. If a cytopathic effect is observed, neutralisation with an antiserum, either *C. sordellii* antitoxin or *C. difficile* antitoxin,

is performed (Carroll and Bartlett 2011; Iv *et al.* 2014). Alternatively, toxigenic culture of CDI detection consists of isolating *C. difficile* from stool samples using selective media, followed by further testing by enzyme immunoassay (EIA) or CCNA methods to confirm *C. difficile* toxin production. These tests have proven superior in terms of their specificity and sensitivity, but they are labour intensive and have a long turnaround time (Iv *et al.* 2014). As a result, their application is now considered limited in routine diagnostic testing. However, toxigenic culture in particular is essential to allow for strain typing in *C. difficile* epidemiology studies following an outbreak, and antibiotic susceptibility testing (Bassetti *et al.* 2012).

EIAs represent the most widely used clinical diagnostic tests (Iv *et al.* 2014). These rapid and relatively inexpensive tests use polyclonal or monoclonal antibodies to directly detect the presence of *C. difficile* toxins. Initial toxin EIAs were limited and had suboptimal sensitivity as they detected TcdA only. Newer EIAs have been developed to detect both toxins. Commercial EIAs produced by several different manufacturers generally show high specificity (90-95%), but low to moderate sensitivity, lying in the 60-90% range. Therefore, these tests are not recommended for standalone use and are often used in two or three-step diagnostic algorithms (Planche *et al.* 2008). Other EIAs detect the present of the common *C. difficile* antigen glutamate dehydrogenase (GDH), which have higher sensitivity (90-95%) but lower specificity (85-90%) than toxin EIAs (Shetty *et al.* 2011).

A more recently available test for CDI is a real-time PCR nucleic acid amplification test (NAAT) that detects *C. difficile* toxin genes (i.e. *tcdA* and *tcdB* that encode TcdA and TcdB, respectively). This test has a rapid turnaround time with a high sensitivity (90%) and specificity (96%) (Deshpande *et al.* 2011). Two NAATs have now been approved for use by the Food and Drug Administration (FDA), but their role in routine clinical practice might require further

evaluation (Carroll and Bartlett 2011). For example, NAATs target DNA encoding *C. difficile* toxins and not the toxins themselves, hence they do not provide evidence that the toxins are actually being expressed *in vivo* (Gilligan 2015). NAATs should therefore be associated with a more specific test that directly detects the expression of *C. difficile* toxins (such as EIAs or toxigenic culture) (Bassetti *et al.* 2012).

At present, no single commercially available test offers both high sensitivity and specificity in combination with rapid turnaround time and low cost. To more effectively distinguish between CDI patients and those without infection, two- or three-step diagnostic algorithms have been developed (Bassetti *et al.* 2012). Indeed, a two-step protocol is currently used in NHS laboratories in England, which comprises an EIA for GDH detection or NAAT, followed by a toxin EIA (Wilcox 2012).

1.2.6 Treatment of CDI

1.2.6.1 Antibiotics

Current treatment guidelines for CDI include supportive care and withdrawal of implicated antibiotics, as well as administration of antimicrobial therapy (Lo Vecchio and Zacur 2012). Metronidazole (250 mg every 6 hours or 500 mg every 8 hours orally) is recommended for treatment of mild-moderate CDI (Leffler and Lamont 2009). Vancomycin is administered to patients with severe CDI or to those who cannot tolerate metronidazole (Leffler and Lamont 2009). Originally, limited use of vancomycin was recommended to prevent the emergence of vancomycin-resistant enterococci (VRE) (Leffler and Lamont 2009). However, studies have shown that metronidazole also selects for VRE (Donskey *et al.* 2000;

Carmeli *et al.* 2002), and in fact, VRE colonisation rates appear similar in patients treated with vancomycin and metronidazole (Al-Nassir *et al.* 2008).

Despite initial response rates of greater than 90% (Wilcox and Howe 1995; Fekety *et al.* 1989), 15-30% of patients experience a relapse in symptoms following successful antibiotic therapy (Fekety *et al.* 1997). Patients who experience a first relapse are retreated with metronidazole or vancomycin (same agent as used originally) (Leffler and Lamont 2009; Heinlen and Ballard 2010), however, up to 50% of these patients go on to experience multiple relapses (Fekety *et al.* 1997). Recently, the number of treatment failures in response to metronidazole have also increased (Aslam *et al.* 2005), suggesting that alternative treatment options are needed to effectively combat CDI.

1.2.6.2 Probiotics

Probiotics are defined as living organisms that confer a health benefit to the host (de Vrese and Schrezenmeir 2008). There is increasing evidence to support the use of probiotics in a range of gastrointestinal diseases, but their role in the treatment and prevention of CDI is unclear (Martin *et al.* 2013; Evans and Johnson 2015). *In vitro* and *in vivo* animal experiments suggest that probiotics such as Lactobacilli and *Saccharomyces boulardii* exhibit inhibitory activity against *C. difficile* (Trejo *et al.* 2006; Castagliuolo *et al.* 1999), and can also modulate the host response (Chen *et al.* 2006; Qamar *et al.* 2001). However, their clinical application is limited by a lack of clinical evidence. A Cochrane review in 2012 (Johnston *et al.* 2012) analysed 20 randomised controlled trials including adult or pediatric patients receiving antibiotics. These trials evaluated the efficacy of any strain or dose of a probiotic in

reducing CDI incidence rates, compared with placebo or no treatment control. Probiotics were shown to reduce incidence of CDI by 66% (pooled relative risk [RR] of 0.34; 95% CI, 0.24-0.49), with no significant adverse effects reported (Johnston *et al.* 2012). However, a recent large, randomised, double-blind, placebo-controlled trial (PLACIDE trial) showed that a multi-strain preparation of Lactobacilli and Bifidobacteria showed no benefit when compared to placebo at preventing AAD or CDI (Allen *et al.* 2013)

There is therefore a lack of sound evidence to support the role of probiotics in the treatment and prevention of CDI, and as such, they are not currently recommended for use (Martin *et al.* 2013; Dickinson and Surawicz 2014). Moreover, treatment with probiotics represents a potential risk factor for fungemia in immunodeficient or critically ill patients (Munoz *et al.* 2005).

1.2.6.3 Immunotherapy

Previous studies have indicated that an inadequate antibody response against *C. difficile* toxins might contribute towards an increased risk of symptomatic and recurrent infection (as already described in *Section 1.2.3.4*). Passive immunity against *C. difficile* toxins might therefore protect against infection. As such, monoclonal antibodies (MAbs) directed against *C. difficile* toxins have been evaluated as an alternative CDI treatment option. Experimental hamster models have demonstrated that MAb administration reduces the severity and duration of diarrhoea, death rate and rate of recurrence following *C. difficile* innoculation (Davies *et al.* 2013). A randomised, double-blind Phase II placebo-controlled trial of two novel, fully human MAbs against *C. difficile* TcdA (CDA1) and TcdB (CDB1) was also able to demonstrate a lower CDI recurrence rate with adminstration of a single infusion of 10 mg/kg

MAb compared to placebo in patients receiving either metronidazole or vancomycin. Recurrent infection developed in 7% of the MAb group compared with 25% of the placebo group, reflecting an RR of 72% (95% CI: 7-29, P < 0.001) (Lowy *et al.* 2010).

However, a number of questions remain regarding the application of MAb therapy to treat CDI. Whilst clinical trials have demonstrated that MAb administration reduces CDI recurrence rates, it has no effect on the duration or severity of diarrhoea (Lowy *et al.* 2010; Safdar 2010). In addition, disease progression varies considerably between different patient populations, i.e. the elderly, so the clinical application of MAb therapy across these populations needs to be further investigated (Parks *et al.* 2010). Additional Phase III human studies now currently underway might provide further clarification to the role of MAb therapy as an effective CDI treatment option (Iv *et al.* 2014; To and Napolitano 2014).

Also likely to emerge in the future is the application of new MAbs (PA-50 and PA-41) that specifically bind to epitopes in the neutralising regions of TcdA and TcdB. These have shown promise in experimental animal models, where the dual administration of PA-50/PA-41 dramatically increased survival rate in a hamster CDI model (long term survival rate of 95% for MAb versus 0% for placebo) (Marozsan *et al.* 2012). In addition, intravenous immunoglobulin (IVIG) prepared from pooled human serum has been studied with respect to both recurrent and severe CDI, but has yielded inconsistent results (Juang *et al.* 2007; Abougergi *et al.* 2010).

1.2.6.4 Fecal microbiota transplantation

Faecal microbiota transplantation (FMT) involves the instillation of the faecal microbiota from a healthy donor into the gut of a CDI patient, usually administered by nasogastric tube, endoscope or enema (Weissman and Coyle 2012). FMT allows the reconstitution of the normal intestinal microbiota, and an increasing number of studies have reported that it is an effective treatment for recurrent CDI (Monier and Barbut 2014). Indeed, case series and systematic reviews of published studies have reported cure rates of more than 90% with no serious adverse effects (Gough *et al.* 2011; Brandt *et al.* 2012; Kelly *et al.* 2012; Kassam *et al.* 2012; Bakken *et al.* 2011). Similar results were also observed in a successful randomised, controlled trial by van Nood and colleagues (van Nood *et al.* 2013). In this study, 94% patients experienced resolution of CDI following an infusion of donor faeces through a nasoduodenal tube, compared to only 31% patients receiving vancomycin alone and 23% patients receiving vancomycin with bowel lavage (van Nood *et al.* 2013).

Due to such promising results regarding its efficacy and safety, FMT has now been approved by the National Institute for Health and Care Excellence (NICE) for its use to treat recurrent CDI, however, the procedure should only be considered for recurrent CDI patients that are non-responsive to antibiotics and other treatments (NICE 2014). It is also worth noting that some concerns exist regarding the long-term outcomes of FMT, as well as the lack of standardardised procedure for donor selection (Borgia *et al.* 2015). As such, further research into FMT is encouraged to establish optimal dosage, mode of administration and choice of donor (NICE 2014).

1.2.7 Animal models of CDI

The continued development of alternative therapeutics is essential for the treatment and prevention of CDI. This, alongside the need to increase the understanding of *C. difficile* pathogenesis, has meant that a number of *C. difficile* experimental animal models have been developed. Indeed, many different animal models have been used to study CDI, including small animals such as hamsters, mice, rats, rabbits, hares, guinea pigs, prairie dogs and quails. In addition, a limited number of studies have utilised larger animals including foals, piglets and monkeys. Zebrafish embryos have also recently been used to study the action of *C. difficile* toxins (Best *et al.* 2012).

Hamster models, in particular Syrian hamsters, have been most extensively utilised for the study of CDI (Best *et al.* 2012). This is because CDI in hamsters mirrors some of the recognised pathophysiological features of CDI observed in humans. Similar to human CDI, infection in hamsters must be induced by disrupting the microbiota through the administration of antibiotics (usually clindamycin), followed by challenge with toxigenic *C. difficile* strains (Hutton *et al.* 2014). This results in an overall deterioration of the animal, alongside changes in the appearance of the gastrointestinal tract, particularly the colon and cecum. Redness, inflammation, fluid accumulation and enlargement of the colon are usually observed, accompanied by a decrease in gut motility (Best *et al.* 2012).

However, certain clinical features of CDI in hamsters differs from that in humans. One potential drawback is that hamsters do not typically develop diarrhoea. Instead, they present with 'wet tail', in which the hamster exhibits lethargy, ruffled fur, hunching, irritability and the refusal of food, which ultimately leads to death (Best *et al.* 2012). Indeed, if left untreated, disease is rapidly fatal in hamsters, and thus, the endpoint of any experiment is the survival

rate in days. Therefore, the hamster model is essentially a prevention of death model (Best *et al.* 2012). This rapidly fatal disease pattern is not characteristic of human CDI. Nevertheless, hamsters are an important and relevant model of *C. difficile* disease, and their main uses have included toxin and strain characterisation, evaluation of new treatment stategies and different diets, colonisation studies, investigation into virulence factors and characterisation of immune responses (Best *et al.* 2012; Hutton *et al.* 2014).

In addition, three mouse models are being increasingly utilised to study CDI. These include the gnotobiotic/germ-free mouse model, the antibiotic cocktail mouse model and the single antibiotic mouse model of CDI (Hutton *et al.* 2014; Lawley and Young 2013).

1.3 ENTEROTOXIGENIC *E.COLI* INFECTION – AN ACUTE DIARRHOEAL DISEASE

Enterotoxigenic *E. coli* (ETEC), one of six recognised diarrheagenic *E. coli*, is a Gram-negative bacterium that colonises the small intestine. ETEC is characterised by the production of one or both of two enterotoxins; heat-labile toxin (LT) and heat-stable toxin (ST), both of which lead to fluid secretion from small intestinal epithelial cells and subsequent diarrhoea (Clements *et al.* 2012). Human ETEC infections occur through consumption of contaminated water and food products or via direct person to person spread from poor hand hygiene (Berger *et al.* 2010; Clements *et al.* 2012).

ETEC was discovered more than 50 years ago when De and colleagues isolated pure *E. coli* culture from patients with symptoms typical of cholera, caused by *Vibrio cholerae*. These *E. coli* isolates also produced a strong cholera-like secretory response in rabbit ileal loops, with similar results found in other studies (De *et al.* 1956). *In vivo* studies also demonstrated

that these *E. coli* strains were responsible for diarrhoeal disease in several animal species (Carlton and Barnum 1969; Smith and Halls 1967) which was confirmed by oral challenge of human volunteers (DuPont *et al.* 1971; Levine *et al.* 1979). Since its initial discovery, there has been a concerted effort to characterise the previously unknown ETEC enterotoxins and virulence factors. Today, ETEC is recognised as a common cause of diarrhoea in adults and children in developing countries, and a major cause of traveller's diarrhoea in people visiting or returning from endemic regions (Qadri *et al.* 2005).

1.3.1 Pathogenesis of ETEC infection

The pathogenesis of ETEC infection can be divided into two important stages; effective colonisation of the intestinal epithelium, followed by successful release of its toxins.

1.3.1.1 Colonisation of the intestinal epithelium

Colonisation of the intestinal epithelium represents an essential step in the pathogenesis of ETEC infection. This is mediated by plasmid-encoded fimbrial colonisation factors (CFs), which bind to enterocytes in the small intestine, allowing the expression of enterotoxins ST and LT in close proximity to the intestinal epithelium (Fleckenstein *et al.* 2010). Studies have also demonstrated that fimbrial CFs possess lectin activity. For example, F17 fimbriae present on *E. coli* K99 exhibits carbohydrate-binding specificity and binds to surface glycolipids containing N-glycolylneuraminic acid (NeuGc) in the form of NeuGcα2-3Galβ4Glc (Sharon 2006). CFs are considered to be key virulence factors as illustrated by a number of *in vivo* animal and human studies which have shown that CF-positive bacteria, but

not spontaneous CF-negative derivatives, colonise the intestinal mucosa and induce diarrhoea (Qadri *et al.* 2005; Evans *et al.* 1978; Levine *et al.* 1979; Svennerholm *et al.* 1990) .

To date, over 25 unique CF types and putative colonisation factors have been characterised, which can be subdivided into five groups based on their genetic and structural homologies (**Table 1**)(Sizemore *et al.* 2004).

Table 1.1. Colonisation factors found in human ETEC strains. Adapted from (Sizemore *et al.* 2004).

Group	Group Members	Characteristics
CFA/1	CFA/1, CS1, CS2, CS4, CS14, CS17, CS19	Highly genetically related. Fimbriae composed of a single structural subunit. Subunit at the tip contains adhesive properties, remaining subunits non-adhesive.
CS5	CS5, CS7, CS13, CS18, CS20	Sequence similarities to fimbriae of animal ETEC strains (particularly porcine isolates)
Bundle-forming	CS8, CS21	Most closely related to Type IV pili expressed by EPEC. Longest pilus >20 μm long.
CS15	CS15, CS22	Antigenically cross-reactive. Closely related to SE14 fimbriae of *Salmonella enteriditis*
Distinct	CS3, CS6, CS10, CS11, CS12	No known homology with each other or any known fimbriae

Whilst there is significant regional variation in CF expression profiles, epidemiological studies have suggested that the most prevalent CFs include CFA/1, CS1-CS7, CS14, CS17 and CS21, which are expressed either alone or in combination (Gaastra and Svennerholm 1996; Isidean *et al.* 2011; Qadri *et al.* 2005). It is worth noting that on approximately 30-50% of ETEC strains worldwide, there are no known CFs detected. This could be due to the true absence of CFs, to loss of CF properties in subculturing, or to a lack of specific tools for their detection (Qadri *et al.* 2005; Isidean *et al.* 2011). Two other factors are also ultilised to characterise ETEC; O serogroups and H serogroups, associated with antigens found on cell wall lipopolysaccharides (LPS) and flagella, respectively (Fleckenstein *et al.* 2010; Qadri *et al.* 2005; Wolf 1997).

1.3.1.2 Release of toxins

Following the successful colonisation of the intestinal epithelium, ETEC mediates pathogencity through the release of at least one of two separate plasmid encoded enterotoxins; LT and ST.

LT (84 kDa) has a similar homology and structure to the cholera toxin, as well as a similar mode of action. Like the cholera toxin, LT is a heterohexameric molecule, composed of an active (A) subunit surrounded by pentameric binding (B) subunits (Fleckenstein *et al.* 2010). Following colonisation and LT secretion, LTB subunits bind irreversibly to GM1 gangliosides on the surface of intestinal epithelial cells whilst internalised LTA activates adenylate cyclase, resulting in an increase of intracellular cyclic adenosine monophosphate (AMP). Subsequent activation of protein kinases then stimulates chloride secretion at crypt cells through the

cystic fibrosis transmembrane regulator channel (CFTR), whilst inhibiting neutral sodium chloride absorption at the villus tips. Ultimately, this leads to a net loss of salt and water into the intestinal lumen, and when these actions exceed the absorptive capacity of the bowel, a purging of watery diarrhoea ensues (**Figure 1.4**) (Fleckenstein *et al.* 2010; Qadri *et al.* 2005).

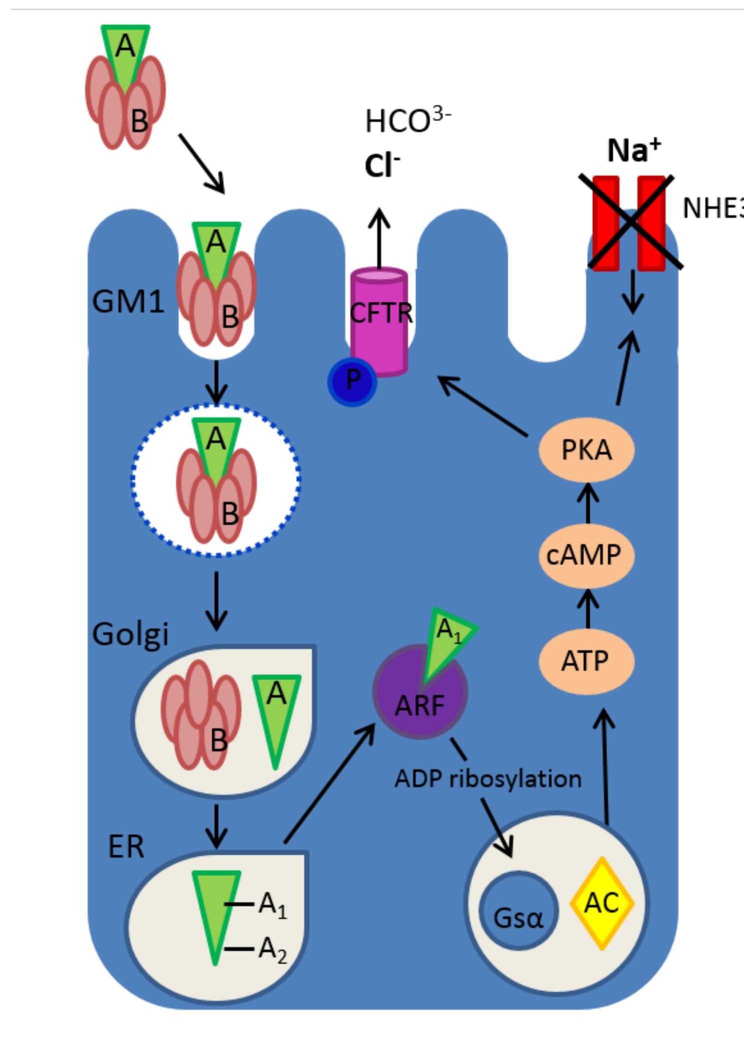


Figure 1.4. Summary of the mechanism of ETEC heat-labile (LT) enterotoxin. Adapted from (Dubreuil 2012).

ST is a nonantigenic low-molecular weight peptide consisting of 18-19 amino acids, and whilst it works independently of LT, it produces a similar physiological effect (Clements *et al.* 2012; Fleckenstein *et al.* 2010). Released in the small intestine, ST binds irreversibly to guanylate cyclase resulting in increased levels of cyclic guanosine monophosphate (GMP). As with LT, this results in increased chloride secretion at crypt cells with inhibition of neutral sodium chloride absorption which leads to an outpour of watery diarrhoea (Fleckenstein *et al.* 2010; Qadri *et al.* 2005)

1.3.2 Epidemiology of ETEC infection

1.3.2.1 Incidence rates

Accurate figures of the incidence of enteric *E. coli* infections are difficult to determine as the causative agents of diarrhogenic infections are often not identified. However, ETEC is reported to be the most commonly isolated enteropathogen in children under 5 years of age in developing countries, accounting for approximately 20% of cases (Qadri *et al.* 2005). It is estimated that 280 million - 400 million ETEC-associated diarrhoea cases occur annually each year in children under five years, with a further 100 million cases in children over 5 years of age, resulting in approximately 300,000 – 500,000 deaths annually (Zhang and Sack 2012). ETEC is also the most common cause of traveller's diarrhoea, accounting for 10-60% of infections depending on the region visited (Clements *et al.* 2012; Gascón *et al.* 1998; Black 1990). Extrapolation of these figures suggests that there may be approximately 10 million cases of traveller's diarrhoea caused by ETEC each year (Clements *et al.* 2012).

1.3.2.2 Relation to presence of LT, ST and colonisation factors

ETEC expressing LT only are more frequently isolated from healthy individuals than from symptomatic patients, and are therefore considered less pathogenic (Qadri *et al.* 2005). This could be related to the low prevalence of CFs expressed on LT-producing ETEC strains when compared with ST- and LT/ST-producing strains (<10% versus >60%, respectively) (Gaastra and Svennerholm 1996; McConnell *et al.* 1991). Indeed, predominant CF types (as discussed in **Section 1.3.1.1**) are also more commonly found on ST- and LT/ST-producing ETEC strains (Qadri *et al.* 2005). Perhaps unsurprisingly, clinic-based studies have also reported that ST- and LT/ST-producing strains cause more serious disease than their LT-expressing counterparts (Qadri *et al.* 2000).

1.3.3 Risk factors of ETEC

1.3.3.1 Age

Infants and young children are much more susceptible to ETEC infection, as evidenced by high incidence rates in this age group. Studies over recent years have demonstrated that ETEC is a frequent cause of diarrhoea in infants younger than 2 years of age (Qadri *et al.* 2000; Rao *et al.* 2003; Steinsland *et al.* 2002). For example, 90% of ETEC cases reported to Bangladeshi hospitals were accounted by children aged 3 months to 2 years old (Qadri *et al.* 2000). The incidence of ETEC infections in developing countries decreases between the ages of 5- to 15 years. Incidence increases again in those over 15 years old, with approximately 25% of infection seen in adults (Merson *et al.* 1980; Qadri *et al.* 2000). Whilst limited epidemiological information exists regarding adult infection, hospitalised adults often present

with more severe forms of ETEC diarrhoea than children and infants (Qadri *et al.* 2005). Further analyses have also indicated that the elderly are also susceptible to more severe ETEC infection requiring hospitalisation, with ETEC identified as the second most frequently isolated (13%) bacterial pathogen after *V. cholerae* (20%) in patients over 65 years of age (Faruque *et al.* 2004). Some explanation as to why ETEC incidence varies between different age groups might be from results obtained from experimental animal models, which indicated indicated age-dependent changes in the presence of intestinal cell surface receptors for *E. coli* K99 fimbrial antigen (Runnels *et al.* 1980).

1.3.3.2 Geographical location

ETEC infection is predominantly confined to developing countries where water supplies and sanitation are inadequate (Clements *et al.* 2012). Surface waters in developing countries, such as Bangladesh, have been found to harbour high levels of ETEC (Begum *et al.* 2005; Ohno *et al.* 1997) As such, ETEC transmission commonly occurs while bathing and/or using this water for food preparation. Studies in communities where personal hygiene, education and living conditions are poor have also shown that infection can spread rapidly within family groups. In one study, it was shown that ETEC spread to 11% of contacts within a 10-day study period (Black *et al.* 1981). Mothers of the family have also been identified as potential resevoirs of infection as they are primary food handlers (Black *et al.* 1981).

In visitors to developing countries, the phenotypes of ETEC strains exhibit country-country variation. For example, LT-producing ETEC was more commonly isolated from visitors to Jamaica (58%) (Jiang *et al.* 2002), LT/ST-producing strains most often seen in visitors to

India (45%) (Jiang *et al.* 2002), whilst ST-producing ETEC was most frequently isolated from visitors to Kenya (51%) (Shaheen *et al.* 2003). Thus, strains that are predominant in a particular country, infecting children and contaminating water and food sources, are likely to determine the particular ETEC strain infecting the travellers (Qadri *et al.* 2005).

1.3.3.3 Malnutrition

Pre-existing malnutrition is considered a risk factor for ETEC infection, and studies have found that it can lead to more severe infection in children (Mathur *et al.* 1985). This might be due to the immunocompromised nature of the host, which might predispose the individual to a greater bacterial load (Brown 2003). Micronutrient deficiency, such as vitamin A and zinc, is also common in developing countries. Indeed, it has been estimated that 40% of Bangladeshi children under the age of 5 years may have a zinc deficiency (Qadri *et al.* 2004; Sarker *et al.* 1985), which has been associated with higher rates of morbidity due to diarrhoeal illness (Rahman *et al.* 2001; Raqib *et al.* 2004).

1.3.4 Clinical presentation of ETEC infection

ETEC infections are classically associated with acute watery diarrhoea, which can range from mildly symptomatic to severe profuse diarrhoea (Fleckenstein *et al.* 2010). Studies have also revealed the existence of short term, assymptomatic carriers of the bacterium (Qadri *et al.* 2005). As the pathophysiology of ETEC diarrhoea is essentially the same as that caused by *V. cholerae*, the two infections cannot be distinguised on clinical grounds (Sack *et al.* 1977). A sudden onset of watery stool leads to the loss of fluids and electrolytes, resulting

in rapid dehydration within a few hours (Finkelstein *et al.* 1976; Sack 1975). Other signs and symptoms of ETEC infection include headache, nausea and vomiting (Fleckenstein *et al.* 2010). Usually diarrhoea lasts only 3-4 days and is self-limited, however, some patients may have prolonged diarrhoea lasting a week or more (Qadri *et al.* 2005; Fleckenstein *et al.* 2010). In these cases, patients are at risk of severe dehydration which results in a dry mouth, rapid pulse, lethargy, decreased skin turgor, decreased blood pressure, muscle cramps and eventually shock in most severe froms, which can be fatal (Centre for Disease Control and Prevention 2004).

1.3.5 Diagnosis of ETEC infection

As ETEC infection is mediated by the release of its enterotoxins, effective diagnosis of disease has long relied on detection of either LT and/or ST. ST was initially detected using the rabbit ileal loop model (De *et al.* 1956), and subsequently the suckling mouse assay (Dean *et al.* 1972). The traditional bioassay for LT involves the use of cell culture, whereby treatment of Y1 adrenal cells and Chinese hamster ovarian (CHO) cells with LT results in LT-specific morphological changes which can be neutralised by antitoxin (Donta and Smith 1974; Guerrant *et al.* 1974). These diagnostic tests were considered gold standard until the development of simpler molecular immunoassays.

During recent years, DNA probes with either radioactive or non-radioactive labelling have become widely used to detect the presence of LT and ST genes. This method has both high sensitivity and specificity, and is useful in detecting ETEC both in clinical and environmental samples (Echeverria *et al.* 1982; Hill *et al.* 1983). Other commonly used

diagnostic tests include multiplex PCR which can simultaneously detect LT- and ST- producing strains, as well as other diarrheagenic *E. coli* (Stacy-Phipps *et al.* 1995; Toma *et al.* 2003; Vidal *et al.* 2004). EIAs that use monoclonal antibodies to detect the presence of ST and LT are also widely used (Qadri *et al.* 2005). DNA probes, EIAs and PCR methods can also be applied to detect the presence of known CFs (Qadri *et al.* 2005; Steinsland *et al.* 2003).

In spite of these available techniques, which are amenable to clinical laboratories in the Western world, there are still no simple, readily available diagnostic tests that can be performed effectively in minimally equipped laboratories. As such, many laboratories conducting research of diarrhoea in endemic countries do not include ETEC in their routine diagnostic testing, and instead, special referral laboratories are required to identify these bacteria. This explains the lack of epidemiological data regarding ETEC infection (Qadri *et al.* 2005).

1.3.6 Treatment of ETEC infection

1.3.6.1 Vaccine development

There is much interest in the development of vaccines for the prevention of ETEC disease. However, ETEC are extremely heterogenous in their nature, and at present, there is no broadly protective vaccine for ETEC suitable for public health use (Zhang and Sack 2012). An ideal broad-coverage ETEC vaccine should contain fimbrial antigens representative of the most prevalent ETEC groups. Unfortunately, the large diversity of ETEC serotypes, particularly with regard to O and H antigens, renders such antigens less attractive as vaccine components (Zhang and Sack 2012). Instead, vaccines have focussed on combining the most prevalent CFs

to achieve broad coverage. CFs are often combined with either cholera toxin or LT toxoids, such as non-toxigenic B subunit LTB or LT mutants, which retain both their anti-toxin and inherent adjuvant properties (Fleckenstein *et al.* 2014). It has been postulated that a multivalent vaccine containing CFA/I and CS1-CS6 in conjunction with an LT toxoid might provide protection against 80-90% of ETEC strains in most geographic areas (Qadri *et al.* 2005). A number of different strategies have been evaluated for the delivery of ETEC fimbrial antigens and toxoids to the human immune system, which include killed whole cell ETEC vaccines (Savarino *et al.* 1998; Tobias and Svennerholm 2012) and live attenuated oral ETEC vaccines (Turner *et al.* 2011), as well as vaccines that contain pure CF and enterotoxoid preparations (Katz *et al.* 2003). To date, these approaches have not yielded a broadly protective vaccine and further research is therefore needed to accelerate development (Fleckenstein *et al.* 2014).

1.3.6.2 Antimicrobials

The use of antibiotic therapy to treat ETEC infection is effective. Indeed, several studies have illustrated that it can reduce both the duration and severity of ETEC-mediated diarrhoea (Black *et al.* 1982; Merson *et al.* 1980). However, in endemic regions, the application of antimicrobial therapy is limited by the lack of simple laboratory tests in place to rapidly diagnose ETEC infection. In regions where sanitation and water supplies are inadequate, diarrhoeal disease can result from infection with a range of different bacterial and viral agents, which all present with similar clinical symptoms (Baldi *et al.* 2009). Furthermore, effective agents might not be widely available where incidence rates are high. Thus, antibiotic treatment is not amenable to most cases of ETEC infection, and instead, oral rehydration therapy is considered more important in managing the disease (Nataro and Kaper 1998).

Antibiotic therapy is more appropriate in the treatment of traveller's diarrhoea, of which ETEC is known to be the most frequent pathogen. The antimicrobial treatment of diarrhoea has changed over the years due to increasing antibiotic resistance of ETEC strains (Nataro and Kaper 1998). At present, it is recommended that adult travellers presenting with ETEC infection undergo a short course of ciprofloxacin (500 mg every 12 hours for one day) or rifaximin (200 mg twice a day for three days) (Hong and Kim 2011). Azithromycin is also used to treat ETEC infection in children (10 mg per kg per day for two days) (Qadri *et al.* 2005). However, if traveller's diarrhoea occurs whilst the individual is still visiting the affected region, antimicrobial treatment is usually not given because diagnosis is not easily made and there may be a low availability of microbial agents (Nataro and Kaper 1998). Therefore, travellers should also be aware of the importance of oral rehydration solutions in managing infection.

1.3.6.3 Nutritional therapy

Studies have indicated that malnutrition is a likely predisposing factor to ETEC infection. Nutritional therapy is therefore an integral part of ETEC treatment, particularly with respect to children and infants. Indeed, it has been shown that breastfeeding decreases the severity and incidence of ETEC infections (Clemens *et al.* 1997; Long *et al.* 1994), which might be due to the presence of secretory immunoglobulin A (IgA) antibodies to CFs and enterotoxins in mothers' breast milk from endemic countries (Cruz *et al.* 1991; Long *et al.* 1994). Whilst not yet studied directly in ETEC, studies have also demonstrated that zinc supplementation increases the adaptive immune response to cholera vaccination in children and adults (Albert

et al. 2003; Karlsen *et al.* 2003; Qadri *et al.* 2004), as well as in children with shigellosis (Raqib *et al.* 2004).

1.3.6.4 Rehydration therapy

As patients infected with ETEC associated diarrhoea often suffer with dehydration, which can be fatal, rehydration is extremely important. Patients suffering dehydration are administered with oral rehydration solutions, which are used until the diarrhoeal episode ceases. For patients with severe dehydration, intravenous fluids are administered until symptoms disappear which is then followed by oral rehydration therapy (Centre for Disease Control and Prevention 2004) . However, it must be remembered that rehydration therapy does not actually treat the cause of infection, but instead only manages the symtoms of disease.

1.3.6.5 Improved sanitation and drinking water

The long term prevention of ETEC is clearly related to improvements in sanitation systems and water supply, meaning that individuals would be less likely to consume contaminated food products and water. However, it is likely to take a long time to implement such drastic improvements on a large scale basis. For example, it has been estimated that it would take US$200 billion to make the improvements neccesary to prevent faecally spread diseases in South America alone (Qadri *et al.* 2005). Other improvements are taking place, but on a much smaller scale. These include building safe-water tube wells, building or improving latrines, as well as the chlorination, filtration or heating of drinking water (Quick *et al.* 1996). These attempts to prevent transmission are effective, but only at a local scale. Therefore, it is

of great importance to develop alternative therapeutic treatments to reduce the public health burden from ETEC.

1.3.7 Animal models of ETEC infection

It has been suggested that the development of vaccines and other therapies to treat human ETEC infection has largely been hampered by a lack of viable small-animal models. A number of animal models have been used in previous studies of ETEC, which include infant and adult mice, rat, rabbits and pigs (Allen *et al.* 2006). However, major drawbacks have been reported in these animal models, such as difficulties in utilisation, as well as animals requiring anaethesia and/or significant surgical manipulation. In addition, some animal models have not been thoroughly evaluated for their viability (Allen *et al.* 2006).

1.4 ULCERATIVE COLITIS – A CHRONIC DIARRHOEAL DISEASE

Alongside Crohn's disease (CD), UC is a major form of IBD, characterised by chronic inflammation that extends proximally from the rectum. First characterised in the 1800s, UC is a cause of significant morbidity worldwide and its incidence and prevalence appears to be increasing with time. Patients with UC typically experience episodes of bloody diarrhoea with or without mucus, abdominal pain, fever and weight loss (Arors and Shen, 2015). Due to the relatively unknown aetiology of UC, as well as its high risk of recurrence and poor prognosis, the disease is a clinical challenge in terms of treatment.

1.4.1 Pathogenesis of UC

UC is thought to result from an aberrant intestinal immune response to bacterial microbiota in a genetically susceptible host (Cooney and Jewell 2009). The exact pathophysiology of UC is not clearly understood, however, it is likely that a complex interaction of genetic, environmental and microbial factors play a role in disease development and progression.

1.4.1.1 Genetic factors

Large scale population studies and twin studies have suggested that there is a significant heriditary component in IBD. However, these studies suggest that such genetic influences might play a greater role in CD than in UC. The relative risk of developing IBD for first-degree relatives of a CD patient is estimated to be 5% in non Jewish and 8% of Jewish patients, whilst the corresponding relative risk of UC is lower, at 1.6% and 5.2%, respectively (Yang *et al.* 1993). Similar results have been found in twin studies, which have reported stronger concordance rates with CD (20-50% for monozygotic twins, 10% for dizygotic twins) than with UC (16% and 4%, respectively) (Halfvarson *et al.* 2003; Orholm *et al.* 2000; Thompson *et al.* 1996).

Due to advances in genetic testing and analysis technologies, a large number of IBD-associated susceptibility loci have now been identified. Thus far, there are a total of 163 IBD-associated loci, of which 110 are associated with both diseases (Waterman *et al.* 2011; Jostins *et al.* 2012), as well as 30 loci specific to CD and 23 loci specific to UC (Zhang and Li 2014; Thompson and Lees 2011). Interestingly, the three strongest gene associations reported are CD-specific (*NOD2/CARD15*, *ATG16L1* and *IRGM*), again suggesting that genetic factors might

play a more important role in CD pathogenesis (Cho 2008). Despite this, a number of UC specific loci have been implicated in dysfunction of the intestinal mucosal barrier, which include *ECM1*, *HNF4A*, *CHD1* and *LAMB1* (Thompson and Lees 2011). Several other susceptibility loci linked with immune system-mediated diseases are also associated with UC, particularly *HLA-DR* and genes that encode cytokine and inflammatory markers, such as IL10, IL7R, IL23R and IFN-ϒ(Danese and Fiocchi 2011; Thompson and Lees 2011).

1.4.1.2 Environmental factors

Whilst studies have identified that genetic factors increase an individual's predisposition to UC, it appears this alone is not sufficient for the onset of inflammation. Indeed, evidence suggests that numerous environmental factors also play an important role in disease pathogenesis (Ananthakrishnan 2015; Molodecky and Kaplan 2010). Smoking remains the most widely studied trigger of IBD, with surprising results. Studies have demonstrated that whilst smoking contributes to a two-fold increased risk of CD and greater likelihood of aggressive disease (Cosnes 2004; Cosnes 2008), it confers protection against UC (Higuchi *et al.* 2012; Mahid *et al.* 2006). The effect of diet on the development of IBD remains a challenging association to monitor due to its time-varying nature, as well as the difficulty in tracking it through the course of childhood and adult life (Ananthakrishnan 2015). However, several large cohort studies in the United States and Europe have shown that diet is likely to be an important IBD factor. In a large prospective cohort study including 170,776 female registered nurses, followed over 26 years, it was shown that intake of fruit fibre in the highest quintile (median 24 grams per day) was associated with a significant reduction in CD risk (HR 0.59, 95%, CI 0.39-0.90), however, this had no effect on UC (Ananthakrishnan *et al.* 2013). In

addition, it has been demonstrated that a diet high in long-chain poly unsaturated fatty acids (PUFA) is associated with a reduced risk of UC (HR 0.72, 95% CI 0.51-1.01) whilst high trans-unsaturated fatty acid intake is associated with a greater UC incidence risk (HR 1.34, 95% CI 0.94 – 1.92). However, neither total fat nor specific fatty acid intake has been reported to have an effect on the incidence of CD (Ananthakrishnan *et al.* 2014). Studies have also shown that Vitamin D deficiency is common in IBD patients, and is associated with an increased susceptibility for the disease (Leslie *et al.* 2008; Zhang and Li 2014). The use of medication can too be a risk factor for the development of IBD; for example, frequent use of non-steroidal anti-inflammatory drugs (NSAIDs) is associated with an increased risk of both UC and CD (Ananthakrishnan *et al.* 2012). Other potential IBD risk factors include elevated stress (Zhang and Li 2014) and air pollution (Kaplan *et al.* 2010), which have both been shown previously to correlate with UC development.

1.4.1.3 Microbial factors

Predisposition to the genetic and environmental factors described above can lead to alterations in barrier function of the intestinal mucosa, as well as changes in the intestinal microbiota. These microbial factors have been strongly implicated in the pathogenesis of UC (**Figure 1.5**).

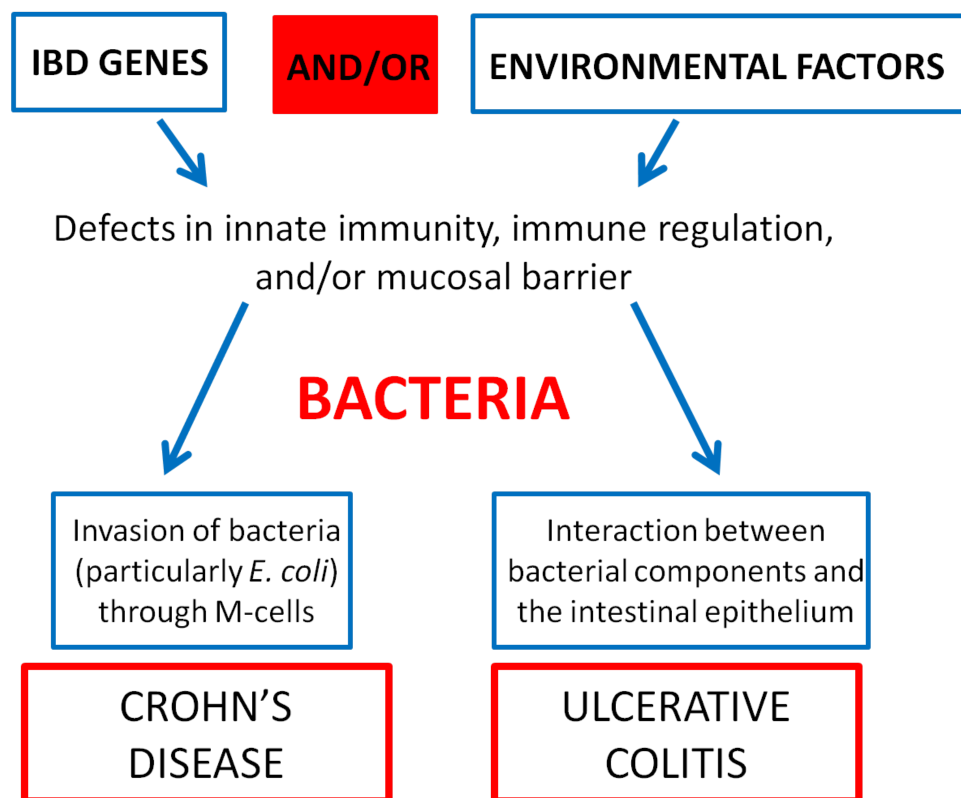


Figure 1.5. Summary of the pathogenesis of IBD. Adapted from (Simpson and Campbell 2015)

1.4.1.3.1 Defects in barrier function

The first physical barrier that intestinal bacteria come into contact with at the mucosal surface is represented by the mucus layer (Torres and Rios 2008). Colonic mucus is secreted by goblet cells, and is mainly composed of secretory MUC2 mucin. Upon secretion from goblet cells, the mucus expands and rapidly forms a stratified, dense inner layer, close to the epithelium. Further from the epithelial surface, a more soluble and less organised mucus layer exists, and studies have shown that this is where gut commensal bacteria reside (Johansson *et al.* 2014).

It has previously been hypothesised that defects in the mucus layer might contribute towards the pathogenesis of UC. Evidence to support this includes the depletion of goblet cells in UC patients, as well as the development of spontaneous colitis in MUC2 deficient mice (Johansson 2014). The erosion of the normally continuous colonic adherent mucus layer results in an increased interaction between bacteria and the intestinal epithelium. One study in particular reported that in the absence of MUC2, bacteria were able to directly adhere and interact with intestinal epithelial cells (Geremia *et al.* 2014). Bacterial antigens can then interact with toll-like receptors (TLRs) or nucleotide oligomerisation domain (NOD) receptors on dendritic cells (DCs) and intestinal epithelial cells, which triggers the activation of the innate and adaptive immune responses (Ordas *et al.* 2012; Subramanian *et al.* 2008).The activation of TLRs results in the subsequent activation of NF-κβ signalling, and release of pro-inflammatory cytokines such as TNF-α, IL-12, IL-23, IL- 6 and IL-1β. It has been shown these cytokines are involved in the synthesis of IL-8, which is a potent neutrophil chemoattractant (Mitsuyama *et al.* 1994). Infiltration by neutrophils is a striking histological feature in the lesions of active UC, and they have been shown to perpetuate the cycle of inflammation typically seen in the disease (Mitsuyama *et al.* 1994).

In addition, IBD patients exhibit an increase in the permeability of the intestinal epithelium due to a redistribution of tight junctions between intestinal epithelial cells (Zhang and Li 2014). Impaired epithelial adhesion processes have also been implicated in UC pathogenesis, and a number of genes encoding proteins involved in key roles in epithelial adhesion have been identified, including hepatocyte nuclear factor-4α, laminin-β1 and E-cadherin (Zhang and Li 2014) Impaired cell adhesion, as well as defective regulation of tight

junctions, can lead to an increase in the permeability of the intestinal epithelium that is concurrent with UC disease.

1.4.1.3.2 Role of the intestinal microbiota

Both quantitative and qualitative changes in the microbial composition have been reported in IBD (Manichanh *et al.* 2012). Many studies have examined the gut flora in CD and UC, in both inflamed and non-inflamed segments, and found that there is a significantly reduced biodiversity in the faecal microbiome of these disease conditions in comparison to healthy controls (Frank *et al.* 2007; Hold *et al.* 2014; Lepage *et al.* 2011) Researchers have previously detected fewer *Bacteroides* and *Clostridium* spp. in the faecal samples of UC patients, with higher levels of *Enterococcus* spp. and *Gammaproteobacteria* (Nemoto *et al.* 2012). Frank and colleagues used culture-independent rRNA sequence analysis of GI tissue samples obtained from CD patients, UC patients and non-IBD controls, where they too detected reduced *Bacteroidetes* and *Firmicutes* in a subset of UC samples (Frank *et al.* 2007). Similar results have been illustrated in a twin study by Lepage and colleagues, where microbiota profiling of mucosal biopsies revealed that UC patients had dysbiosis characterised by a reduction in the number of *Bacteroidetes*, with an increase in the level of *Actinobacteria* and *Proteobacteria* (Lepage *et al.* 2011). Other studies have demonstrated a reduction in the level of beneficial butyrate producing bacteria in UC patients, which includes *Roseburia hominis* and *Faecalibacterium prausnitzii* (Kumari *et al.* 2013; Sokol *et al.* 2006; Sokol *et al.* 2009). Indeed, Varela and colleagues found that low levels of *F. prausnitzii* were associated with short-term UC remission and relapse, whilst levels increased during remission (Varela *et al.* 2013). Furthermore, a reduction in SCFA-producing Firmicutes has also been shown to be

associated with an increased abundance of opportunistic pathogens including *Fusobacterium* spp., *Helicobacter* spp., *Campylobacter* spp. and *Clostridium difficile* (Rajilic-Stojanovic *et al.* 2013). An increased presence of these enteric bacteria in the colonic mucosa of UC patients has also been reported elsewhere, where they have been implicated in the induction of inflammation (Chen *et al.* 2014a).

Several independent studies have now also reported a specific increase of adherent and invasive *E. coli* (AIEC) in CD (Darfeuille-Michaud *et al.* 1998; Martin *et al.* 2004) as well as in UC (Kotlowski *et al.* 2007; Mylonaki *et al.* 2005; Sokol *et al.* 2006; Swidsinski *et al.* 2002) and colon cancer (Swidsinski *et al.* 1998). Similar *E. coli* strains have also been shown to be associated with granulomatous colitis in boxer dogs (Simpson *et al.* 2006). AIEC are present in both non-inflamed and inflamed mucosa (Martin *et al.* 2004; Kotlowski *et al.* 2007), suggesting that they are not just recruited as a consequence of inflammation, but instead play an important role in the pathogenesis of disease. Although there is evidence of tissue invasion by *E. coli* in CD, whereby AIEC have been identified within mucosal macrophages and inside granulomata (Ryan *et al.* 2004), there is a lack of evidence to suggest that they invade the mucosa in UC (Martin *et al.* 2004). Instead, it is thought that mucosally associated UC *E. coli* mediate IL-8 release from colonic epithelial cells via an interaction between *E. coli* flagellin and TLR5 (Subramanian *et al.* 2008). Flagellin receptor TLR5 is expressed on the basolateral aspect of intestinal epithelial cells. As such, in the healthy intestinal mucosa, it is unlikely that bacterial components could interact with TLR5 without direct invasion of UC *E. coli.* However, impaired barrier function in UC is likely to be sufficient to allow flagellin to access basolateral TLR5 *in vivo*, triggering an aberrant immune response (Subramanian *et al.* 2008).

1.4.2 Epidemiology of UC

1.4.2.1 Incidence rates

The occurrence of UC has increased on a worldwide basis in recent years. IBD is common in the industrialised world, particularly with respect to North America and Western Europe. However, reports suggest that incidence rates are also beginning to increase in some developing countries such as Asia, Latin America and Eastern Europe (da Silva *et al.* 2014). The incidence rate of UC may vary from 0.5 to 31.5 per 100,000 people each year, depending on the studied population (Burisch and Munkholm 2015). The majority of UC patients are in the age group of 30-40 years at diagnosis (Cosnes *et al.* 2011). UC is typically less common in children, but recent studies have illustrated an increase in the number of cases seen in pediatric patients and adolescents. For example, Pant and colleagues demonstrated that in the United States, between 2000 and 2009, the number of pediatric hospitalisations due to UC increased from 4171 to 7127 per year (Pant *et al.* 2013). Similar results have also been observed in the UK; in Scotland, UC incidence rates in individuals under 16 years of age increased from 1.59 per 100,000 per year in 1990-1995 to 2.06 per 100,000 per year in 2003-2008 (95% CI: 1.70-2.47; P = 0.023) (Henderson *et al.* 2012). Gender studies have reported mixed findings, but most have illustrated male predominance or an equal distribution between genders (Cosnes *et al.* 2011).

1.4.3 Clinical presentation of UC

The hallmark clinical symptom of UC is bloody diarrhoea, with or without mucus. Depending on the severity of disease, diarrhoea can be accompanied by abdominal pain,

urgency, tenesmus, fever, malaise and weight loss (Feuerstein and Cheifetz 2014). The onset of disease is typically gradual, whilst the clinical course is marked by exacerbations and remissions that may occur spontaneously in response to treatment changes or illness (Kornbluth *et al.* 2010; Danese and Fiocchi 2011).

The severity of UC can be characterised as mild, moderate, severe or fulminant. Mild disease is characterised as fewer than 4 stools (with or without blood) per day without systemic signs of toxin effects and normal inflammatory markers. Moderate disease is described as 4 or more bloody stools per day with minimal signs of toxic effects. Severe disease is classified as more than 6 bloody stools per day with some evidence of toxic effects, which include fever, tachycardia, anaemia or elevated inflammatory markers. Fulminant disease is classified as more than 10 bloody stools and clear clinical signs of toxin effects, including abdominal distension, severe loss of blood requiring transfusion and colonic dilation (Feuerstein and Cheifetz 2014). UC can also be categorised on the basis of the extent or location of disease; proctitis (inflammation limited to the rectum), proctosigmoiditis (inflammation affecting the rectum and sigmoid colon), left sided colitis (inflammation that does not extend beyond the splenic flexure) and extensive colitis (inflammation that extends beyond splenic flexure) (Danese and Fiocchi 2011; Kornbluth *et al.* 2010).

In addition, UC is also associated with a number of extraintestinal manifestations that can primarily affect the skin, joints, eyes and liver (Feuerstein and Cheifetz 2014). Arthritis represents the most common extraintestinal manifestation affecting joints (Dorofeyev *et al.* 2009), whilst erythema nodosum and pyoderma gangrenosum are the two most common skin lesions associated with UC (Feuerstein and Cheifetz 2014; Huang *et al.* 2012).

1.4.4 Diagnosis of UC

An accurate diagnosis of UC is made on the basis of typical symptoms, as well as endoscopic evidence of the extent and severity of colonic inflammation (Dignass *et al.* 2012; Kornbluth *et al.* 2010). Colonoscopy reveals mucosal changes characteristic of UC, which include loss of vascular pattern, granularity and friability. These changes typically affect the distal rectum and proceed proximally in a symmetric and continuous fashion to involve all or part of the colon. Erosions or micro-ulcerations are also evident in moderate disease, whereas shallow ulcerations with spontaneous bleeding are observed in severe cases (Simpson and Papadakis 2008; Kornbluth *et al.* 2010). Colonoscopy aids in differentiating UC from CD, which is characterised by a cobblestone-patterned mucosa, rectal sparing, skip lesions and apthous ulcers (Danese and Fiocchi 2011).

Histological findings obtained from biopsy specimens are commonly used to distinguish UD from infectious colitis or CD (Kornbluth *et al.* 2010). Typically, UC inflammation is limited to the mucosal layers with infiltrates varying in density and composition depending on whether the patient is experiencing active disease or a period of remission. Predominant inflammatory infiltrates include lymphocytes, plasma cells and granulocytes (Loddenkemper 2009). Other histological features include distortion, separation and atrophy of crypts, as well as goblet cell depletion and Paneth cell metaplasia (Loddenkemper 2009). To rule out an infectious cause, it is also recommended that stool cultures be assessed for the presence of *C. difficile*, *Campylobacter* spp. and *E. coli* (Kornbluth *et al.* 2010). Laboratory tests for elevated inflammatory markers are also used to assess disease severity, and include erythrocyte sedimentation rate, as well as levels of C-reactive protein, faecal calprotectin and faecal lactoferrin (Vermeire *et al.* 2006; Sipponen 2013).

1.4.5 Treatment of UC

1.4.5.1 Mesalazine

The first line therapy for mild-moderate UC limited to the rectum is mesalazine (5-aminosalicylate; 5-ASA) (Dignass *et al.* 2012; Lichtenstein *et al.* 2006). Mesalazine can be administered orally, topically or both. Topical administration of mesalazine is more effective than oral mesalazine. Indeed, a Cochrane systematic review of 38 clinical trials confirmed the superiority of this therapy over placebo for inducing symptomatic, endoscopic and histological improvement and remission (Marshall *et al.* 2010). Combining topical mesalazine with oral mesalazine or topical steroid is more effective than either alone, and as such, should be considered for treatment escalation in non-responders (Marteau *et al.* 2005; Mulder *et al.* 1996). Mesalazine intolerance occurs in up to 15% of patients. Diarrhoea (3%), headache (2%), nausea (2%), rash (1%) and thrombocytopenia (<1%) are reported, however, systematic reviews have demonstrated that newer 5-ASA agents cause fewer adverse effects (Loftus *et al.* 2004).

1.4.5.2 Glucocorticoids

Patients that do not respond to mesalazine are candidates for glucocorticoid treatment, either in combination with mesalazine, or alone (Kornbluth *et al.* 2010). Steroids are effective at inducing remission, but are not acceptable as a means to maintain remission (Feuerstein and Cheifetz 2014). If UC is limited to proctosigmoiditis, steroid enemas are effective at improving symptoms (Kornbluth *et al.* 2010). UC patients with severe disease who fail to respond to maximal doses (oral or topical) of 5-ASA and corticosteroids warrant

hospitalisation for the administration of intravenous corticosteroids, typically prednisone. Intravenous steroids are effective at inducing remission in up to 70% of patients (Turner *et al.* 2007). Long-term therapy with corticosteroids is associated with significant complications, and as such, they are not used to maintain remission in UC (Lichtenstein *et al.* 2006). Therefore, any patient treated with steroids should be bridged to a medication that is proven to maintain remission (Dignass *et al.* 2012).

1.4.5.3 Thiopurines

Thiopurines (azathioprine and mercaptopurine) are the most commonly used immunosuppressants in UC. Rather than induce remission, they are used to maintain remission in quiescent disease induced by other treatments, particularly glucocorticoids (Sands 2006). This might be explained by their slow onset of action, which is typically 6 – 12 weeks (Sandborn 2001). Despite their widespread use, the evidence base to support their efficacy is not strong. A recent systematic review and meta-analysis identified only three RCTs involving 127 patients. Pooled data illustrated that azathioprine was significantly more effective than placebo at preventing relapse (RR=0.60; 95% CI = 0.37-0.95). The meta-analysis also identified two RCTs in 130 active UC patients, and perhaps unsurprisingly, azathioprine had no significant effect (RR = 0.64, 95% CI = 0.34 – 1.23) (Khan *et al.* 2011). Potentially serious adverse effects of thiopurine therapy include myelosuppression and associated opportunistic infections, acute and chronic effects on liver function, and hypersensitivity reactions, including pancreatitis. Long-term use may also be associated with an increased risk of cancer (Ford *et al.* 2013)

1.4.5.4 Anti-tumour necrosis factor (TNF) agents

Anti-TNF agents are monoclonal antibodies that are directed against tumour necrosis factor α (TNF-α) (Kornbluth *et al.* 2010). Currently, three main anti-TNF agents are used to treat moderate to severe UC that include infliximab, adalimumab, and more recently, golimumab (Kornbluth *et al.* 2010; Lowenberg *et al.* 2014). All three agents have similar efficacy and safety profiles, but it has been suggested that Infliximab has a superior clinical effect (Mei *et al.* 2015). A systematic review of the efficacy of infliximab for treating patients with moderate to severe UC refractory to corticosteroids and/or immunosuppressants concluded that it was effective for inducing clinical remission, clinical response, promoting mucosal healing and reducing the need for colectomy in the short term (Lawson *et al.* 2006). Similar results were also found in two large Active Ulceration Colitis Trials (ACT1 and ACT2, respectively) involving 728 patients with moderate-severe UC. Results demonstrated that patients treated with infliximab at 0, 2 and 6 weeks and every 8 weeks thereafter were more likely to have a clinical response at weeks 8, 30 and 54 than those receiving placebo (Rutgeerts *et al.* 2005). Anti-TNF therapy is considered a relatively safe treatment option, but common side effects include injection site reactions, nausea, vomiting, arthralgia and myalgia (Lichtenstein *et al.* 2012). Whilst serious adverse effects are rare, all biological agents carry a risk of serious infection and demyelinating disease. Additionally, long-term treatment in combination with immunosuppressants causes an increased risk of lymphoma (Deepak *et al.* 2013).

1.4.5.5 Other therapies targeting the microbiota

Whilst a considerable number of UC patients can maintain remission when treated with the standard therapies described above, a significant number experience persistent disease activity and ultimately require a colectomy (Chen *et al.* 2014b; Danese 2011). The development of alternative effective therapies is therefore extremely important. It is becoming evident that the interaction between the enteric microbiota and the host immune system drives inflammation in UC; therefore, the manipulation or restoration of intestinal dysbiosis or mucosal barrier might represent an attractive therapeutic approach.

The administration of probiotics and/or prebiotics sounds an attractive concept for the treatment of UC. Indeed, probiotic mixtures such as VSL3 (consisting of 4 strains of Lactobacilli, 3 strains of Bifidobacteria and 1 strain of *Streptococcus thermophiles*) are effective at maintaining and inducing remission in both child and adult patients with mild-moderate active UC, either in combination with standard therapies or alone (Miele *et al.* 2009; Sood *et al.* 2009). Similar results have been seen with *E. coli* Nissle 1917 (Kruis *et al.* 2004) and *Lactobacillus* GG (Zocco *et al.* 2006). Prebiotic agents such as germinated barley foodstuff (GBF), inulin and pysillium have also been shown to effectively maintain, and sometimes induce, remission in patients with mild-moderate active UC (Scaldaferri *et al.* 2013; Bamba *et al.* 2002; Kanauchi *et al.* 2002; Casellas *et al.* 2007; Kanauchi *et al.* 2013; Fernandez-Banares *et al.* 1999). Whilst this approach would be safe and cost-effective, studies are still in their infancy and clinical evidence is limited by a lack of human studies involving only a small number of patients.

Other approaches include FMT, which aims to reverse the intestinal dysbiosis implicated in disease pathogenesis. Whilst studies indicate clinical improvement following the procedure

(Borody *et al.* 2003; Kunde *et al.* 2013), some report that this effect is only short-term and that FMT cannot induce remission in UC patients (Kump *et al.* 2013) (Angelberger *et al.* 2013). More recent studies suggest that FMT is effective in inducing remission in some, but not all UC patients (Moayyedi *et al.* 2015; Rossen *et al.* 2015). As such, further studies are needed to assess the effectiveness of FMT in treating UC, as well as to address the potential safety issues associated with the procedure (Rubin 2013).

Phosphatidylcholine (PC), a major mucus phospholipid significantly reduced in UC patients and easily obtained from a variety of dietary sources (such as egg yolk, soy beans etc.) (Torres *et al.* 2013) has also been investigated as a treatment for UC, with promising results. One study reported that 90% of patients receiving a retarded-release PC preparation reached clinical remission or significantly improved by over 50% compared with only 10% in the placebo group (Stremmel *et al.* 2005). In an open-label follow up treatment, continuous remission was maintained in 33% of PC treated patients versus 10% of controls. Results from a recent Phase IIb placebo-controlled dose-finding study using an optimised highly enriched PC preparation have also proved promising results (Torres *et al.* 2013), however, larger studies will be needed to confirm its effectiveness in maintaining UC remission.

1.4.5.6 Animal models of colitis

In recent years, a large number of experimental animal models have led to a greater understanding of the pathogenesis in IBD. In addition, their study has also enabled the identification of disease targets and evaluation of novel treatments in pre-clinical testing. Experimental colitis models with epithelial barrier defects and innate immunity defects have

both been extensively employed (Khanna *et al.* 2014). Here, the former will be discussed as UC pathogenesis is largely mediated by dysfunction of the intestinal epithelial barrier.

Administration of chelating agent dextran sulphate sodium (DSS) in the drinking water of mice causes acute colonic inflammation, which presents as weight loss, diarrhoea and gross rectal bleeding (Okayasu *et al.* 1990). The acute DSS model shares a number of pathological features with UC, including mucin depletion, epithelial degeneration, neutrophil infiltration, formation of crypt abscesses and mucosal erosions (Valatas *et al.* 2015). Other experimental colitis models include the 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced colitis mouse model, which employs the intra-rectal administration of TNBS and induces severe colonic inflammation that is similar to the clinical and histopathological findings observed in CD (Scheiffele and Fuss 2001). Muc2$^{-/-}$ mice, which exhibit defective mucin production, spontaneously develop colitis that reflects the clinical and cellular features of UC (Van der Sluis *et al.* 2006). The murine multiple drug resistance 1 a (*Mdr1a*) gene is implicated in the regulation of epithelial transcellular permeability, and as such, Mdr1a$^{-/-}$ mice also develop spontaneous, flora-dependent colonic inflammation typical of UC (Panwala *et al.* 1998).

1.5 BACTERIAL-EPITHELIAL INTERACTIONS MEDIATE THE PATHOGENESIS OF DIARRHOEAL DISEASE

Adhesion of pathogenic bacteria to host tissues represents an early, but critical step in the pathogenesis of virtually all infections (Lehmann *et al.* 2006). With respect to diarrhoeal gut pathogens *C. difficile* and ETEC, it is thought that their close proximity to the intestinal

epithelium is critical for the subsequent release of their respective toxins. As such, epithelial adhesion represents an essential mediator of disease pathogenesis.

With regards to UC, it is becoming increasingly evident that enteric bacteria play an important role in the mediation of inflammation. Indeed, the loss of protective intestinal barrier function results in direct contact between bacteria, bacterial components such as flagellin and LPS and the surface epithelium, with a major potential for interaction with TLRs and consequent release of pro-inflammatory cytokines.

Therefore, the ability to prevent these harmful bacterial-epithelial interactions is an attractive therapeutic strategy.

1.5.1 Soluble plant fibres (non-starch polysaccharides) can inhibit the epithelial adhesion of a range enteric pathogens

During studies in our own laboratory, we have previously demonstrated that soluble plant fibre, or non-starch polysaccharides (NSPs), can disrupt potentially harmful interactions between bacteria and the gut epithelium.

As part of this work, a range of soluble fibres were first investigated for their ability to block the *in vitro* adhesion and invasion of a range Crohn's associated AIEC isolates to intestinal epithelial cells. Here, it was shown that soluble NSP extracted from plantain bananas (Musa *spp*.) exhibited particular efficacy, and moreover, it could significantly inhibit bacterial translocation across *ex-vivo* explants of villous or follicle-associated epithelium (FAE) mounted on Ussing chambers (Martin *et al.* 2004; Roberts *et al.* 2010; Roberts *et al.* 2013).

Interestingly, it seems that soluble plantain fibre can also inhibit the adherence of a range of other gut pathogens, such as *Salmonella* spp., and *Shigella sonnei* (Roberts *et al.* 2013). Importantly, this has also been demonstrated *in vivo*, where supplementation of a commercial feed with soluble plantain fibre was shown to significantly block *Salmonella* colonisation in the chicken (Parsons *et al.* 2014). Furthermore, preliminary results have also suggested that soluble plantain fibre can significantly inhibit the epithelial adhesion of diarrhoeal pathogens *Clostridium difficile* and ETEC (Roberts *et al.* 2013).

Overall, our previous findings raise the possibility that dietary supplementation with specific soluble plant NSPs could be beneficial for the maintenance of intestinal health and prevention of diarrhoeal disease. Previous clinical studies also provide evidence to support the beneficial role of soluble plant fibres, where banana flakes supplemented to enteral feed have been shown to control diarrhoeal episodes (Emery *et al.* 1997). Additionally, the juice from boiled green bananas, that would contain soluble fibre, has been reported to reduce the severity and duration of persistent diarrhoeas (Rabbani *et al.* 2004; Rabbani *et al.* 2001), including shigellosis (Alvarez-Acosta *et al.* 2009).

Chapter 2

Hypothesis

2.1 HYPOTHESIS

Soluble NSPs may have a beneficial effect on intestinal health by their ability to inhibit harmful interactions between bacteria and the human intestinal epithelium.

Chapter 3

Aims

3.1 AIMS

1. To evaluate soluble plantain NSP, as well as a range of other soluble fibres for their efficacy at inhibiting the epithelial adhesion of *C. difficile* and ETEC

2. To evaluate whether soluble plantain NSP can inhibit the epithelial adhesion of *C. difficile* spores

3. To evaluate whether soluble plantain NSP can reduce the epithelial damage induced by purified *C. difficile* toxins

4. To evaluate whether soluble plantain NSP can reduce the epithelial damage induced by mucosally associated UC *E. coli*, purified flagella and LPS

5. To characterise the polysaccharide component present in plantain NSP that confers its inhibitory activity

6. To characterise the molecular mechanism of the inhibitory action of plantain NSP

Chapter 4

Materials and Methods

4.1 MATERIALS

Unless indicated otherwise, all chemicals used herein were obtained from Sigma Aldrich (Gillingham, UK). Bacto™ agar, Bacto™ tryptone and Bacto™ yeast extract for use in bacterial culture were supplied by BD Biosciences (Oxford, UK), whilst all other bacterial culture media was supplied by Oxoid (Hampshire, UK). All plastics for human cell culture were obtained from Corning/Costar (High Wycombe, UK), as were plastics for liquid (broth) bacterial culture. Solid (agar) bacterial culture was performed using plastics obtained from Sterilin (Caerphilly, UK). For recipes of commonly used buffers and reagents, see *Appendix 1*.

4.2 CELL CULTURE

4.2.1 Human cell lines used in the study

4.2.1.1 Caco2

A human derived colorectal adenocarcinoma cell line, obtained from the European Collection of Authenticated Cell Cultures (ECACC) (Wiltshire, UK; EACC Number 86010202). The Caco2 cell line was selected for the majority of these studies as it represents an established *in vitro* model of the intestinal epithelial barrier. Used at sub confluent levels, these cells are models of isolated colonic epithelial cells. Upon reaching confluence, the cells undergo spontaneous differentiation to express several morphological and biochemical characteristics typically observed in the mature enterocyte (Sambuy *et al.* 2005).

4.2.1.2 HT29

A human derived colorectal adenocarcinoma cell line with epithelial morphology, obtained from the ECACC (ECACC Number 85061109) (Kimball *et al.* 1981).

4.2.1.3 SW620

A human derived colorectal adenocarcinoma cell line isolated from a metastatic lymph node, also obtained from the ECACC (ECACC Number 87051203) (Leibovitz *et al.* 1976).

4.2.2 Cell line maintenance

Caco2, HT29 and SW620 cells were maintained Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) FBS, 100 U/mL penicillin, 100 µg/mL streptomycin and 8 mM glutamine (complete DMEM), in T150 tissue culture flasks until 70 – 80% confluent. Cells were maintained at 37°C in a humidified atmosphere of 5% CO_2, 95% air.

To passage cells, media was removed and the cells washed with pre-warmed (37°C) sterile phosphate-buffered saline (PBS; pH 7.4). Cells were then detached from their flasks by treatment with 5 mL of 1X trypsin-EDTA solution (0.25% (v/v) trypsin, 0.02% (w/v) EDTA in PBS), which was neutralised by the addition of an equal volume of complete DMEM. The cell suspension was then split at a ratio of 1:5 into new T150 flasks alongside 20 mL of fresh culture media. To reduce potential passage number effects, cells were only maintained between passages 1 – 30 (Briske-Anderson *et al.* 1997).

4.2.3 Suppression of galectin-3 expression in SW620 cells

Colonic SW620 cells were transfected with short hairpin RNA (shRNA) or the empty vector control to generate stable galectin-3 knockdown cells ($SW620^{Gal3-}$) and galectin-3 expressing cells ($SW620^{Gal3+}$), respectively. Transfection was performed by Paulina Sindrewicz (Gastroenterology Research Unit, Department of Cellular & Molecular Physiology, University of Liverpool), as described in Duckworth *et al.* (Duckworth *et al.* 2015).

4.3 SOLUBLE NON STARCH POLYSACCHARIDES (NSPs)

Soluble NSP fibres were provided by Dr Niamh O'Kennedy (Provexis plc; Windsor, UK). A range of NSP fibres were selected to represent common monocotyledon (i.e. having one seed leaf, or cotyledon) or dicotyledon (i.e. plants with two seed leaves) source of dietary fibre. Apple, bean, blueberry, leek, pear, strawberry and tomato NSP are classified as monocots, whilst oat and plantain are classified as dicots. The monosaccharide composition of the NSP fibres used in the study is summarised in *Appendix 2*.

4.3.1 Preparation of soluble non starch polysaccharides (NSPs)

Our preferred source of soluble non-starch NSP was from green plantain (ripeness stage 1) flour produced in Ecuador from locally grown cultivars Musa AAB (Horn) variation Domino (See *Appendix 2*). The plantain NSP preparations used in this study were from Confoco plantain flour (Trobana Green Plantain flour; Confoco International Ltd; Ripley, UK) and were prepared by Provexis Plc (Windsor, UK) at the Teagasc Food Research Centre

(Moorepark, Ireland), as described previously (Parsons *et al.* 2014; Roberts *et al.* 2010; Simpson and Campbell 2014)

Briefly, dry plantain flour was homogenised in reverse-osmosis purified water (ratio 1:2) and then heated to 90°C – 100°C for 10 min with continuous high-shear mixing to effect starch swelling and gelatinisation. The homogenate was then cooled to 25°C and treated with fungal α-amylase Fungamyl® (Novozymes; Bagsvaerd, Denmark) for 2 h (pH 6-7) to hydrolyse native starch. The mixture was then heated to 72°C for 20 min to fully inactivate the Fungamyl® enzyme. Insoluble NSP was removed by centrifugation and subsequently, low molecular weight components (<300 Da), including starch degradation products, were removed from the soluble NSP by nanofiltration. The concentrated retentate (containing in addition up to 45% by weight plantain-derived maltodextrin carrier as part of the bulk-manufacturing process to counter difficulties in freeze drying/resolubilisation) was spray-dried to a fine dry powder with a particle size distribution of 50 – 100 µm and a bulk density of 175 g/L. Other soluble NSPs used in the study were prepared to the same specification.

4.3.2 Preparation of acidic and neutral polysaccharide fractions of plantain NSP

Acidic and neutral polysaccharide fractions of soluble plantain NSP were prepared in two batches by Q-Sepharose anion-exchange chromatography, as previously described (Parsons *et al.* 2014).

4.3.2.1 Small scale, analytical strong anion-exchange fractionation

Initially, acidic and neutral polysaccharide fractions were prepared by small scale, analytical fractionation, using a HiPrepTM Q-Sepharose FF 16/10 column on the AKTA Prime

Plus chromatography system. Briefly, 1.6 g soluble plantain NSP was dissolved in 50 mL equilibration buffer (50 mM Tris-HCl, pH 7.4) and loaded onto the column at a flow rate of 5 mL/min. The column was then washed with 40 mL equilibration buffer to collect the unbound, neutral polysaccharide fraction of plantain NSP. The bound, acidic polysaccharide fraction of plantain NSP was eluted step-wise with 0.1M, 0.5M and 1M NaCl in equilibration buffer. Subsequently, both neutral and acidic fractions were desalted on PD MidiTrap G-10 columns (GE Healthcare Sciences, Buckinghamshire, UK), performed according to the manufacturer's instructions. These columns have an exclusion limit of > 700 Da, and as approximately 95% plantain NSP is < 5 kDa in size, they are suitable for desalting samples containing soluble plantain fibre.

4.3.2.2 Scaled-up, preparative scale strong anion-exchange fractionation

Based on the results from the small-scale analytical fractionation of plantain NSP, a bulk preparation of purified neutral and acidic fractions of soluble plantain fibre was performed. This was also carried out by strong anion-exchange chromatography, but in the absence of a column (Clark 1976), performed using Q-Sepharose FastFlow beads (GE Healthcare Life Sciences) in large 2.5 L mixing vessels. Each 300 mL batch of Q-Sepharose was reconstituted in 1 L deionised water, transferred into the vessel and rotated for 15 min, which was repeated three times. The Q-Sepharose beads were then equilibrated twice with 600 mL sterile-filtered 50 mM Tris-HCl buffer (pH 7.4).

Twenty-five grams of soluble plantain NSP was added to 780 mL 50 mM Tris-HCl, pH 7.4. The 'solution' was left overnight at 4°C to settle and the majority, clear upper layer was removed and filtered under vacuum through a scintered glass separating funnel and

Whatmann #1 filter paper. This resulted in a volume of approximately 700 mL. The solubilised plantain NSP was then added to the 2.5 L vessel containing 300 mL Q-Sepharose and rotated for 1 h. The unbound, neutral polysaccharide fraction of soluble plantain NSP was collected and filtered using a scintered glass separating funnel. To ensure the removal of all unbound material, the Q-Sepharose beads underwent two 15 min washes with 300 mL equilibration buffer, which was then pooled with the Q-Sepharose unbound fraction.

The Q-Sepharose-bound acidic polysaccharide fraction from plantain NSP was eluted with 400 mL 0.5 M NaCl in equilibration buffer, rotated overnight in 4°C. Any unbound acidic polysaccharides were further eluted by two 15 min washes with 200 mL 0.5 M NaCl, and an overnight wash with 400 mL 1M NaCl. Finally, Q-Sepharose was cleaned overnight with 400 mL 1M NaOH. Only the 0.5M NaCl overnight fraction and washes were pooled prior to desalting.

4.3.2.3 Desalting of neutral and acidic polysaccharide plantain fractions

The neutral and acidic fractions were both desalted using multiple PD MidiTrap G-10 gravity mini-columns (GE Healthcare Sciences), as per the manufacturer's instructions (at 1 mL sample per column). To determine the G-10 column fractionation elution profiles for neutral and acidic fractions, total hexose content was determined using a phenol-sulphuric acid assay. Phenol red (354 Da) was added as a molecular size elution marker (**Figure 4.1**).

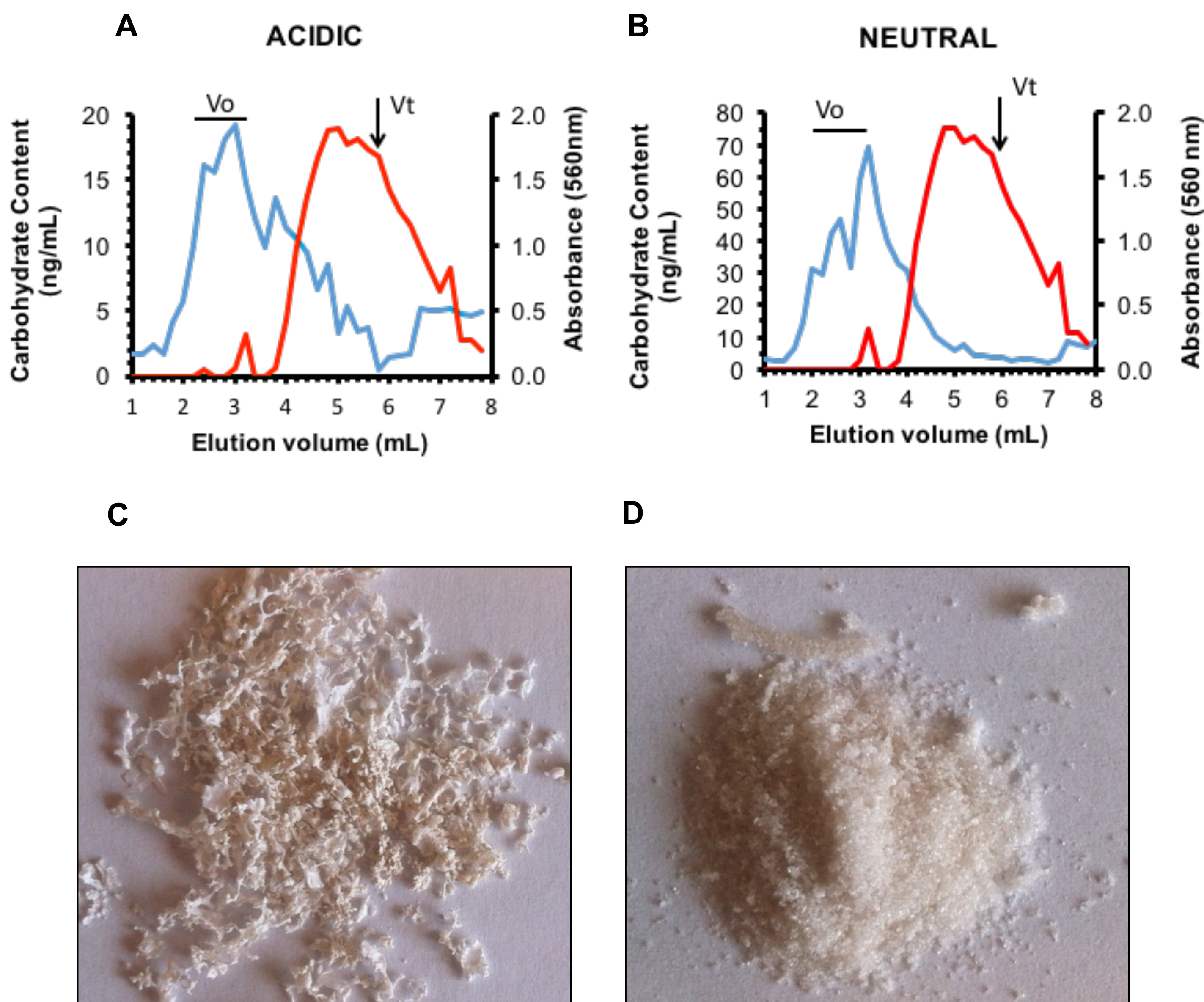


Figure 4.1 Desalting of Q-Sepharose® anion-exchange chromatography fractions from soluble plantain NSP. Following preparative anion-exchange chromatography, sodium chloride-eluted **A)** acidic polysaccharides and **B)** unbound neutral polysaccharide fractions were desalted into water using multiple PD MidiTrap™ G-10 columns. Carbohydrate content of eluted fractions was measured using a phenol-sulphuric acid assay (blue line). Columns were pre-calibrated with the low molecule size marker phenol red (354 Da), measured as A_{560} (red line). The arrow indicates total column volume (Vt). The solid bar indicates the elution fraction collected for lyophilisation and bioassay (Vo indicates the void volume of the column). Desalted acidic and neutral fractions were then freeze dried and lyophilised, as depicted in images **C)** and **D)**, respectively.

4.3.2.4 Hexose Assay

Fractions collected from the G-10 desalting columns were assayed for total hexose using a modified method of Dubois (1956) (DuBois *et al.* 1956). Briefly, 10 µL fractions were added to 96-well microtitre plates in triplicate, and 100 µL of 4% (w/v) phenol dissolved in deionised water was added, at room temperature for 5 min. Concentrated H_2SO_4 (150 µL) was then rapidly added to wells and aspirated to generate heat. Plates were left to cool for 20 min. Optical density (OD) was then measured at 560 nm. D-glucose standards (0-20 ng/mL) and water blanks were also included. Hexose content of samples was determined using a D-glucose standard calibration curve (**Figure 4.2**).

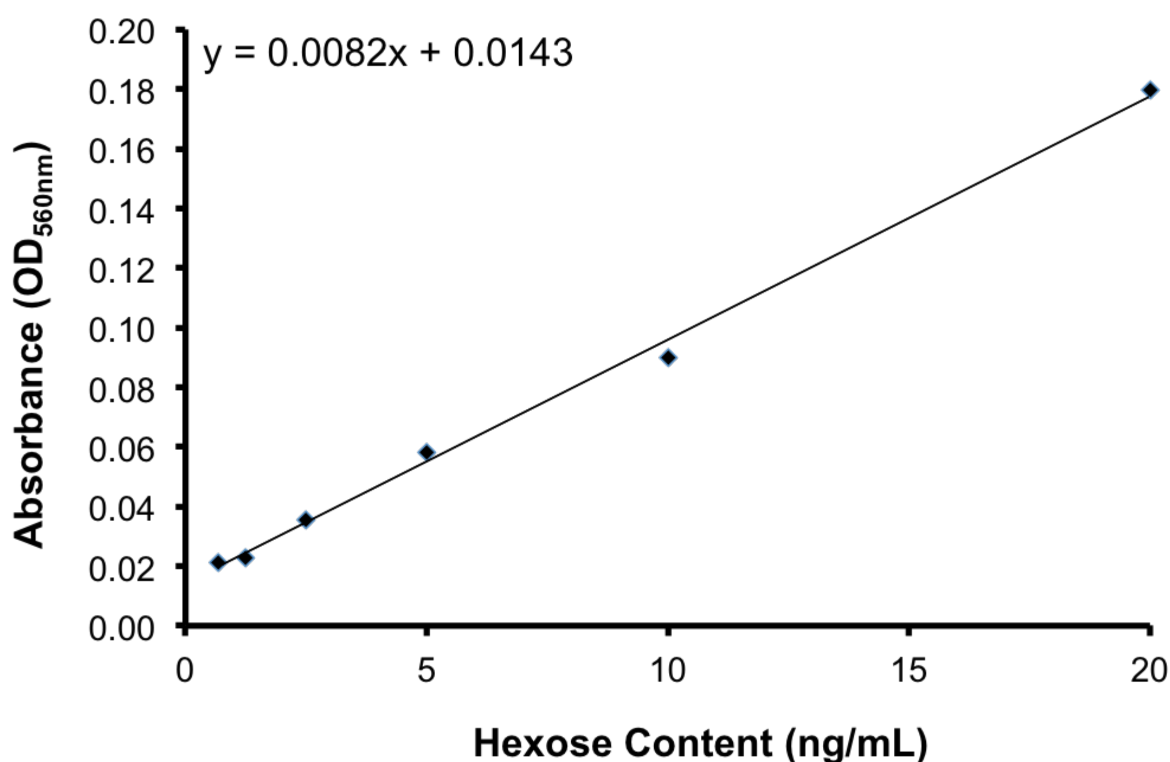


Figure 4.2 D-Glucose calibration curve used to determine the unknown hexose content of neutral and acidic polysaccharide fractions of plantain NSP. D-Glucose standards (0-20 ng/mL) were added in triplicate and hexose content determined using a phenol-sulphuric acid assay, with the absorbance measured spectrophotometrically at optical density (OD) 560 nm (N=1, n=3)

4.3.2.5 Lyophilisation of neutral and acidic plantain NSP fractions

Desalted neutral and acidic fractions were freeze-dried overnight in round-bottom flasks (Edwards Modulyo 4K Freeze Drier; operating temperature -55 ± 5°C; maximum vacuum atmospheric pressure 215 psi). To facilitate freeze drying, the sample was shell-frozen over a large surface area of each round bottom flask by immersion and rapid rotation in a bath of ethanol and dry ice. Flasks were stored for at least 20 min at -80°C before lyophilisation.

4.3.2.6 Yield projections

Whilst only a portion of neutral and acidic fractions had been desalted and lyophilised at the time of writing, initial yields from total starting material of 75 g plantain NSP can be extrapolated. From 800 mL of the NaCl Q-Sepharose eluted acidic fraction (total volume 3.6 L), the yield of material was 1.16 g. Therefore, a projected yield of acidic fraction is estimated at 5.21 g. From the 120 mL of the Q-Sepharose unbound neutral fraction (total volume 2.1 L), the yield of material was 0.72 g, giving a projected total yield estimated at 12.60 g. Additional material within the Q-Sepharose unbound wash step and 1 M NaOH clean-up fractions have yet to be determined.

4.4 BACTERIAL CULTURE

4.4.1 Bacterial strains used in the study

4.4.1.1 C. difficile isolates

Clinical laboratory reference strain *C. difficile* 080042 (Ribotype [Rb] 027a) was kindly donated by Dr Godfrey Smith (Medical Microbiology, Royal Liverpool and Broadgreen

University Hospitals NHS Trust, Merseyside, UK). Eleven further *C. difficile* clinical isolates were obtained from Dr Fabio Miyajima (Institute of Translational Medicine, University of Liverpool, UK) and Mr Paul Roberts (Royal Liverpool and Broadgreen University Hospitals NHS Trust) A further *C. difficile* isolate 1342 (Rb 005) was generously provided by Dr Gillian Douce and Dr Anthony Buckley (Institute of Infection, Immunity and Inflammation, University of Glasgow, UK). The characteristics (source, toxinotype and ribotype) of the *C. difficile* clinical isolates used in this study are summarised in **Table 4.1.**

Table 4.1 Bacterial characteristics of *C. difficile* clinical isolates used in this study

Strain Number	Ribotype	Toxin A	Toxin B	Binary Toxin	Origin
98011	027	+	+	+	Liverpool
108536	027	+	+	+	Japan
98078	078	+	+	+	Liverpool
98231	078	+	+	+	Liverpool
108526	018	+	+	-	Japan
108906	012	+	+	-	Malawi
98359	106	+	+	-	Liverpool
108963	017	-	+	-	Liverpool
108519	017	-	+	-	Japan
108520	368	-	+	-	Japan
98220	010	-	-	-	Liverpool
1342	005	-	-	-	Glasgow

4.4.1.2 Enterotoxigenic E. coli

ETEC C410 (serotype 0160, LT+/ST+) was a kind gift from Dr Godfrey Smith (Medical Microbiology, Royal Liverpool and Broadgreen University Hospitals NHS Trust, UK).

4.4.1.3 Mucosally-associated E. coli isolated from patients with UC

E. coli strains (HM250, HM295, HM378, HM380 and HM387) were previously isolated from colonic mucosal biopsies of five patients with UC, as described elsewhere (Martin *et al.* 2004) (Subramanian *et al.* 2008). Their characteristics are summarised in *Appendix 3*.

4.4.1.4 Salmonella enteric serovar. Typhimurium

S. Typhimurium LT2 was generously donated by Professor Craig Winstanley (Department of Clinical Infection, Microbiology and Immunology, Institute of Global Health and Infection, University of Liverpool, UK).

4.4.2 Bacterial growth conditions

For most purposes, bacterial strains were grown from frozen stocks, which were stored down on Protect beads at -80°C (Lab M; Lancashire, UK). *C. difficile* isolates were routinely grown on Brain Heart Infusion (BHI) agar supplemented with 0.1% (w/v) L-cysteine and 5 mg/mL yeast extract (BHIS agar) in anaerobic conditions, at 37°C for 48 h. Anaerobic conditions were generated by incubating *C. difficile* in sealed 2.5 L AnaeroJars (Oxoid) containing an AnaeroGen sachet (Oxoid). All other bacterial strains were grown on Luria Burtani (LB) agar in aerobic conditions, at 37°C for 24 h.

Prior to infection of cultured epithelial cells, approximately 5 bacterial colonies were removed from solid phase culture and suspended in sterile phosphate-buffered saline (PBS, pH 7.4). The bacterial culture was centrifuged at 10,000 x g for 5 minutes and washed 3 times before being resuspended to an OD_{600nm}, which was equivalent to 1×10^9 colony forming units (CFU)/mL.

4.5 *C. difficile* SPORE PURIFICATON

Early attempts at *C. difficile* spore purification included the use of an individual heat treatment (60°C for 20 min) or alcohol treatment (100% ethanol for 1 h) (Burns and Minton 2011). However, Schaeffer and Fulton endospore staining (Schaeffer and Fulton 1933) revealed significant vegetative *C. difficile* contamination, suggesting that these are ineffective purification methods (**Figure 4.3**). *C. difficile* spores were therefore purified according to methods described previously by Permpoonpattana and colleagues (Permpoonpattana *et al.* 2011), with some modifications.

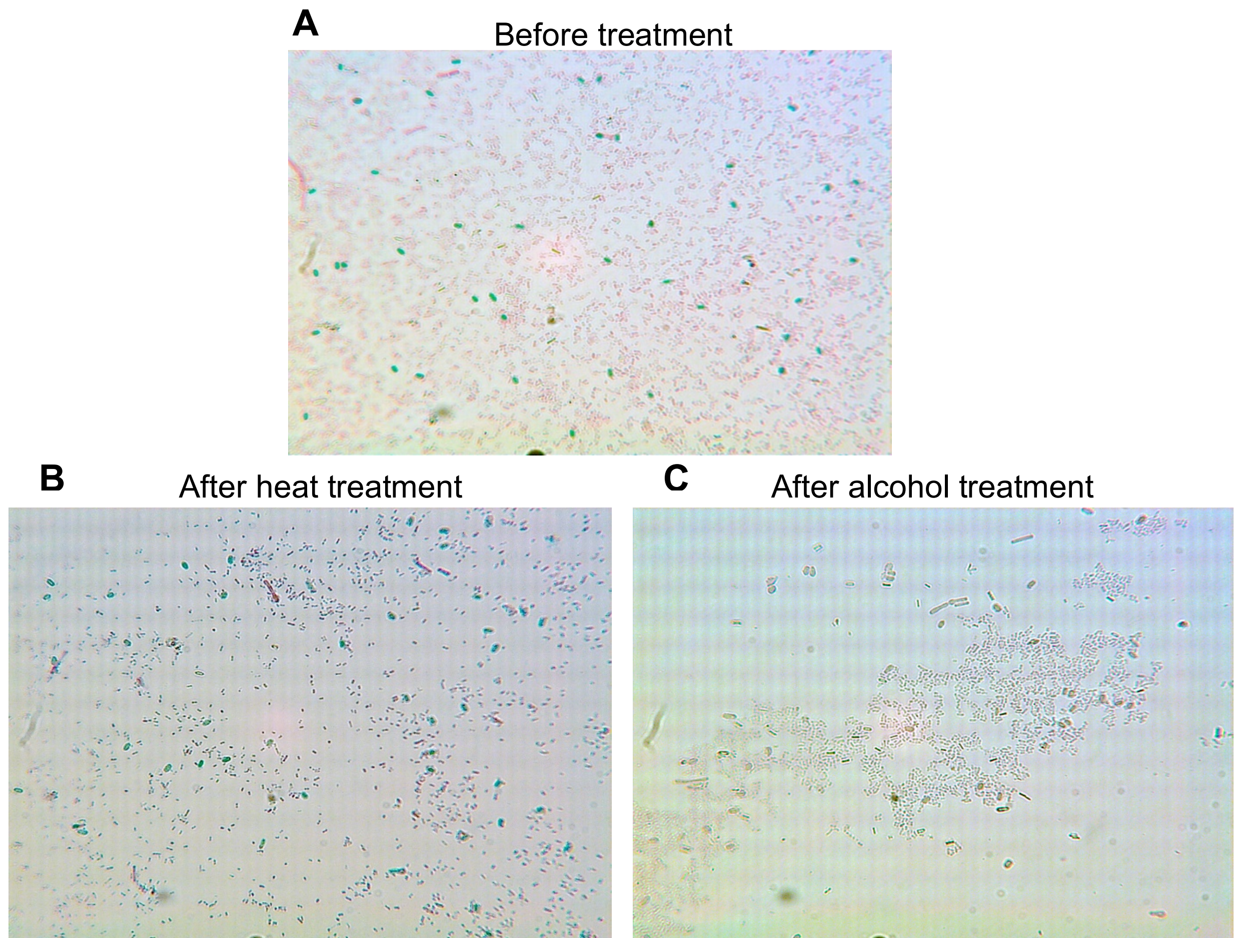


Figure 4.3 Purification methods using only heat or alcohol treatment do not generate a pure *C. difficile* spore sample. A representative Schaeffer and Fulton endospore stain of *C. difficile* 98011 vegetative cell: spore suspension **A)** before and after **B)** heat treatment (60°C for 20 min) or **C)** alcohol treatment (100% ethanol for 1 h). Cells were then stained with malachite green and safranin red, subsequently resulting in red vegetative cells and green spores.

4.5.1 Promotion of *C. difficile* sporulation

C. difficile isolates were routinely cultured on BHIS agar in anaerobic conditions at 37°C, for 48 h. Following incubation, one single bacterial colony was removed from the agar plate and used to inoculate 10 mL Tryptone Glucose Yeast (TGY) broth. After overnight incubation, TGY culture medium was sub-cultured (1:100) into Sorbitol MacConkey (SMC) broth. The SMC culture medium was incubated at 37°C until an OD_{600nm} of 0.5 was reached.

Subsequently, 100 µL of this culture broth was used to inoculate SMC agar plates, which were incubated in anaerobic conditions at 37°C for 7 days.

4.5.2 Purification of *C. difficile* spores

After the extended *C. difficile* incubation period, 5 mL sterile water was added to each agar plate and bacterial growth was harvested by carefully scraping the surface of the agar to dislodge *C. difficile* spores. Spores were then washed twice with sterile water (4500 x g for 15 min) and suspended in 2 mL lysis buffer (PBS containing 125 mM Tris, 200 mM EDTA, 0.3 mg/mL proteinase K and 1% (v/v) sarcosyl). The suspension was incubated with gentle shaking at 37°C for 2h, and spores were then centrifuged (4500 x g for 20 min), The pellet was next resuspended in cold (2 - 5°C) sterile water, which was repeated a further 10 times. After the final wash, spores were heat-treated (60°C for 20 min) to kill any residual vegetative cells. Spores were then frozen at -20°C until required. Schaeffer and Fulton endospore staining confirmed the presence of a pure *C. difficile* spore sample, free from vegetative cell contamination (**Figure 4.4**).

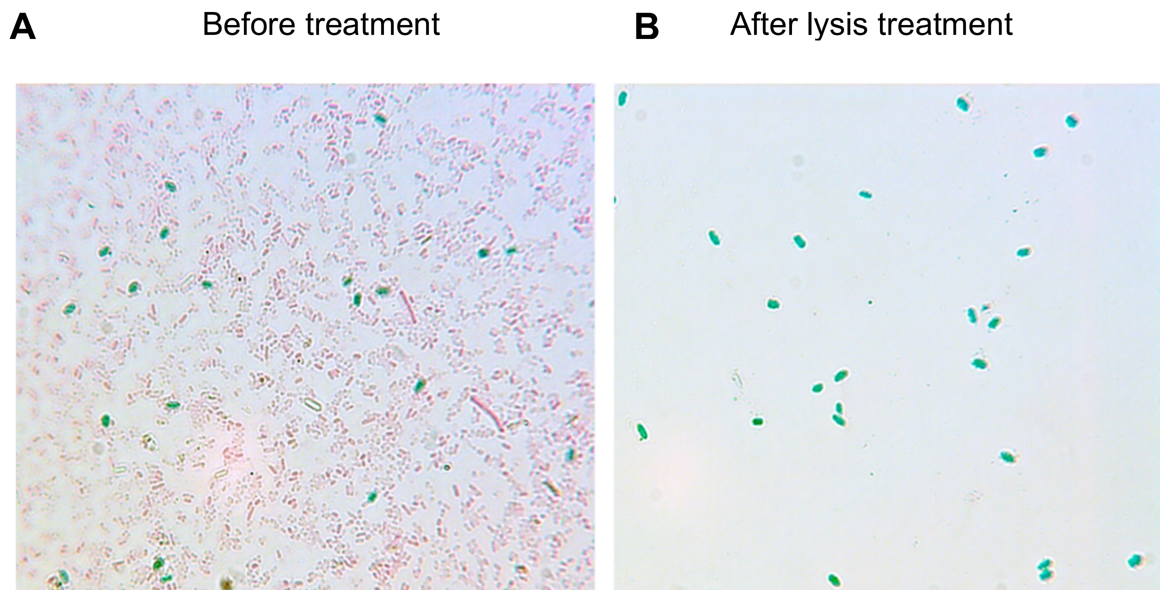


Figure 4.4 Purification methods involving vegetative cell lysis, serial washing and a heat treatment generate a pure *C. difficile* spore sample. A representative Schaeffer and Fulton endospore stain of *C. difficile* 98011 vegetative cell: spore suspension **A)** before and after **B)** purification, which involved incubation with a lysis buffer (2 h), serial washing with cold (2 - 5°C) sterile water and a heat treatment (60°C for 20 min) to kill any residual vegetative cells. Cells were then stained with malachite green and safranin red, subsequently resulting in red vegetative cells and green spores.

4.5.3 Schaeffer and Fulton endospore stain

As already mentioned, an Schaeffer and Fulton endospore stain was performed to confirm purity of the spore sample (Schaeffer and Fulton 1933). Briefly, 100 µL was extracted from the spore sample and smeared on a glass slide. The sample was then air dried and fixed with a gentle heat. A piece of tissue paper was placed on top of the slide and flooded with malachite green. The slide was then placed on a heat block set at 100 °C, and steamed for 5 min. The wet tissue paper was then removed and the slide washed under a running tap, before being counterstained with safranin red for 30 seconds. The slide was washed once again with water and air-dried. Images were captured at 100x magnification using a Leica DMLA microscope, then analysed using ImageJ software (Schneider *et al.* 2012).

4.5.4 Enumeration of purified *C. difficile* spores

To enumerate spore yield, spore aliquots were serially diluted in PBS and plated onto BHIS agar supplemented with 0.1% (w/v) sodium taurocholate. After 48 h, spore titre was enumerated by counting vegetative bacterium CFUs. Sporulation efficiency was then determined by comparing CFU/mL between treated and untreated samples (**Table 4.2**).

Table 4.2 The sporulation characteristics of five *C. difficile* clinical isolates used in the study. To determine spore yield, spores were serially diluted and plated onto BHIS agar supplemented with 0.1% (w/v) sodium taurocholate. After 48h, germinated spores were enumerated by quantifying bacterial CFU. Sporulation efficiency was determined by comparing CFU/mL between treated and untreated samples (data expressed as mean ± SEM; for each isolate N=2, n=3).

C. difficile isolate	Sporulation efficiency (%)	Spore yield (x10^9 CFU/mL)
98011	82.7 ± 6.9	4.6 ± 0.3
98220	71.6 ± 5.7	4.4 ± 0.4
108536	75.9 ± 4.9	5.9 ± 0.3
108906	83.0 ± 5.2	6.7 ± 0.4
1342	76.3 ± 1.3	8.0 ± 0.1

4.6 BACTERIAL ADHESION ASSAYS

Soluble NSPs were evaluated for their ability to inhibit the epithelial adhesion of *C. difficile* and ETEC, as well as *Salmonella* Typhimurium, which was used as a positive control (Parsons *et al*. 2014). In addition, soluble plantain NSP was evaluated for its ability to inhibit the epithelial adhesion of purified *C. difficile* spores.

Intestinal epithelial cell-lines were seeded at 1 x 10^5 cells/well in 24-well tissue culture plates, and incubated in complete DMEM at 37°C in a humidified atmosphere of 5% CO_2 and 95% air, for 24 h. The following day, confluent cell monolayers were washed three times with pre-warmed sterile PBS, before undergoing a 30 min pre-treatment with or without soluble NSP (0-20 mg/mL in antibiotic-free DMEM). The soluble NSP concentrations that were tested were within the range of effective luminal concentrations in the human distal colon that would be readily achievable with dietary supplementation (Parsons *et al.* 2014; Roberts *et al.* 2010; Roberts *et al.* 2013).

Cell monolayers were then infected with *C. difficile* bacteria or spores (2 h), ETEC (1.5 h) or *S.* Typhimurium (1.5 h) at a multiplicity of infection (MOI) of 100. Following infection, cells were washed three times with sterile PBS to remove any non-adherent bacteria and then lysed using sterile 1% (v/v) Triton X-100 in deionised water. The epithelial adhesion of bacteria and spores was enumerated by performing serial dilutions of cell lysates, then plating out 20 µL lysate on agar in triplicate. *C. difficile* bacteria and spores were plated out on BHIS agar and BHIS agar supplemented with 0.1% (w/v) sodium taurocholate, respectively, before incubation for 48 h at 37°C in anaerobic conditions. All other bacteria were plated onto LB agar and incubated for 24 h at 37°C in aerobic conditions. Bacterial CFUs were then quantified to assess bacterial adhesion.

4.7 BACTERIAL GROWTH ASSAYS

To assess the effect of plantain NSP on bacterial growth, plantain NSP was incubated with bacteria in the absence of intestinal epithelial cells. Plantain NSP was solubilised in antibiotic-free DMEM to make final concentrations of 0, 5, 10 and 20 mg/mL. 1mL of each concentration was then added to a 24-well culture plate in triplicate. Subsequently, *C. difficile* or ETEC was added to each well at 1 x 10^5 CFU/mL, and the plate incubated for 2 h and 1.5 h, respectively. At specific time points, 200 µL media was removed from each well (*C. difficile* - 0, 60 and 120 min; ETEC – 0, 30 and 90 min). Serial dilutions were then performed and 20 µL of media plated on agar. *C. difficile* was grown on BHIS agar in anaerobic conditions at 37°C for 48 h, whilst ETEC was grown on LB agar in aerobic conditions for 24 h. Bacterial numbers were then assessed by quantifying CFU/mL.

4.8 TREATMENT OF CACO2 CELLS WITH *C. difficile* TcdA AND TcdB

Plantain NSP was assessed for its ability to reduce epithelial cell damage and inflammation induced by purified native *C. difficile* toxins TcdA and TcdB, which were generously provided by Dr Fabio Miyajima (Institute of Translational Medicine, University of Liverpool). The purified *C. difficile* toxins, derived from reference *C. difficile* strain vpi10463, were originally obtained through collaboration with Dr Clifford Shone from Public Health England (formerly Health Protection Agency; Porton, UK) (Maynard-Smith *et al.* 2014).

Caco2 cells were seeded at 2 x 10^5 cells/well in 6-well tissue culture plates and maintained in complete DMEM at 37°C with 5% CO_2, 95% air, for 24 h. Cell monolayers were then washed three times with pre-warmed sterile PBS before undergoing a 30 min pre-

treatment with or without 10 mg/mL plantain NSP. Subsequently, cells were treated with *C. difficile* TcdA or TcdB at concentrations ranging from 1-100 ng/mL for 0-48 h. Cell culture media was then harvested for future analysis.

4.9 TREATMENT OF HT29 CELLS WITH MUCOSALLY ASSOCIATED *E. coli* FLAGELLIN

Plantain NSP was assessed for its ability to reduce the epithelial cell damage and inflammation induced by bacterial components such as flagellin. Cells were seeded in 24-well plates at 2 x 10^5 cells/well and incubated for 24 h to form confluent monolayers. Cells were then washed three times with pre-warmed sterile PBS and pre-incubated for 30 min with 10 mg/mL plantain NSP.

Five UC *E. coli* strains (HM250, HM295, HM378, HM380 and HM387) were grown as described in *Section 4.4.2* and suspended in sterile water following three washes. Bacteria were pooled to generate a bacterial suspension of OD 0.125 at 550_{nm} and *E. coli* were then filtered using a sterile 0.2 µm filter. The resultant bacteria-free supernatant, containing flagellae (flagellin) and outer membrane vesicles (OMVs), as shown by Subramanian and colleagues (Subramanian *et al.*, 2008) was then used to treat cells at a volume of 50 µL/well. In parallel, cells were individually treated with each UC *E. coli* isolate at an MOI of 10, or with 1 µg/mL purified flagellin (or vehicle control), for 4 h at 37°C. Cell culture media was then harvested for future analysis.

4.10 TREATMENT OF CACO2 CELLS WITH BACTERIAL LIPOPOLYSACCHARIDE (LPS)

Plantain NSP was assessed for its ability to inhibit the epithelial cell damage and inflammation mediated by bacterial LPS. Caco2 cells were seeded at 1 x 10^5 cells/mL in 24-well tissue culture plates, and incubated for 24 h at 37°C in humidified conditions of 5% CO_2 and 95% air. The next day, cell monolayers were washed three times with sterile PBS and pre-treated for 30 min with or without 10 mg/mL plantain NSP. Cells were then treated with LPS (*E. coli* 0111: B4; Sigma-Aldrich) at 1, 100 or 1000 µg/mL (or saline vehicle control) and incubated for 24 h. Cell culture media was then harvested and stored at -20°C for future analysis.

4.11 MODIFIED BACTERIAL ADHESION ASSAYS

To determine if the inhibitory action of plantain NSP against diarrhoeal pathogens occurred via an action of the epithelial monolayer or through direct interaction with bacteria, modifications were made to the bacterial adhesion assays described in *Section 4.6* (Parsons *et al.* 2014).

To test if soluble plantain NSP exhibited its inhibitory effect via interaction with the intestinal epithelium, plantain NSP was added to cell monolayers 30 min prior to infection as earlier described, but then removed by three washes with pre-warmed sterile PBS (1 min each; 37°C). Monolayers were then provided fresh antibiotic-free DMEM and infected with bacteria as normal. To test if soluble plantain NSP exhibited its inhibitory effect via a direct interaction with bacteria, plantain NSP was pre-incubated with bacteria for 30 min, followed by centrifugation, re-suspension of bacteria in antibiotic-free media and inoculation of

epithelial cell monolayers. These alternative conditions were performed according to the methods described in *Section 4.6*.

4.12 CHLORIDE CHANNEL ACTIVITY ASSAYS

Studies were performed to assess if the inhibitory action of plantain NSP against diarrhoeal pathogens was mediated by increased electrogenic chloride secretion. Cellular chloride secretion was assessed using a simple colorimetric assay that measures cellular iodide efflux as a means of monitoring chloride channel activity, as previously described (Tang and Wildey 2004).

4.12.1 Chloride channel activation

Plantain NSP was first evaluated for its ability to activate epithelial chloride channel activation alongside known chloride channel activators, forskolin and RP107 (Noel *et al.* 2006) which were used as positive controls. Caco2 cells were seeded in a 96-well plate at 2.5 x 10^4 cells/well and incubated for 24 h at 37°C. Cells were then loaded with 100 µL pre-warmed iodine loading buffer (containing 150 mM NaI; see *Appendix 1* for full recipe) and incubated for 4 h at 37°C (iodine loading step). Subsequently, cells were washed three times with sterile PBS (pH 7.4), and incubated for 30 min with 100 µL plantain NSP (2.5 - 10 mg/mL), forskolin (12.5 – 200 µM), RP107 (12.5 – 200 µM) or saline vehicle control. Following incubation, cells were lysed with 1% (v/v) Triton-X-100 in deionised water. Intracellular iodine concentration was determined using the modified Sandell-Kolthoff (SK) reaction, in which 100 µL cell lysate was mixed in a 1:1 ratio with detection buffer 2. Detection buffer 1 (100 µL) was then added

to the mixture, and incubated for 5 min at room temperature before measuring absorbance at OD 410nm. For full recipes of the reagents used in chloride channel activation assays, see *Appendix 1.*

4.12.2 Chloride channel inhibition

Chloride channel antagonists, cystic fibrosis conductance regulator (CFTR) inhibitor 172 (CFTR-inh-172) and 5-nitro-2-(-3-phenylpropyl-amino) benzoic acid (NPPB), were next evaluated for their ability to block cellular chloride channel activation (Keeling *et al.* 1991; Linsdell 2014). Caco2 cells were treated with 100 µL CFTR-inh-172 (12.5 – 200 µM) or NPPB (50 – 800 µM) during the iodine-loading step. As previously described, cells were then incubated for 4 h before undergoing 30 min incubation with plantain NSP, forskolin or RP107. Cells were subsequently lysed and the intracellular iodine concentration determined using the SK reaction.

4.12.3 Use of chloride channel modulators in bacterial adhesion assays

Following confirmation that chloride channel modulators had a measurable effect on cellular chloride channel activity, these drugs were used in bacterial adhesion assays to assess their effect on bacterial adhesion. Bacterial adhesion assays were carried out as previously described in *Section 4.6*, with some modifications.

To assess the effect of chloride channel activation on bacterial adhesion, cell monolayers were pre-treated with or without 10 mg/mL plantain NSP, and in parallel, 100 µM

RP107 or 100 µM forskolin, for 30 min prior to infection with bacteria. The remainder of the bacterial adhesion assay was performed as usual.

To assess the effect of chloride channel inhibition on bacterial adhesion, cell monolayers underwent an additional pre-treatment with 200 µM CFTR-inh-172 or 800 µM NPPB for 1 h prior to treatment with plantain NSP or chloride channel activators and subsequent bacterial inoculation.

4.13 FLUORESCENCE MICROSCOPY

4.13.1 Phalloidin/4',6-diamidino-2-phenylindole (DAPI) staining to assess cellular cytotoxicity

Cell rounding was assessed using fluorescence microscopy. Briefly, Caco2 cells were seeded at 1 x 10^{5} cells/mL in a 24-well culture plate on 13mm coverslips, and incubated for 48h. Following toxin treatment, cells were fixed with 2% (v/v) paraformaldehyde (PFA) for 10 min, and then washed 3 times with sterile PBS. Actin filaments were visualised by adding AlexaFluor®488-phalloidin (Life Technologies Ltd, Paisley, UK) (Excitation/Emission: 350/470 nm) to cells at a ratio of 1:100 for 30 min. Cells were washed three times with sterile PBS and the coverslips then air dried. Cell nuclei were visualised by counterstaining with 4'6-diamidino-2-phenylindole (DAPI), in which coverslips were mounted on glass slides using VectaShield + DAPI mounting media (Vector Laboratories Ltd, Peterborough, UK) (Excitation/Emission: 495/519). Images were captured using an Olympus BX51 fluorescent microscope.

4.13.2 Immunocytoflourescence to assess nuclear translocation of β-catenin

The intracellular localisation of β-catenin was visualised by immunocytofluorescence microscopy. Cells were seeded at 1 x 10^5 cells/mL in a 24-well culture plate on 13mm coverslips, and incubated for 48h at 37°C. Following treatment, cells were washed three times with sterile PBS and fixed with 100% methanol for 15 min at -20°C. To aid cell permeabilisation, cells were treated with 1 mL PBS containing 5% (w/v) BSA and 1% (v/v) Triton-X100 for 15 min. Cells were then washed three times with sterile PBS and blocked overnight with PBS containing 5% (w/v) BSA at 4°C. Subsequently, cells were incubated with primary mouse monoclonal IgG anti human β-catenin antibody (Clone 14; BD Biosciences; Oxford, UK) (1:1000) followed by secondary fluorescein isothiocyanate (FITC)-conjugated rabbit polyclonal anti-mouse Ig antibody (Abcam; Cambridge, UK) (1:2000), each for 1 h at room temperature. Three 5 min washes with sterile PBS washes were performed in-between and after each antibody incubation. Finally, coverslips were mounted onto glass slides using VectaShield + DAPI mounting media (Vector Laboratories Ltd). Images were captured using an Olympus BX51 fluorescent microscope.

4.14 GIEMSA MICROSCOPY

Giemsa microscopy was performed to visually assess bacterial adhesion to intestinal epithelial cells. First, intestinal epithelial cells were grown on 13 mm glass coverslips in 12-well tissue culture plates at 2 x 10^5 cells/well. The plate was incubated at 37°C for 24 h and washed three times with sterile PBS. Cells were then pre-incubated with or without 10 mg/mL soluble plantain NSP for 30 min prior to infection with bacteria at an MOI of 100. Following incubation with bacteria (*C. difficile*, 2 h; ETEC, 1.5 h), cells were washed three times with

sterile PBS and then fixed with 70% (v/v) ethanol for 20 min at room temperature. The coverslips were washed three times with sterile PBS and then stained with 10% (v/v) Giemsa solution (Sigma Aldrich) for 30 min at room temperature. Coverslips were mounted with cell monolayers face down, onto glass slides using DPX (Distrene, Plasticiser, Xylene) mounting media (Leica Microsystems; Milton Keynes, UK). Images were captured at 100x magnification using a Leica DMLA microscope, then analysed using ImageJ software (Schneider *et al.* 2012).

4.15 QUANTIFICATION OF PRO-INFLAMMATORY INTERLEUKIN 8 (IL-8) RELEASE BY ELISA

Harvested media from cells was measured for the presence of IL-8 using a solid phase sandwich ELISA (IL-8 Human Elipair Kit; Abcam; ab48483). 50 µL capture antibody specific for IL-8 (B-K8) was diluted in 10 mL 0.5M carbonate-bicarbonate buffer (pH 9.6). 100 µL diluted capture antibody was then coated into 96 well high-binding γ-irradiated ELISA plates (Corning/Co-star) overnight at 4°C. Following three washes with 120µL PBS-0.1% (v/v) Tween-20 buffer (pH 7.4), capture antibody coated plates were blocked using 250µL 1% (w/v) BSA in PBS-Tween for 2h at room temperature. Triplicate treatment samples (100µL) of cell free medium were then incubated overnight at 4°C, plates washed three times with 120 µL PBS-0.1% (v/v) Tween-20 and 50 µL anti-IL8 biotinylated detection antibody added for 2 h at room temperature. Subsequently, 100 µL HRP-streptavidin was added to each well, incubated for 20 min at room temperature and then washed three times with 120 µL PBS-Tween. The ELISA was developed with *o*-phenylenediamine (OPD) substrate (SigmaFast), as per manufactures instructions, incubated in the dark for 10 min and stopped with 100µL 4M H_2SO_4. Optical density was then measured at OD 492 nm. Recombinant IL-8 standards (0-2000 pg/mL) and water blanks were also included on each ELISA plate. IL-8 concentration of cell culture

samples was determined against the IL-8 standard calibration curve (**Figure 4.5**). The within-assay of co-efficient was 7.8% (n=8) and the between assay co-efficient of variation was 7.3% (N=3).

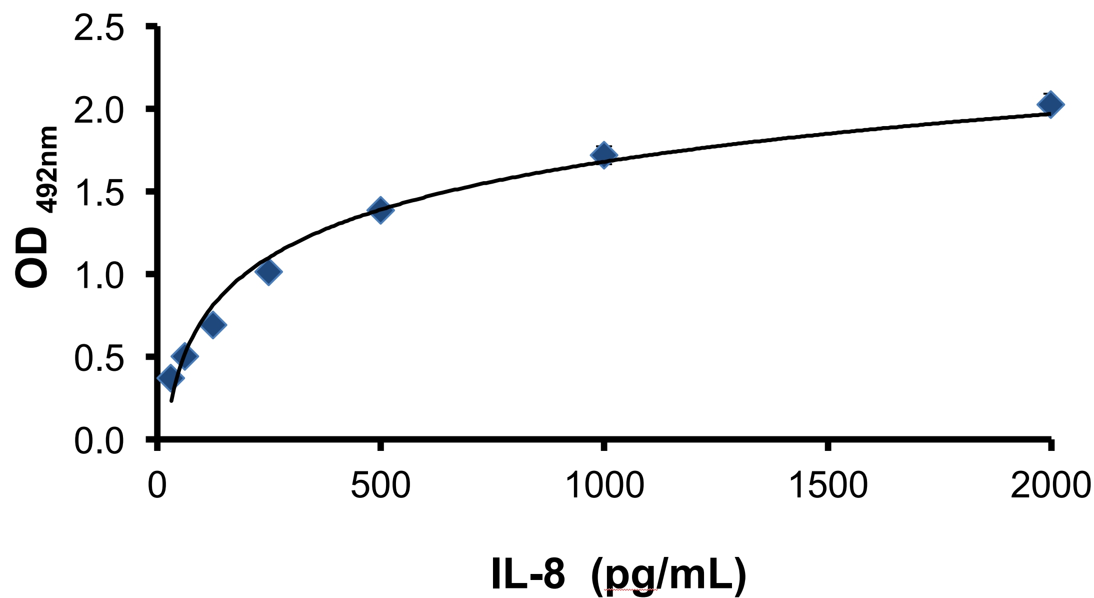


Figure 4.5 IL-8 calibration curve used to determine the unknown IL-8 concentration of culture medium harvested from intestinal epithelial cells. Recombinant IL-8 standards (0-2000 pg/mL in cell culture medium) were added in triplicate and IL-8 concentration determined using an IL-8 Human Elipair Kit (Abcam) (Representative figure; N=1, n=3).

4.16 MEASUREMENT OF CASPASE-3 ACTIVATION

Caco2 cells were seeded at 1 x 10^4 cells/mL in clear bottomed, white walled 96-well tissue culture plates (Corning/Costar) and cultured overnight at 37°C in humidified conditions of 95% air, 5% CO_2. Following treatment, cell monolayers were assayed for active caspase-3 using a commercial Caspase-Glo® 3/7 Assay (Promega; Southampton, UK) as per manufacturer's instructions. Luminescence was detected using the Tecan Infinite F200 plate reader (Tecan, Reading, UK). Experiments were performed in triplicate, with background luminescence determined from wells containing culture medium without cells subtracted from results.

4.17 MEASUREMENT OF ADENYLATE KINASE

Media harvested from cell monolayers and was assessed for the presence of adenylate kinase (AK) using Toxilight Bioassay Kit (Lonza; Slough, UK). The assay was performed according to the manufacturer's instructions, using white walled 96-well tissue culture plates with flat clear bottoms. Luminescence was detected using the Tecan F200 plate reader (as above *Section 4.16*). Experiments were performed in triplicate and results were expressed as a percentage of total cell adenylate kinase (AK) released from untreated cells, lysed using Toxilight 100% Lysis Reagent kit (Lonza).

4.18 IMMUNOBLOTTING

Intestinal epithelial cells were lysed in ice cold 20 mM Tris-HCl buffer, p.H 7.4 and resultant whole cell lysates then resuspended in 250 µL sample buffer. The suspension was then vortexed for 30 seconds to ensure a homogenous sample and boiled at 90°C for 5 min.

Whole cell lysates (25 µL) were separated by SDS-PAGE using 4 – 15% Mini-PROTEAN® TGX™ precast protein gels (Biorad; Hemel Hempstead, UK) for 2 h, at 150 V, followed by electrotransfer to nitrocellulose (1 h, 100 V). Nitrocellulose membranes were blocked using 1% (v/v) bovine serum albumin (BSA) in PBS containing 0.1% (v/v) Tween-20 overnight at 4°C, then probed with the primary antibody; anti-Rac1 102 (BD Transduction Lab; Oxford, UK), which has been shown to lose affinity for Rac1 following monoglucosylation, or anti-Rac 238a (Abcam; Cambridge, UK), which recognises both unglucosylated and mono-glucosylated Rac1 (Gerhard *et al.* 2008). Both antibodies were diluted 1:1000 in PBS-0.1% (v/v) Tween-20 and incubated for 2h at room temperature, followed by three 5 min washes with the same buffer.

Blots were then incubated with an appropriate rabbit anti-mouse Ig horseradish peroxidase (HRP)-conjugated secondary antibody (1:2000) for 1 h at room temperature. Membranes were again washed three times with PBS-0.1% (v/v) Tween-20 and protein bands visualised with SuperSignal West Dura Extended Duration Substrate (Fischer Scientific UK Ltd; Loughborough, UK). Using Image Lab Software v. 3.0.1 (Biorad), images were subjected to quantitative densitometric analysis of protein bands.

4.19 STATISTICAL ANALYSIS

Data is expressed as mean ± standard error of the mean (SEM). N numbers indicate the total number of independent experiments performed, where each experiment was performed with at least n=3 replicates for any individual treatment group, unless otherwise indicated. Independent sample groups were first assessed for normality and equality of variance using Shapiro Wilks and F-test. Two-sample comparisons were performed using unpaired Students t-test or Mann Whitney U as appropriate. Multiple treatment groups were analysed using either the one-way analysis of variance (ANOVA) or non-parametric Kruskal-Wallis ANOVA, followed by pair-wise comparisons of treatment means (Stats-Direct v2.6.6; Sale, UK). Differences were considered significant when $P<0.05$.

Chapter 5

Soluble plant fibres inhibit the *in vitro* epithelial adhesion of *C. difficile* and Enterotoxigenic *E. coli* bacteria, as well as *C. difficile* spores

5.1 INTRODUCTION

C. difficile and ETEC represent two significant diarrhoeal pathogens that cause significant morbidity and mortality worldwide. Indeed, incidence and mortality rates associated with CDI have increased due to the emergence of hyper-virulent strains, such as the BI/NAP1/027 strain (Freeman *et al.* 2010). In addition, there has been a considerable increase in recurrence and relapse rates associated with CDI (Kelly 2012), thought to be due to the production of robust *C. difficile* endospores. Prevention of ETEC infection is clearly related to improvements in sanitation and food preparation in endemic regions where prevalence is high. However, such improvements would require significant investment and likely take a long time (Qadri *et al.* 2005). With a lack of broadly protective ETEC vaccines (Zhang and Sack 2012) and a low availability of effective microbials in endemic regions (Nataro and Kaper 1998), ETEC therapy mainly relies on use of oral rehydration, which only manages symptoms rather than treating the cause of infection (King *et al.* 2003) Therefore, it is of paramount importance that alternative therapeutic strategies are developed to effectively combat these diarrhoeal diseases.

Adhesion of pathogenic bacteria to host tissues represents an early, but critical step in the pathogenesis of virtually all infections (Lehmann *et al.* 2006). To mediate this process, *C. difficile* and ETEC possess adhesins, which recognise and bind to complementary carbohydrates on the surface of host cells, promoting bacterial-epithelial adherence (Sharon 2006). Indeed, close proximity of *C. difficile* and ETEC to the intestinal epithelium is likely to be essential for the subsequent release of their respective toxins. In addition, studies have also shown that *C. difficile* spores interact with the intestinal epithelium, leading to their persistence within the colonic tract (Paredes-Sabja and Sarker 2012).

It is clear that *C. difficile* and ETEC adhesins, as well as *C. difficile* spores, play a key role in the pathogenesis of infection. Interrupting these potentially harmful bacteria-epithelial interactions could therefore represent a promising therapeutic strategy to prevent and treat CDI and ETEC infection.

Recent studies from our laboratory have demonstrated that soluble dietary plant NSPs can block the epithelial adhesion of a range of diarrhoeal pathogens (including *S.* Typhimurium, *Shigella sonnei* and *E. coli* pathovars) both *in vitro* and *in vivo* (Parsons *et al.* 2014; Roberts *et al.* 2010; Roberts *et al.* 2013). Out of all the soluble fibres tested, NSP from plantain bananas (*Musa* spp.) has shown the greatest efficacy. Preliminary results have also indicated that plantain NSP is effective at inhibiting the *in vitro* epithelial adhesion of diarrhoeal pathogens *C. difficile* and ETEC (Roberts *et al.* 2013).

5.2 HYPOTHESIS

Soluble plant fibres, including those from plantain bananas, can inhibit the *in vitro* epithelial adhesion of *C. difficile* and ETEC bacteria, as well as *C. difficile* spores

5.3 AIMS

1. To evaluate soluble plantain fibre, as well as a range of other soluble dietary fibres, for their efficacy at inhibiting *C. difficile* and ETEC adhesion to intestinal epithelial cells

2. To evaluate soluble plantain fibre for its ability to inhibit the epithelial adhesion of eleven *C. difficile* clinical isolates, which vary in their toxin expression and ribotype status

3. To evaluate soluble plantain fibre for its ability to inhibit the epithelial adhesion of purified *C. difficile* spores

5.4 METHODS

An initial inhibitory screen was first performed to evaluate a range of different soluble plant fibres for their ability to inhibit *C. difficile* and ETEC adhesion to intestinal epithelial Caco2 cells. Apple, bean, blueberry, leek, pear, strawberry and tomato NSP were selected as they represent common sources of dietary fibre from monocots (monocotyledon plants, i.e. having one seed leaf, or cotyledon), whilst oat and plantain NSP are classified as dicots (dicotyledon plants with two seed leaves). NSP fibres that exhibited the highest efficacy in the inhibitory screen were selected for further evaluation in dose-response studies. In addition, soluble NSP was assessed for its ability to inhibit the epithelial adhesion of eleven *C. difficile* clinical isolates of differing ribotype and toxin type, as well as five purified *C. difficile* spore samples, prepared by the methods described in *Section 4.5*.

The inhibitory activity of soluble dietary fibre was assessed using bacterial adhesion assays, for which detailed methods can be found in *Section 4.6*. Briefly, Caco2 cell monolayers were pre-incubated for 30 min with or without soluble NSP (2.5 – 20 mg/mL) and then infected with *C. difficile* 080042 (Ribotype 027a) (MOI of 100; 2 h) or ETEC C410 (serotype 0160, LT+/ST+) (MOI 100; 1.5 h) versus uninfected controls. Following incubation, cells were washed three times to remove any non-adherent bacteria, then lysed and grown overnight on bacterial agar plates to enumerate bacteria adherent to the epithelial monolayer. *C. difficile* and ETEC were cultured on BHIS and LB agar plates, respectively. The same method was used for *C. difficile* spore adhesion assays, but cell lysates were plated on BHIS agar supplemented with 0.1% (w/v) sodium taurocholate to aid spore germination and enumeration of resultant vegetative cells.

5.5 RESULTS

5.5.1 Evaluation of various soluble dietary fibres for their ability to inhibit the *in vitro* epithelial adhesion of *C. difficile* and ETEC

A screen of inhibitory activity was first performed, in which a number of soluble dietary fibres (apple, bean, blueberry, broccoli, celery, leek, oats, pear, pepper, plantain, strawberry and tomato NSP) were assessed for their ability to inhibit the epithelial adhesion of *C. difficile*. Pre-incubation of intestinal cells with 5 mg/mL bean, broccoli, leek, pepper, plantain and tomato NSP all resulted in significant inhibition of bacterial adhesion. In particular, plantain NSP affected the highest level of blockade, reducing *C. difficile* adhesion by 77.0 ± 1.7% in comparison to the untreated control ($P < 0.001$; Kruskal-Wallis; N=3, n=4). In addition, broccoli and leek NSP also resulted in considerable levels of inhibition, reducing *C. difficile* adhesion by 43.6 ± 12.8 % ($P < 0.001$) and 54.8 ± 9.7% ($P < 0.01$), respectively (**Figure 5.1**).

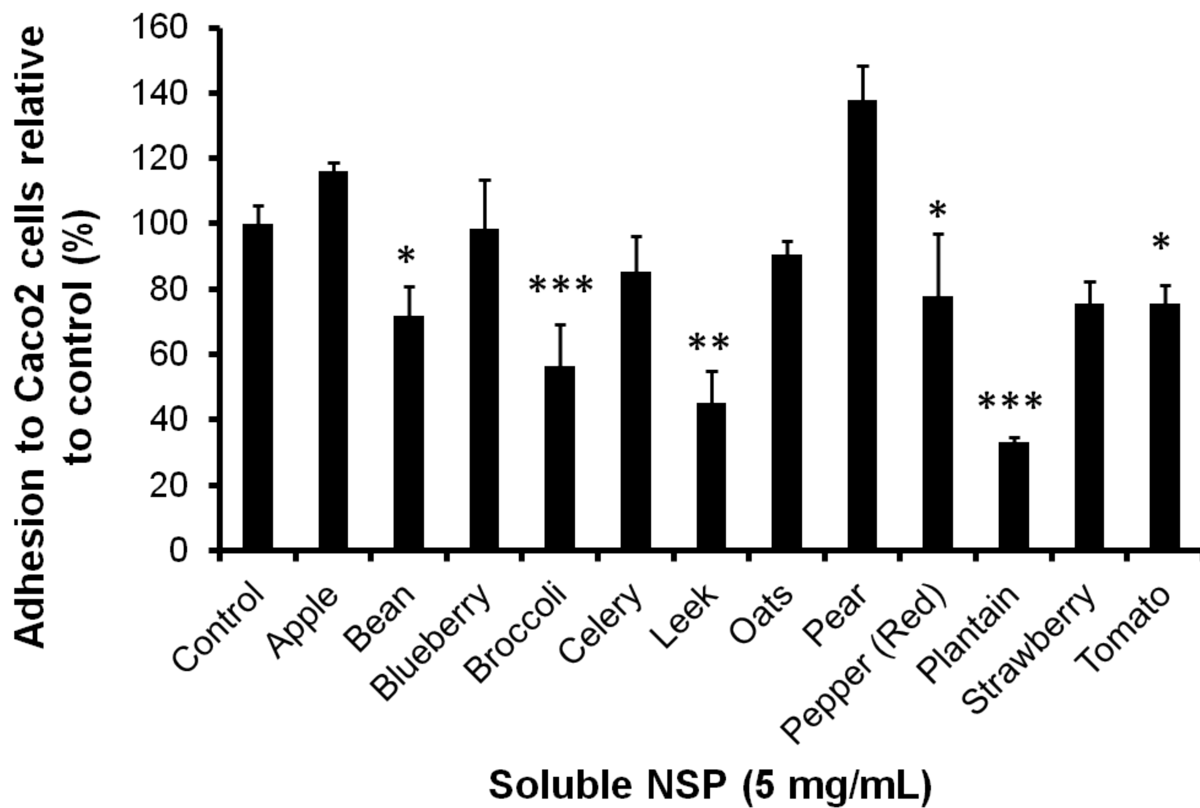


Figure 5.1. Evaluation of a range of soluble NSPs for their ability to inhibit the *in vitro* epithelial adhesion of *C. difficile*. Adhesion of *C. difficile* to confluent Caco2 cell monolayers was significantly inhibited in the presence of 5 mg/mL bean, broccoli, leek, pepper, plantain and tomato NSP. Adhesion was measured as relative to CFU/mL quantified in the absence of any NSP (set as 100%) (All N=3, n=3-4 excepting #N=1, n=3; * P < 0.05, ** P < 0.01, *** P < 0.001; Kruskal-Wallis).

Due to a limited availability of sufficient quantities of alternative dietary fibres, a smaller number of soluble NSPs were selected and assessed for their ability to inhibit the epithelial adhesion of ETEC (including apple, broccoli, leek, oat and plantain NSP only). Pre-incubation of Caco2 cells with 5 mg/mL plantain and leek NSP significantly inhibited bacterial adhesion by 40.9 ± 9.3% ($P < 0.001$) and 53.7 ± 13.6% ($P < 0.01$), respectively. Pre-incubation of Caco2 cells with broccoli, apple and oat NSP did not have a significant effect on the epithelial adhesion of ETEC (**Figure 5.2**).

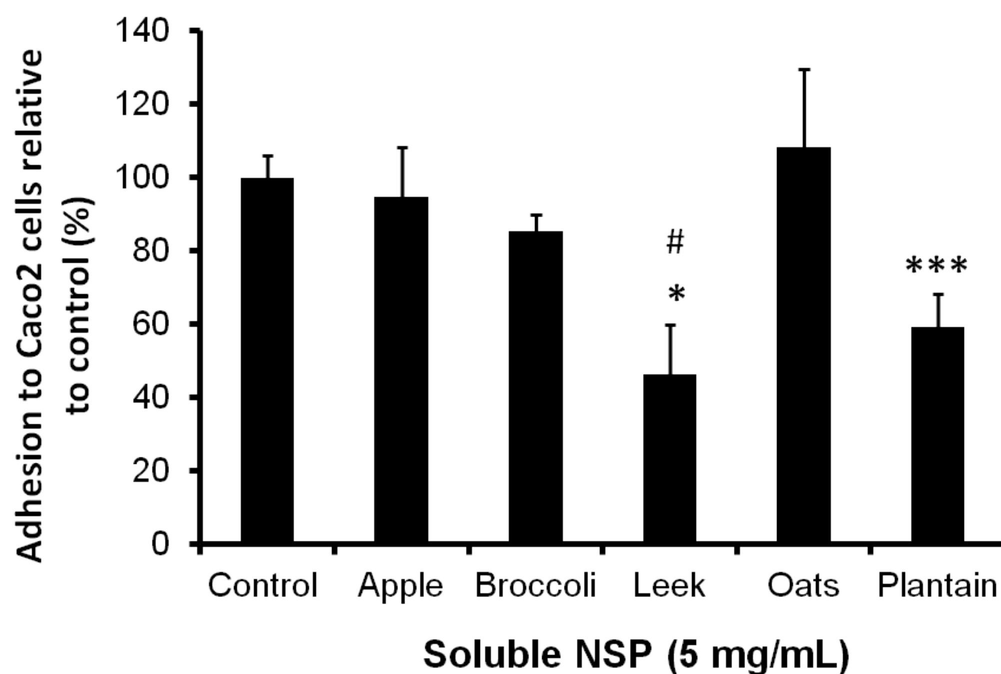


Figure 5.2. Evaluation of a range of soluble NSPs for their ability to inhibit the *in vitro* epithelial adhesion of ETEC. Adhesion of ETEC to confluent Caco2 cell monolayers was significantly inhibited in the presence of 5 mg/mL leek and plantain NSP. Adhesion was measured as relative to CFU/mL quantified in the absence of any NSP (set at 100%) (N=3, n=3 excepting #N=1, n=3; * $P < 0.05$, *** $P < 0.001$; One way ANOVA).

5.5.2 Soluble plantain NSP inhibits the epithelial adhesion of *C. difficile* and ETEC in a dose-dependent manner

Due to the promising results seen with plantain NSP in the initial screen, the soluble fibre was selected for further analysis in dose-response studies. Plantain NSP exhibited a dose-dependent inhibitive effect on *C. difficile*-epithelial cell adhesion, in which NSP concentrations of 2.5, 5, 10 and 20 mg/mL all significantly reduced epithelial interaction of adherent bacterial numbers. The optimum dosage of plantain NSP was 10 mg/mL, which reduced *C. difficile* adhesion by 68.7 ± 8.7% in comparison to the untreated control ($P < 0.001$; Kruskal-Wallis; N=3, n=3). Whilst still significant, a decreased inhibitive effect was observed in the presence of 20 mg/mL plantain NSP, decreasing *C. difficile* adhesion by 42.1 ± 11.4% ($P < 0.01$) (**Figure 5.3**).

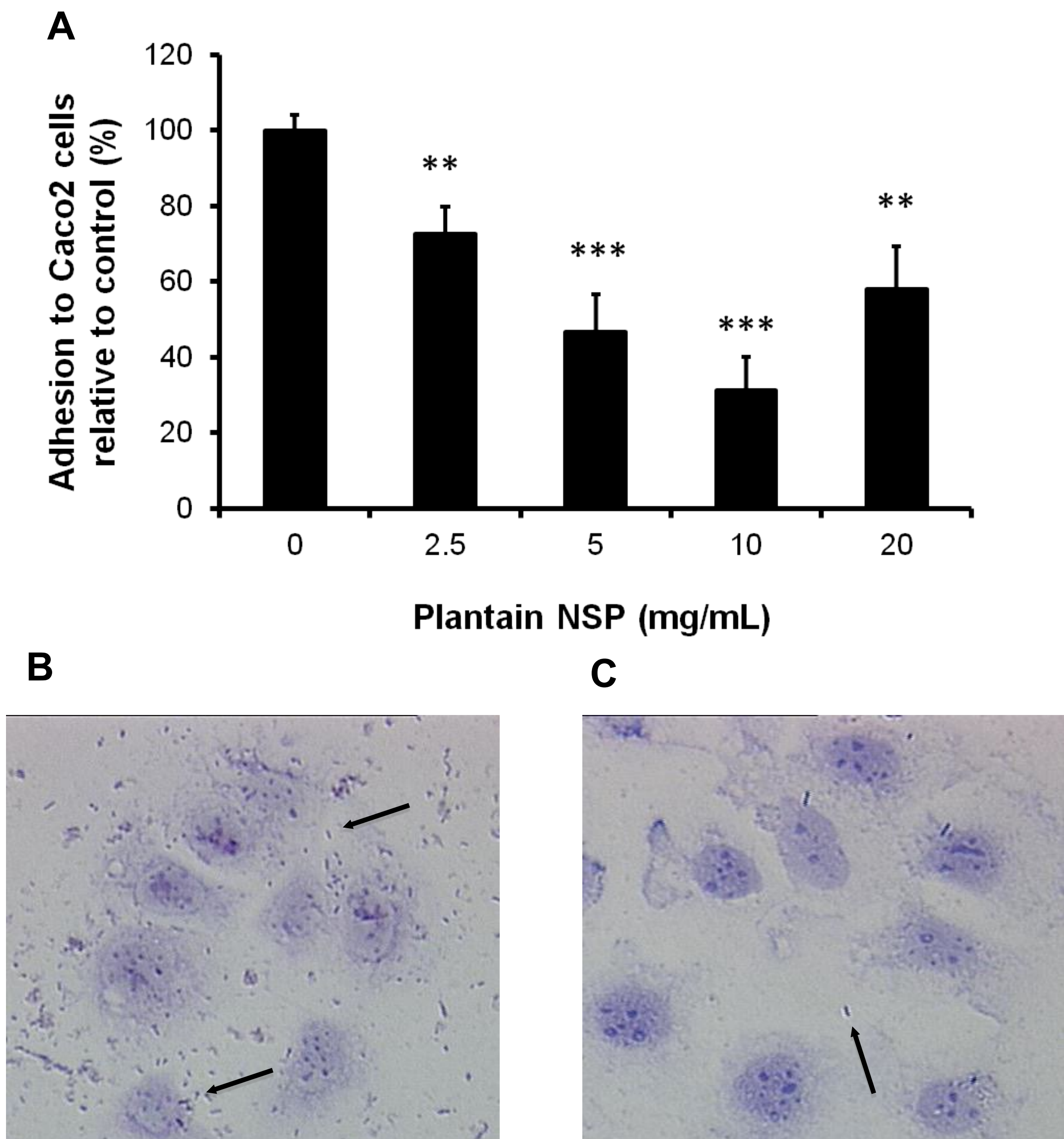


Figure 5.3. Soluble plantain NSP inhibits *C. difficile* adhesion to intestinal epithelial cells in a dose-dependent manner. A) Adhesion of *C. difficile* to confluent Caco2 cell monolayers is significantly inhibited in the presence of 2.5, 5, 10 and 20 mg/mL plantain NSP. Adhesion is expressed relative to CFU/mL found in the absence of any fibre (set as 100%) (N=3, n=3; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; Kruskal-Wallis) **B)** Giemsa stained *C. difficile* infected Caco2 cells in the absence and **C)** presence of 10 mg/mL plantain NSP. Black arrows indicate adherent bacteria.

Plantain NSP also had a dose-dependent inhibitive effect on ETEC adhesion. In the presence of 5 mg/mL plantain NSP, there was significant blockade of bacterial epithelial adhesion, which decreased by 74.7 ± 13.3% ($P < 0.05$; One-way ANOVA; N=3, n=3). As seen with *C. difficile*, higher concentrations of plantain NSP (10 and 20 mg/mL) also exhibited decreased inhibitive activity against ETEC adhesion (**Figure 5.4**).

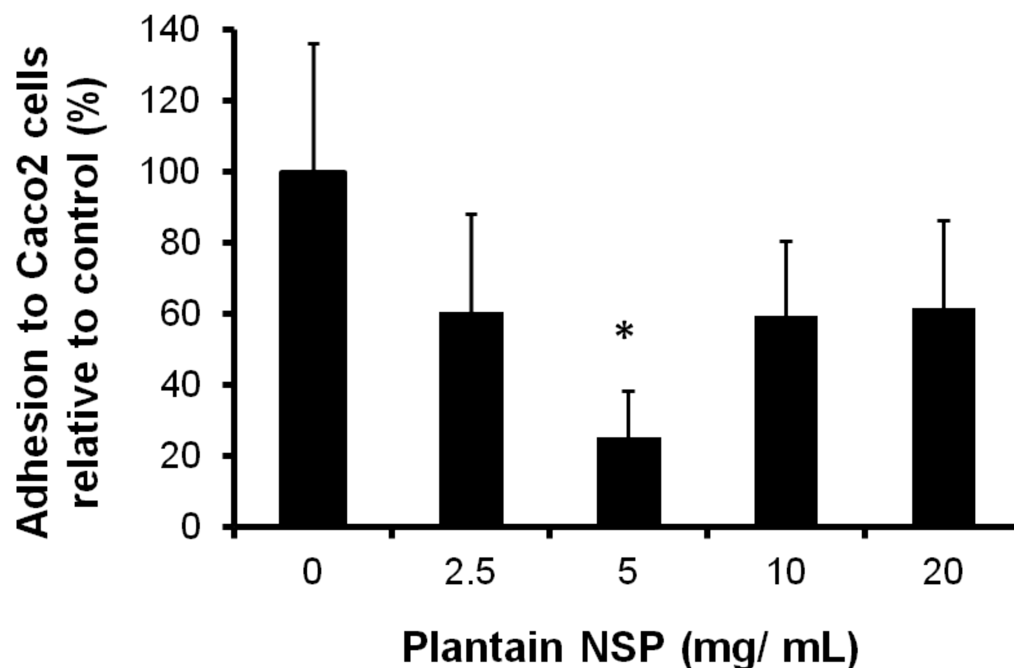


Figure 5.4. Soluble plantain NSP inhibits ETEC adhesion to intestinal epithelial cells in a dose-dependent manner. Adhesion of ETEC to confluent Caco2 cell monolayers was significantly inhibited in the presence of 5 mg/mL plantain NSP. Adhesion was expressed relative to CFU/mL found in the absence of plantain NSP (set as 100%) (N=3, n=3; * $P < 0.05$; One-way ANOVA)

5.5.3 Soluble plantain NSP increases bacterial pathogen growth in a time and concentration dependent manner

In order to investigate the decreased inhibitive effect seen with higher concentrations of plantain NSP (**Figure 5.3**, **Figure 5.4**), an experiment was performed without Caco2 cells to directly determine the effect of plantain NSP on *C. difficile* and ETEC growth (**Figure 5.5A**).

Results indicated that plantain NSP enhances bacterial growth in a time and dose-dependent manner. In the absence of plantain NSP, there was an 8.0 ± 1.4-fold increase in *C. difficile* growth observed between 0 and 2 hours. However, in the presence of 10 and 20 mg/mL plantain NSP, bacterial growth over the same period was enhanced by 10.6 ± 1.5 fold and 13.1 ± 1.9 fold respectively ($P < 0.05$; One-way ANOVA) (**Figure 5.5A**).

Similar results were observed for ETEC. In the absence of plantain NSP, bacterial growth increased by 8.1 ± 1.3 fold, but in the presence of 10 and 20 mg/mL plantain NSP, growth between 0 to 2 hours was enhanced by 11.6 ± 1.2 fold and 12.1 ± 1.1 fold respectively ($P < 0.05$; Kruskal-Wallis) (**Figure 5.5B**)

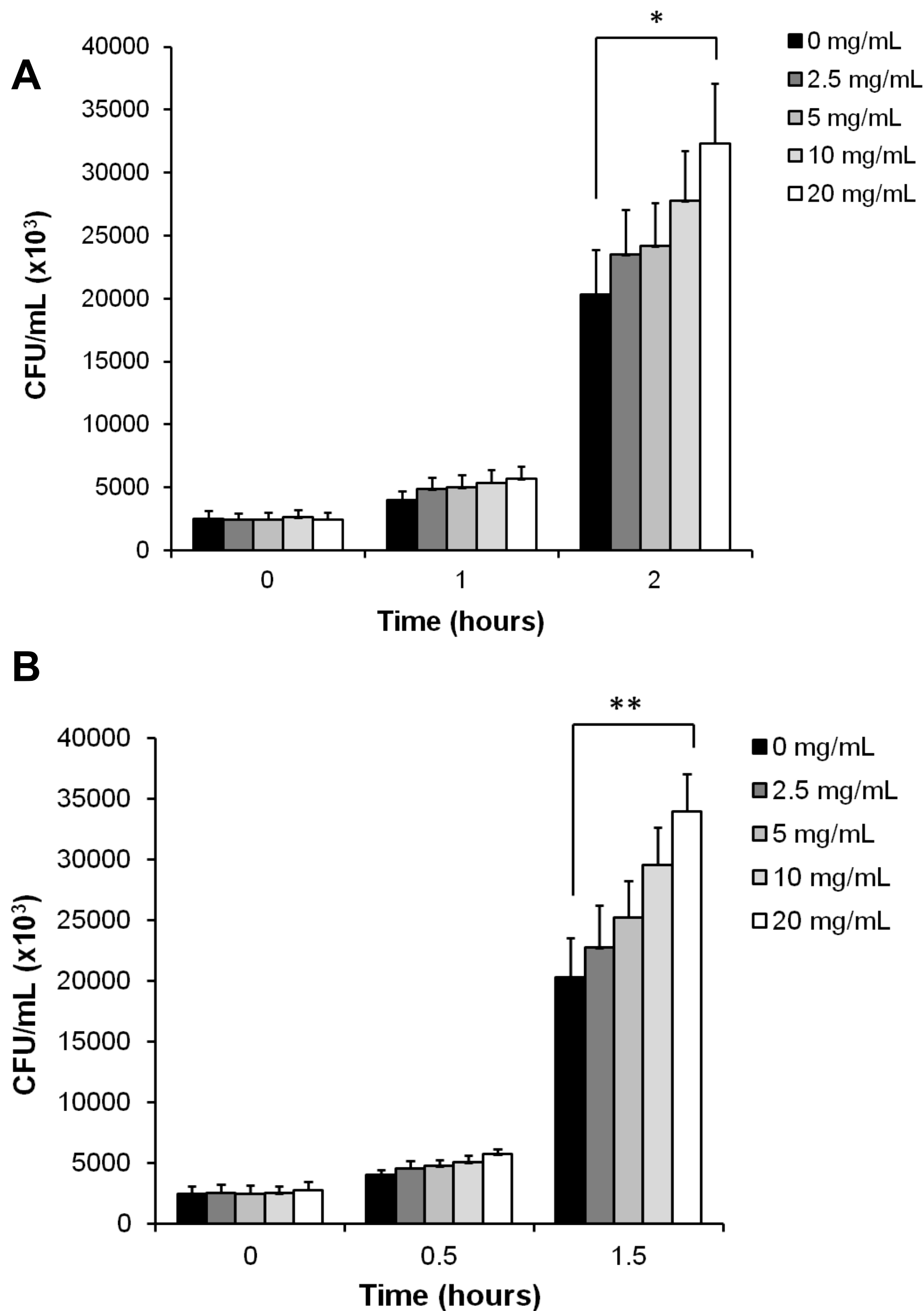


Figure 5.5. Soluble plantain NSP increases bacterial pathogen growth in a time and concentration dependent manner. Whilst there was a natural increase in **A)** *C. difficile* and **B)** ETEC CFU/mL during their respective incubation periods of 2h and 1.5h, this was significantly enhanced in the presence of plantain NSP (N=3, n=2; * P < 0.01, ** P < 0.01, *** P < 0.001; Kruskal-Wallis).

5.5.4 Soluble broccoli and leek NSP also inhibit *C. difficile* epithelial adhesion in a dose-dependent manner, but not as effectively as plantain NSP

Due to the promising results observed in the inhibitory screen, broccoli and leek NSP were also evaluated for their dose-dependent effects on *C. difficile* epithelial adhesion. These indicated a dose-dependent inhibitory effect for leek NSP on epithelial adhesion, with peak effect seen at 20 mg/mL (57.5 ± 6.5%; $P < 0.001$; **Figure 5.6A**). Broccoli was similarly effective at 20 mg/mL (56.9 ± 7.2 %; $P < 0.001$; **Figure 5.6B**). Both of these soluble dietary fibres had a lower efficacy than that seen with plantain NSP; used as a positive control at 10 mg/mL, the epithelial adhesion of *C. difficile* was reduced by 81.8 ± 4.6 ($P < 0.001$; N=2, n ≥ 3).

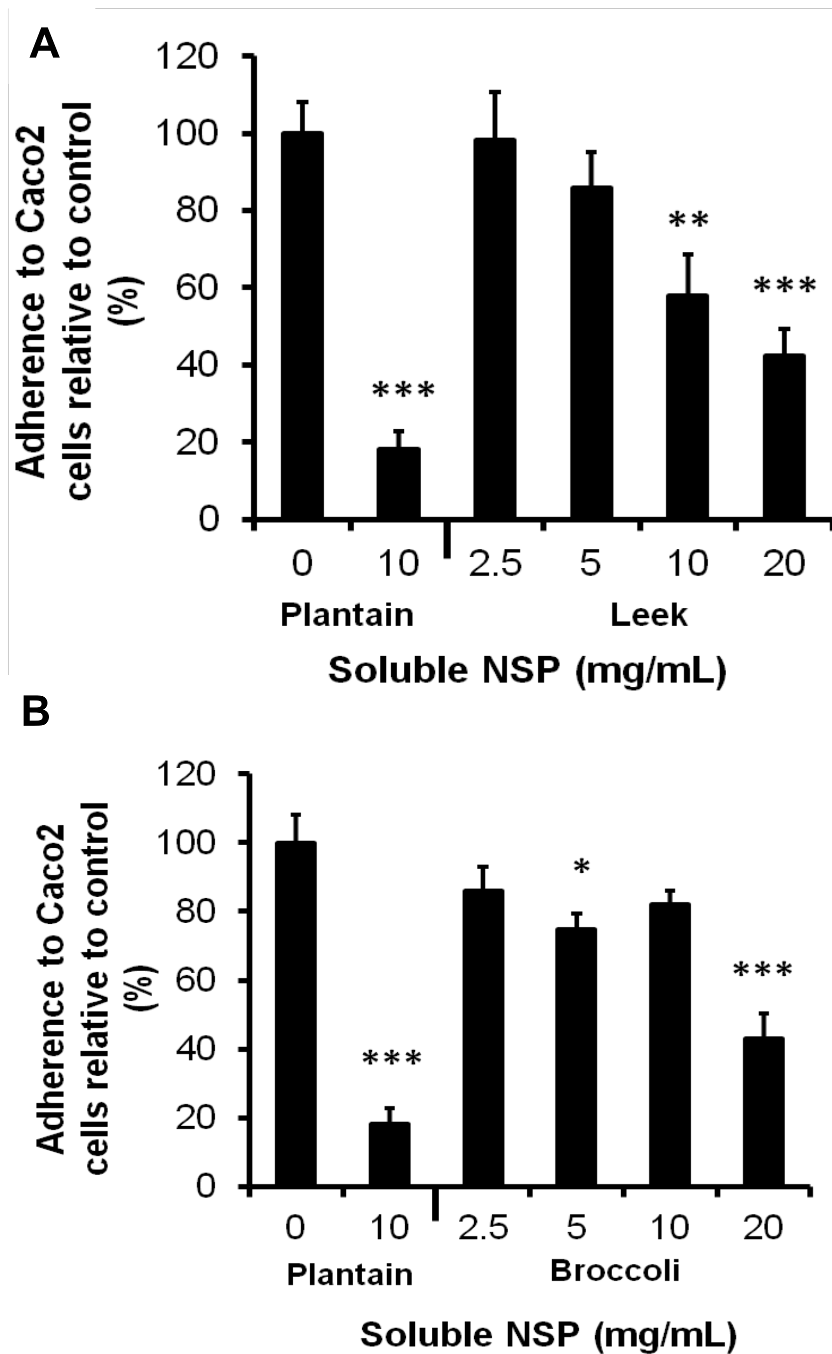


Figure 5.6. Soluble leek and broccoli fibre also inhibit *C. difficile* epithelial adhesion in a dose-dependent manner. The epithelial adhesion of *C. difficile* was inhibited in the presence of **A)** leek and **B)** broccoli NSP. Plantain NSP was used as a positive control. Adhesion was expressed relative to CFU/mL found in the absence of any fibre (set as 100%) (N=2, n ≥ 3; * P < 0.05, ** P < 0.01, *** P < 0.001; Kruskal-Wallis).

3.5.3 Plantain NSP inhibits the epithelial adhesion of a panel of *C. difficile* clinical isolates that vary in their toxin expression and ribotype status

Out of all of the soluble fibres tested, soluble plantain NSP exhibited the most efficacious and significant inhibitive effect against *C. difficile* and ETEC epithelial adhesion. Therefore, soluble plantain fibre was selected for further validation experiments where it was evaluated for its ability to block the adhesion of eleven *C. difficile* clinical isolates differing according to their toxin expression and ribotype status (**Table 4.1**; *Section 4.4.1*).

These studies illustrated that pre-incubation of Caco2 cells with 10 mg/mL plantain NSP (previously determined to be the optimum dose; **Figure 5.3**) significantly inhibited the epithelial adhesion of all eleven *C. difficile* clinical isolates, reducing adhesion by range of 52.2 ± 3.0 to 98.6 ± 1.0%, with a median reduction of 73.8% in comparison to the vehicle-treated control monolayers (**Figure 5.7A-D**; for all; $P < 0.001$; Kruskal-Wallis). These results demonstrate that the inhibitory effect of plantain NSP is independent of *C. difficile* toxin expression and ribotype status.

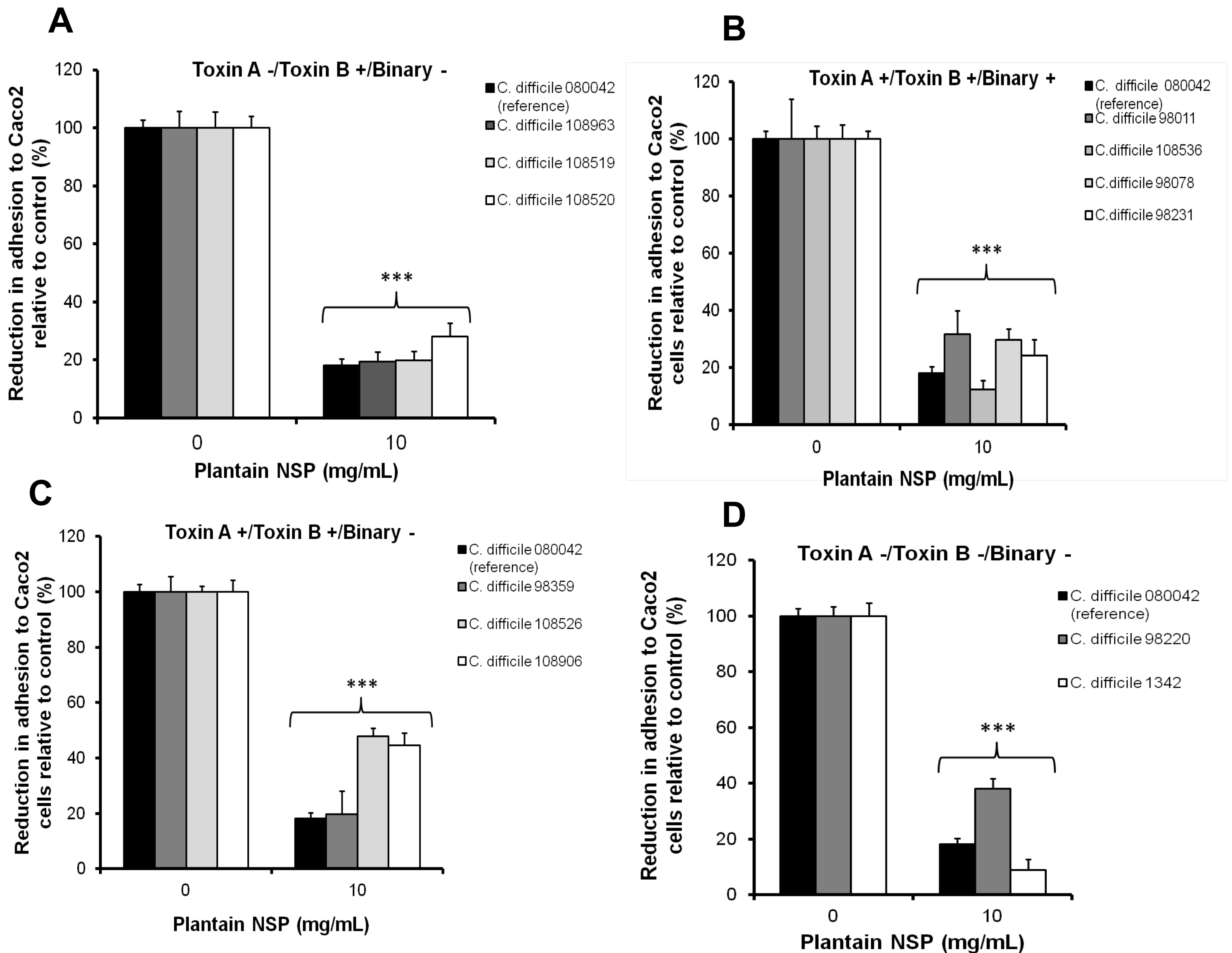


Figure 5.7. Soluble plantain NSP inhibits the epithelial adhesion of a range of *C. difficile* clinical isolates, which differ in their toxin expression and ribotype status. Pre-incubation of Caco2 cells with the optimum dosage of 10 mg/mL plantain NSP significantly inhibited the epithelial adherence of **A)** $TcdA^{+}$ $TcdB^{+}$ $Binary^{+}$ *C. difficile* strains, **B)** $TcdA^{+}$ $TcdB^{+}$ $Binary^{-}$ *C. difficile* strains, **C)** $TcdA^{-}$ $TcdB^{+}$ $Binary^{-}$ *C. difficile* strains, as well as **D)** $TcdA^{-}$ $TcdB^{-}$ $Binary^{-}$ *C. difficile* strains. Our original clinical *C. difficile* isolate (080042, Rb 027) was also used as a positive control. Adhesion is expressed relative to CFU/mL found in the absence of any fibre (set as 100%) (N=3, n = 3; *** $P < 0.001$; Kruskal-Wallis).

5.5.5 Soluble plantain NSP inhibits the epithelial adhesion of purified *C. difficile* spores

Soluble plantain NSP, at 10 mg/mL, significantly reduced the adhesion of *C. difficile* spores purified from clinical isolate CD98011 ($TcdA^+$/$TcdB^+$/$Binary^+$) (**Figure 5.8**) and all four other *C. difficile* isolate spore preparations tested (**Table 5.1**). Inhibition of *C. difficile* spores ranged from 27.9 ± 5.4% to 33.8 ± 6.9% (For all; $P \leq 0.05$; Kruskal-Wallis; **Table 5.1**). Blockade of spores was less than that observed with the corresponding vegetative *C. difficile* bacterium from which the spores were purified, at the same MOI 100, where pre-incubation with 10 mg/mL plantain NSP resulted in inhibition ranging from 78.9 ± 3.1 to 97.9 ± 1.5% (For all isolates; $P < 0.001$, Kruskal-Wallis). It is of significant note, however, that *C. difficile* spores (from all five clinical isolates tested) showed markedly greater adhesion to Caco2 cells (85 ± 7.3% spore adherence versus 4 ± 0.6% vegetative adherence of the original inoculum); therefore, the level of inhibition may have greater significance (**Figure 5.9**).

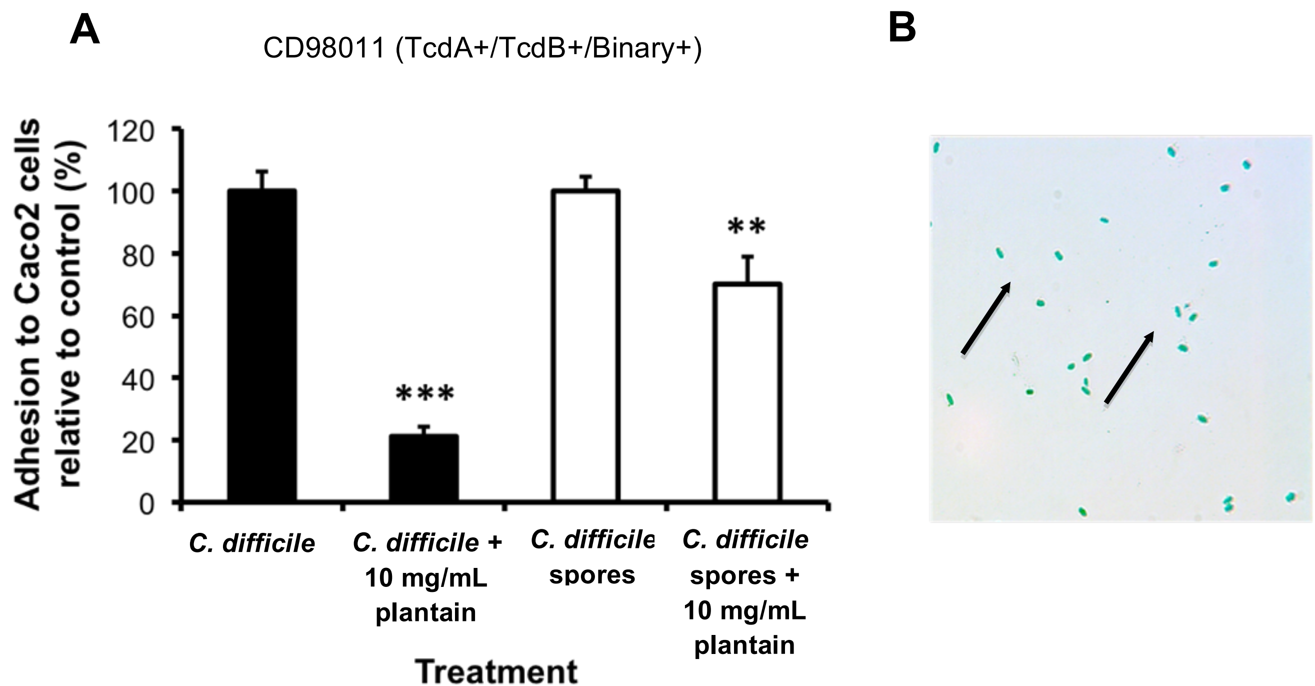


Figure 5.8. Soluble plantain NSP inhibits the *in vitro* epithelial adhesion of *C. difficile* spores purified from clinical isolate CD98011. A) Pre-incubation of Caco2 cells with 10 mg/mL plantain NSP significantly inhibited the adhesion of purified CD98011 spores and corresponding vegetative CD98011, used as a positive control. Adhesion is expressed relative to CFU/mL found in the absence of any fibre (set as 100%) (N=3, n=3; ** $P < 0.01$, *** $P < 0.001$; Kruskal-Wallis). **B)** Schaeffer and Fulton endospore stain of the purified CD98011 spores used in bacterial adhesion assays. Arrows indicate *C. difficile* spores, stained green.

Table 5.1 Soluble plantain NSP inhibits the *in vitro* epithelial adhesion of *C. difficile* spores purified from five clinical *C. difficile* isolates. Pre-incubation of Caco2 cells with 10 mg/mL plantain NSP inhibited the epithelial adhesion of all *C. difficile* spores and their corresponding vegetative *C. difficile* bacterium, used as a positive control.

***C. difficile* isolate**	**Reduction in bacterial adhesion to Caco2 cells (%)**	***P value**	**Reduction in spore adhesion to Caco2 cells (%)**	***P value**
CD98011	93.4 ± 1.9	< 0.001	30.3 ± 5.2	< 0.05
CD108906	92.6 ± 4.3	< 0.001	33.8 ± 6.9	< 0.05
CD108536	86.7 ± 2.0	< 0.001	27.9 ± 5.4	< 0.01
CD98220	78.9 ± 3.1	< 0.001	29.9 ± 8.9	< 0.01
CD1342	97.9 ± 1.5	< 0.001	32.8 ± 14.9	< 0.01

* Statistical significance was determined using Kruskal-Wallis; N ≥ 2, n=3

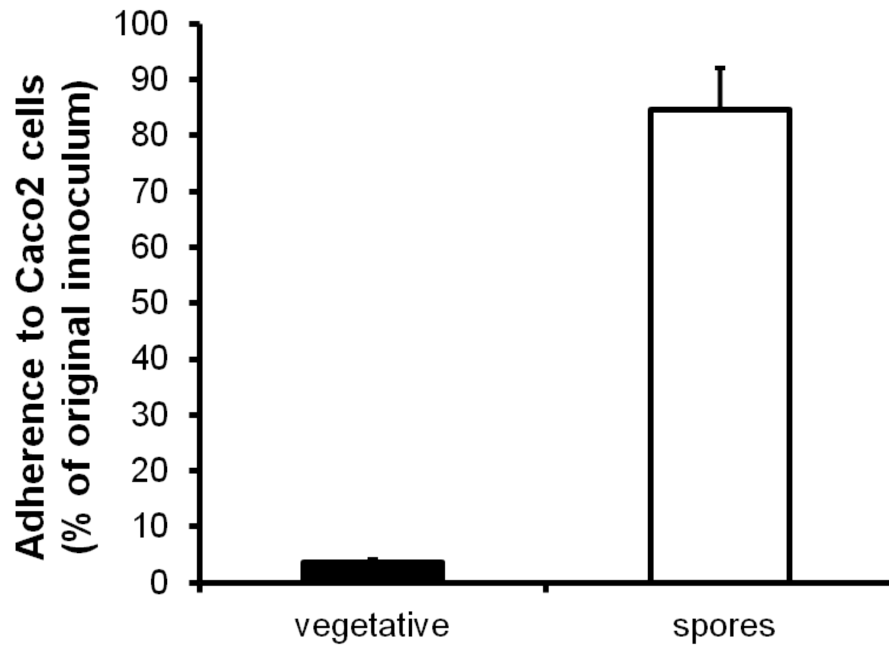


Figure 5.9 *C. difficile* spores are more adhesive to intestinal epithelial cells than their vegetative *C. difficile* counterparts. Vegetative bacterium and spore adhesion is expressed as a percentage of the original inoculum (1 x 10^7 CFU/mL, set as 100%).

5.6 SUMMARY OF RESULTS

1. A range of soluble dietary fibres significantly inhibit *C. difficile* and ETEC adhesion to intestinal Caco2 cells. Out of all fibres tested, soluble plantain NSP exhibits the highest efficacy, with an optimum inhibitive concentration of 10 mg/mL

2. Plantain NSP increases *C. difficile* and ETEC growth in a concentration and time-dependent manner, suggesting that it may be utilised as an energy source for these bacteria

3. Plantain NSP inhibits the epithelial adhesion of eleven *C. difficile* clinical isolates, irrespective of their toxin expression or ribotype status

4. Plantain NSP significantly inhibits the epithelial adhesion of five purified *C. difficile* spore preparations, but to a lesser extent than that observed with vegetative *C. difficile*. However, *C. difficile* spores are over 20 times more adhesive to Caco2 cells so inhibition could be under-represented

5.7 DISCUSSION

Dietary fibre is widely recognised as being beneficial for intestinal health. A high dietary fibre intake increases faecal bulking and viscosity, resulting in increased transit time through the colon (Lattimer and Haub 2010). Furthermore, its fermentation by resident gut microbiota produces short chain fatty acids (SCFAs), such as butyrate, that act as energy sources for the gut epithelium (Macfarlane *et al.* 2006) and possess anti-carcinogenic properties (Lattimer and Haub 2010). Fibre has also attracted considerable attention due to its role in the prebiotic effect, where it may selectively promote the growth of resident health promoting bacteria, such as Lactobacilli and Bifidobacteria (Hamer *et al.* 2008). Our studies suggest an alternative role of soluble fibre in the 'contrabiotic' effect, where it also has the ability to promote health by disrupting potentially harmful interactions between bacteria and the gut epithelium.

Here, we provide evidence towards the inhibitory effect of plantain NSP against diarrhoeal pathogens *C. difficile* and ETEC. Whilst a range of soluble plant fibres (such as broccoli and leek NSP) do significantly inhibit the epithelial adhesion of these bacteria, plantain NSP exhibits a much more significant effect. Furthermore, plantain NSP also significantly inhibits the epithelial adhesion of a range of clinically relevant *C. difficile* isolates, irrespective of their toxin or ribotype status. Indeed, these results imply that soluble plantain fibre could be developed as a potential prophylaxis or treatment for CDI and ETEC infection.

Importantly, we illustrate here that soluble plantain fibre can also inhibit the epithelial adhesion of *C. difficile* spores, which represent the main vehicle of transmission of CDI. Whilst the level of *C. difficile* spore inhibition appears to be less than compared with vegetative *C. difficile*, we observed in our studies that *C. difficile* spores are much more adhesive to

intestinal epithelial cells. Therefore, the level of inhibition may be under-represented and thus hold greater significance. These results are in agreement with previous studies, which have also demonstrated that *C. difficile* spores are much more adhesive to the gut than vegetative cells (Dingle *et al.* 2010; Paredes-Sabja and Sarker 2012), and provide further evidence to suggest that spore adhesion might be exploited by *C. difficile* as a means of persistence in the host (Paredes-Sabja and Sarker 2012). *C. difficile* spore persistence within the colon of CDI patients not only complicates treatment options, but also increases recurrence rates associated with the disease. Indeed, 25 – 85% of all CDI recurrences are attributed to the *C. difficile* strain that caused the initial infection (Barbut *et al.* 2000; Oka *et al.* 2012). Therefore, the ability to inhibit *C. difficile* spore interactions enhances the therapeutic potential of soluble plantain fibre, and suggests that its dietary supplementation might also help maintain remission from CDI.

Plantain banana (*Musa* spp.) is traditionally cooked as a vegetable, and forms an important part of the staple diet in parts of the world such as Africa, India and Central America (Imam and Akter 2011), where the incidence of IBD and colorectal cancer is lower than that observed in Westernised nations. Other evidence to support a protective role of plantain NSP against diarrhoeal disease include a study using banana flakes supplemented to enteral feed to control diarrhoeal episodes (Emery *et al.* 1997), and another controlled trial in Bangladesh in which green boiled banana pulp or pectin was shown to be effective in treating persistent childhood diarrhoea (Rabbani *et al.* 2004; Rabbani *et al.* 2001). Other clinical studies have reported that the juice from boiled green bananas was effective in reducing the severity and duration of persistent diarrhoeas (Alvarez-Acosta *et al.* 2009).

The results presented here support previous findings from our group showing that soluble plantain NSP can block the intestinal interactions of a range of other gut pathogens. Indeed, studies have illustrated that soluble plantain NSP can inhibit the *in vitro* epithelial adhesion, invasion and M-cell translocation of Crohn's mucosa associated AIEC, *S.* Typhimurium and *S. sonnei* (Parsons *et al.* 2014; Roberts *et al.* 2013). The inhibitory effect of soluble plantain fibre has also been confirmed in *ex-vivo* studies*,* whereby plantain NSP significantly reduced AIEC and *S*. Typhimurium translocation across isolated human ileal follicle-associated epithelium (FAE) and villous epithelium (VE) mounted in Ussing chambers (Roberts *et al.* 2010; Roberts *et al.* 2013). Moreover, inhibitory activity has been demonstrated *in vivo* where supplementation of poultry feed with plantain NSP effectively reduces salmonellosis in the chicken (Parsons *et al.* 2014).

We suggest that the diverse array of oligosaccharides present in plantain NSP allow it the capability to inhibit a wide range of bacterial epithelial interactions. However, it is important to note that plantain NSP does not exhibit inhibitory activity against all adherent gut bacteria. Indeed, previous studies demonstrated that plantain NSP lacks the ability to inhibit the adhesion of Enteropathogenic *E. coli* (EPEC) (Roberts *et al.* 2013), which is a major cause of neonatal gastroenteritis (Ochoa and Contreras 2011). Studies have illustrated that EPEC binds intimately to intestinal epithelial cell lines (including Caco2) using key adhesins α1-bundlin and intimin, which are blocked specifically by N-acetyllactosamine (LacNac) (Hyland *et al.* 2008). This might suggest that oligosaccharides bearing this glycoform are perhaps not present within soluble plantain fibre, and therefore this very specific interaction may not inhibited (Roberts *et al.* 2013).

Here, we report that *C. difficile* and ETEC interact with plantain NSP and use this as an energy source. This could provide an explanation as to why higher concentrations of plantain NSP exhibit decreased inhibitory activity against bacterial epithelial adhesion. This has also been observed with respect to *E. coli* (Roberts *et al.* 2010) and *Salmonella* spp., including *S*. Typhimurium (Parsons *et al.* 2014) and *S*. Gallinarum (Parsons *et al.* 2014). In the gut lumen however, this would not be harmful, as bacterial adhesion to the intestinal epithelium would be inhibited. As such, bacteria would simply 'flush through' the gut lumen without releasing their respective toxins. Additionally, the level of blockade of bacterial adhesion is also being under-estimated if only expressed relative to the original inoculum and not those bacterial numbers actually in the medium at the time of sampling. These results suggest that soluble plantain fibre might also act as a fermentable substrate for bacteria in the large intestine, however, modelling of soluble plantain NSP breakdown using mixed faecal microbiota obtained from healthy volunteers has shown that 25-75% of ingested plantain NSP is likely to avoid fermentation in the human colon (Backman 2009; Roberts *et al.* 2010). *In vitro* studies have suggested that Bacteroides are the major fermenters of plantain NSP, whereas species tested from key bacterial groups such as Bifidobacteria, Lactobacolli, Streptococci and Ruminococci cannot easily ferment this soluble dietary fibre source, suggesting little or no prebiotic effect for soluble plantain NSP (Backman 2009).

Soluble plantain fibre, at concentrations of 5 mg/mL or higher, is shown here exhibit inhibitory activity against bacterial adhesion. Importantly, this concentration of fibre is readily achievable in the distal colon by dietary supplementation, even after partial fermentation. Assuming passage of 1 litre of fluid daily into the caecum, oral dosing of humans with 5 g plantain NSP twice daily, with 25% fermentation, would produce effective luminal

concentrations of 10 mg/mL and 7.5 mg/mL in the caecum and rectum, respectively (Roberts *et al.* 2010).

Overall, these results provide convincing evidence to suggest that dietary supplementation with soluble plantain fibre could have a protective effect against intestinal pathogens causing antibiotic-associated diarrhoea and traveller's diarrhoea.

Chapter 6

Soluble plantain fibre reduces the epithelial cell damage and inflammation mediated by *C. difficile* and its toxins

6.1 INTRODUCTION

Following successful colonisation of the intestinal epithelium, *C. difficile* mediates epithelial cellular damage and inflammation through the secretion of two main virulence factors, TcdA and TcdB. These potent exotoxins are involved in the monoglucosylation and subsequent inactivation of small regulatory Rho-GTPases, which includes Rho, Rac and Cdc42 (Voth and Ballard 2005). The irreversible modification of Rho proteins prevents their interaction with downstream signalling proteins such as Rho-kinase (Fujisawa *et al.* 1998), citron K (Madaule *et al.* 1998) and phosphatidylinositol 4-phosphate 5-kinase (Chong *et al.* 1994). This results in a number of toxin-mediated cytopathic effects in the actin cytoskeleton, including loss of actin stress fibres, increased cell rounding and disruption of intracellular tight junctions (Sehr *et al.* 1998; Voth and Ballard 2005).

In addition to modulating cytoskeletal function, *C. difficile* toxins can activate a variety of intracellular signalling cascades responsible for the induction of gene transcription and the production and release of inflammatory mediators, leading to the induction of the pro-inflammatory response (Shen 2012). Indeed, TcdA and TcdB have been shown to mediate the production of several pro-inflammatory cytokines such as IL-8 (Kim *et al.* 2006), which is a potent neutrophil chemoattractant (Sun and Hirota 2015), as well as other pro-inflammatory mediators such as growth-related oncogene alpha (GRO-α) and monocyte-chemotactic protein 1 (MCP-1) (Sun and Hirota 2015; Sun *et al.* 2010). Other toxin-mediated effects include cell cytotoxicity, which results in eventual cell death. Cellular apoptosis is triggered by the activation of executioner caspase-3, via intrinsic and extrinsic apoptotic pathways (Gerhard *et al.* 2008; Sun *et al.* 2010).

To induce their cytotoxic and pro-inflammatory effect, both *C. difficile* TcdA and TcdB must first be internalised into host epithelial cells (Voth and Ballard 2005). Receptor binding is an essential step in toxin internalisation, and as such, this plays an important role in the pathogenesis of infection (Just and Gerhard 2004). Studies have demonstrated that *C. difficile* toxins express lectin activity, whereby they bind specifically to carbohydrate structures present on the host-cell surface (Just and Gerhard 2004; Voth and Ballard 2005). Indeed, TcdA has been shown to possess binding affinity for the disaccharide Galβ1-4GlcNAc, which is expressed on I, Lewis^{X} and Lewis^{Y} carbohydrate antigens present on the human intestinal epithelium (Tucker and Wilkins 1991).

The results from Chapter 5 demonstrate that soluble plantain fibre can disrupt potentially harmful interactions between *C. difficile* bacterium and the intestinal epithelium. As both *C. difficile* and its toxins possess lectin activity, it is possible that soluble plantain fibre will also inhibit the interaction between *C. difficile* toxins and the intestinal epithelium, thus blocking their action and resulting in a protective effect.

6.2 HYPOTHESIS

Soluble plantain fibre can inhibit the epithelial cell damage and inflammation mediated by *C. difficile* and its toxins.

6.3 AIMS

1. To determine whether soluble plantain fibre can inhibit cellular Rac1 monoglucosylation and associated morphological changes mediated by purified *C. difficile* TcdA and TcdB

2. To determine whether soluble plantain fibre can inhibit the pro-inflammatory, cytotoxicity and apoptotic response mediated by *C. difficile* and purified *C. difficile* TcdA and TcdB

6.4 METHODS

Detailed methods can be found in Chapter 4. Briefly, Caco2 cell monolayers were pre-incubated for 30 min with or without 10 mg/mL plantain NSP. Cells were then treated with purified native *C. difficile* TcdA or TcdB (0 – 100 ng/mL over 48h), and in parallel, infected with *C. difficile* clinical isolates CD98011 ($TcdA^+/TcdB^+$) or CD108519 ($TcdA^-/TcdB^+$) at a MOI 100 for 4h. To assess if soluble plantain fibre could block the epithelial action of *C. difficile* toxins, a number of toxin and bacterium-mediated cellular effects were then monitored, which included cytopathic effects such as Rac1 monoglucosylation and cell rounding, as well as induction of the pro-inflammatory, apoptotic and cytotoxicity response.

Cellular Rac1 monoglucosylation was monitored by immunoblotting (see *Section 4.18*) using two monoclonal anti-Rac1 antibodies; clone 102 (BD Transduction Lab), which has previously been shown to lose affinity for Rac1 following its glucosylation (Genth *et al.* 2006), and clone 238a (Abcam), which recognises Rac1 in both its glucosylated and unglucosylated form (Genth *et al.* 2006). Cell rounding was assessed by fluorescence microscopy (*Section 4.13.1*), where actin filaments were visualised by staining Caco2 cells with Phalloidin conjugated to the fluorophore Alexa Fluor 488 (Life Technologies) at a ratio of 1:100 for 30 min. Cell nuclei were visualised using Vectashield mounting media containing DAPI (Vector Laboratories).

To monitor the induction of the inflammatory response, harvested culture media from bacterium- and toxin-treated Caco2 cells were measured for the presence of pro-inflammatory cytokine IL-8, using a solid-phase sandwich ELISA (Abcam) (*Section 4.15*). Cellular apoptosis was measured using a Caspase-Glo® 3/7 Assay (Promega) (*Section 4.16*).

To assess the cytotoxicity response, harvested cell culture media was also measured for the presence of the enzyme AK using a Toxilight™ bioassay kit (Lonza) (*Section 4.17*).

6.5 RESULTS

6.5.1 Soluble plantain fibre does not block *C. difficile* TcdA and TcdB mediated Rac1 monoglucosylation

Peak responses were first determined by treating Caco2 cells with 10 ng/mL TcdA or TcdB over a time-course of 48 h (**Figure 6.1**). Treatment of 10 mg/mL TcdA revealed elevation of cellular levels of mono-glucosylated Rac1 (as indicated by a loss of antibody clone 102 binding to Rac1) 10 h post-toxin treatment, with peak Rac1 mono-glucosylation observed at 24 h compared to vehicle treated control cells ($P < 0.01$; Kruskal-Wallis; N=3, n=3; **Figure 6.1A**). Treatment of cells with 10 ng/mL TcdB also revealed elevated cellular levels of mono-glucosylated Rac1, but this was only significant 48 h post-toxin treatment ($P < 0.01$; Kruskal-Wallis; N=3, n=3; **Figure 6.1B**).

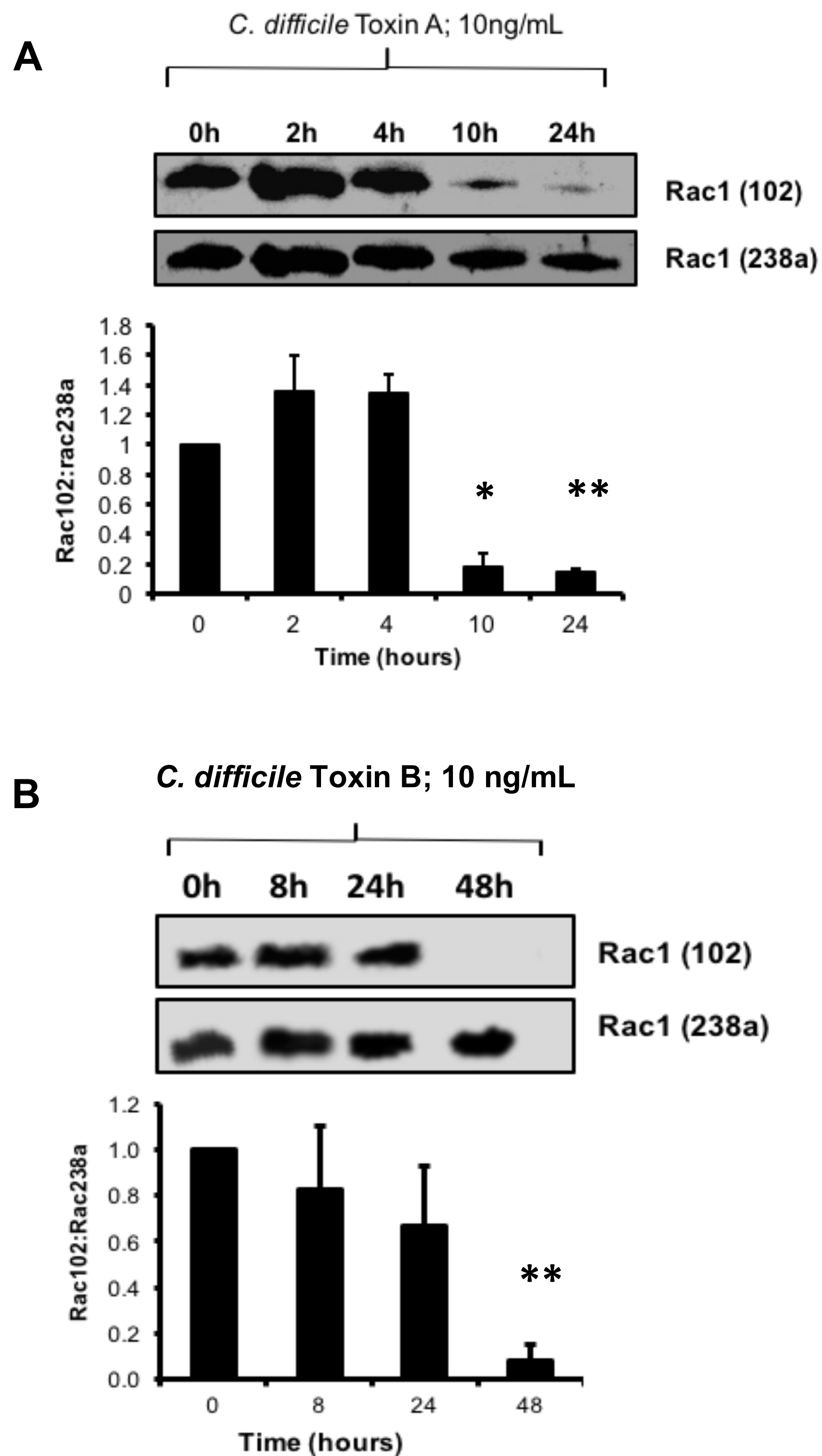


Figure 6.1. ***C. difficile*** **TcdA and TcdB mediate increased Rac1 glucosylation in intestinal epithelial cells.** Immunoblot analysis shows an increase in Rac1 glucosylation following treatment with **A)** TcdA for 24 h and **B)** TcdB for 48 h. Protein bands were analysed densitometrically. Glucosylated Rac1 levels were normalised to total Rac1 (238a) (N=3, n=3; ** P < 0.01; Kruskal-Wallis; representative blots shown).

Caco2 cells were then treated with 0-100 ng/mL *C. difficile* TcdA or TcdB for 24 h and 48 h, respectively, in the absence or presence of 10 mg/mL plantain NSP (**Figure 6.2**). Elevated cellular levels of mono-glucosylation Rac1 were observed following treatment with 1, 10 and 100 ng/mL TcdA, indicated by a loss of anti-Rac1 clone 102 antibody binding to Rac1 (100 ng/mL TcdA; $P < 0.01$; Kruskal-Wallis). However, this was not reversed in the presence of 10 mg/mL plantain NSP (**Figure 6.2A**).

Treatment of Caco2 cells with 1, 10 and 100 ng/mL TcdB also resulted in increased cellular levels of mono-glucosylated Rac1, with peak levels of activation observed at 100 ng/mL TcdB. ($P < 0.01$; Kruskal-Wallis; N=3, n=3). Similarly, this was not reversed in the presence of 10 mg/mL plantain NSP (**Figure 6.2B**). These results therefore suggest that plantain NSP does not block the TcdA and TcdB mediated monoglucosylation of Rac1 in Caco2 cells.

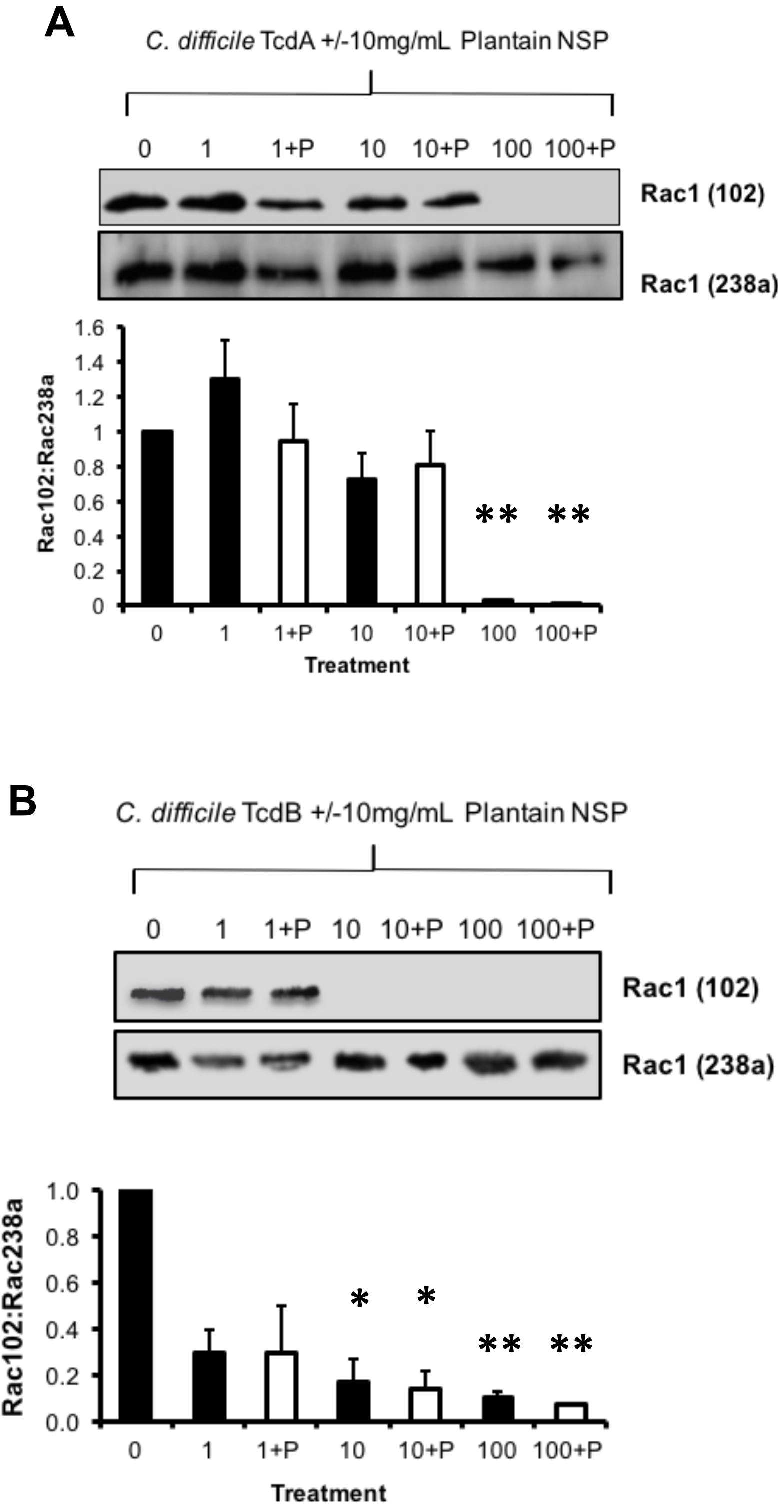


Figure 6.2. ***C. difficile*** **TcdA and TcdB mediated Rac1 glucosylation is unaffected by pre-treatment of Caco2 cells with soluble plantain fibre.** Immunoblot analysis shows an increase in Rac1 glucosylation following treatment with **A)** 100 ng/mL TcdA for 24h or **B)** 10 and 100 ng/mL TcdB for 48h, which is unaffected by pre-treatment of Caco2 cells with 10 mg/mL soluble plantain NSP. Protein bands were analysed densitometrically. Glucosylated Rac1 levels were normalised to total Rac1 (238a) (N=3, n=3; * $P < 0.05$, ** $P < 0.01$; Kruskal-Wallis; representative blots shown).

6.5.2 Soluble plantain NSP has no effect on the morphological changes observed in *C. difficile* toxin-treated cells

To assess the effect of soluble plantain NSP treatment on the morphological change observed in *C. difficile* toxin-treated cells, cellular actin filaments were visualised using fluorescent microscopy. Untreated Caco2 cells had a normal morphology with a uniform actin cytoskeleton (**Figure 6.3A**), whilst cells treated with 100 ng/mL *C. difficile* TcdA for 24 h exhibited clear morphological changes. In toxin-treated cells, there was a loss of the actin cytoskeleton, which shrank around cell nuclei resulting in obvious cell rounding (**Figure 6.3B**). These morphological effects were not reversed following pre-incubation of Caco2 cells with 10 mg/mL plantain NSP (**Figure 6.3C and D**).

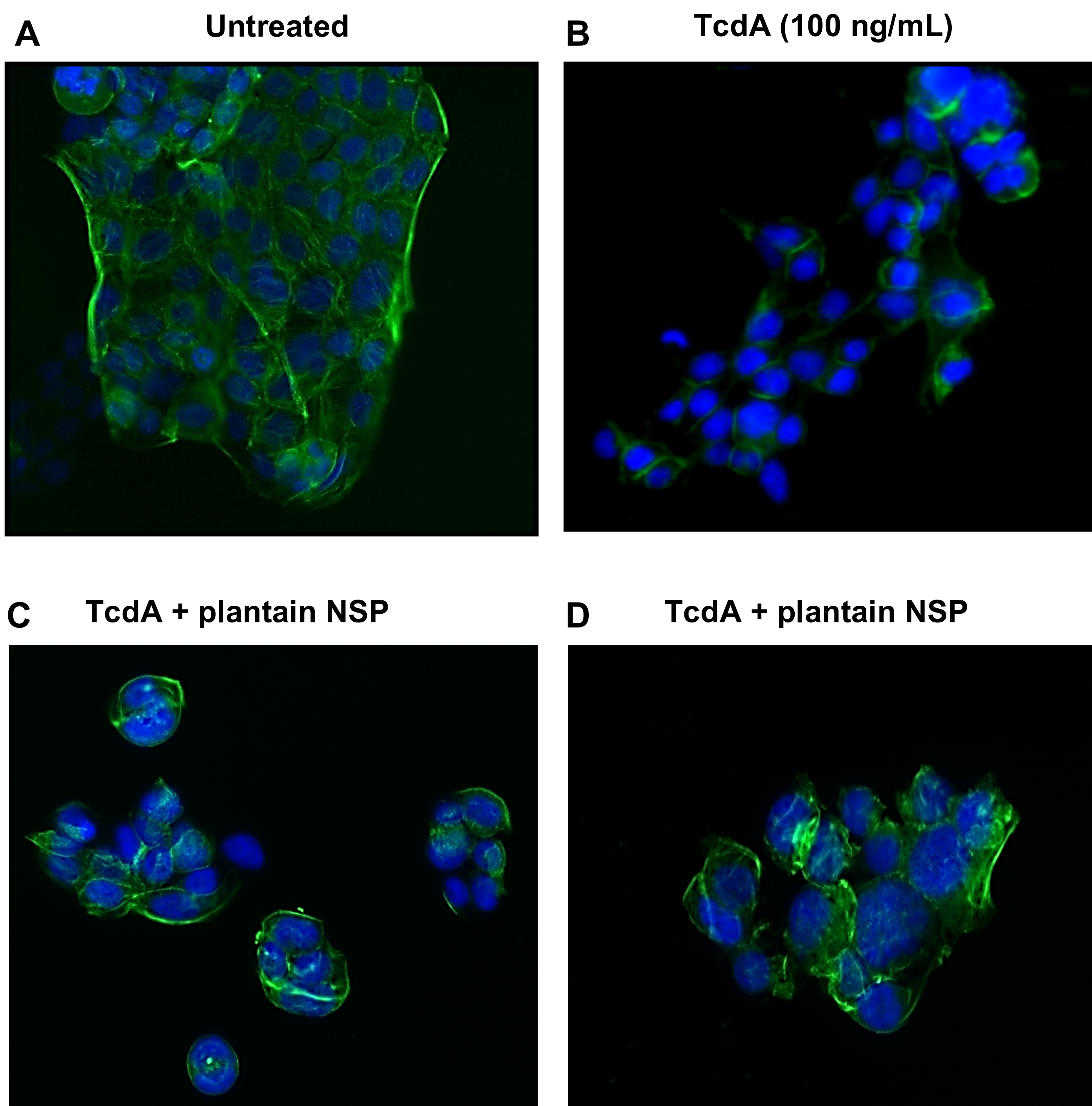


Figure 6.3. Plantain NSP has no effect on the morphological changes observed in TcdA-treated cells, as assessed by fluorescence microscopy. A) Untreated Caco2 cells, **B)** TcdA-treated Caco2 cells and **C)** TcdA-treated Caco2 cells that underwent pre-incubation with 10 mg/mL plantain NSP were all fixed with 2% (v/v) paraformaldehyde (PFA) for 10 min and washed three times with sterile PBS. Actin filaments were visualised by treating fixed cells with AlexaFluor®488-phalloidin (1:100; 30 min). Cell nuclei were visualised by counterstaining with DAPI (N=1, n=3; representative images shown).

6.5.3 Soluble plantain NSP reduces *C. difficile* bacterium and *C. difficile* toxin-mediated IL-8 release from intestinal epithelial cells

Treatment of Caco2 cells with 1, 10 and 100 ng/mL TcdA and TcdB for 24 h elicited dose-dependent increases in pro-inflammatory IL-8 release, with peak responses seen at 100 ng/mL for both toxins. Interestingly, the IL-8 response was observed to be higher in those Caco2 cells that had been treated with TcdA (At 100 ng/mL, TcdA-elicited IL-8 response was 1.9-fold higher than that induced by TcdB; $P < 0.01$; Kruskal-Wallis; **Figure 6.4A and B**). The toxin-mediated IL-8 response was blocked by pre-treatment of Caco2 cells with 10 mg/mL plantain NSP. Plantain NSP decreased IL-8 response to 100 ng/mL TcdA from 438.6 ± 30.9 pg/mL to 270.9 ± 21.1 pg/mL, and also reduced IL-8 response to 100 ng/mL TcdB from 228.2 ± 18.5 to 96.0 ± 3.3 pg/mL (both $P < 0.001$; N=2, n=3, Kruskal-Wallis; **Figure 6.4A and B**)

Treatment of Caco2 cells with *C. difficile* isolate CD98011 (TcdA+/TcdB+), at MOI 100 for 4 h, also resulted in increased IL-8 release from basal levels of 206.7 ± 7.8 pg/mL to 915.6 ± 20.6 pg/mL. In the presence of 10 mg/mL plantain NSP, *C. difficile* mediated IL-8 release was significantly decreased to 683.6 ± 63.7 ($P < 0.05$; **Figure 6.4A**). Similarly, 10 mg/mL plantain NSP also decreased *C. difficile* isolate CD108519 (TcdA-/TcdB+) mediated IL-8 release from Caco2 cells (2.8-fold decrease; $P < 0.001$; **Figure 6.4B**). Notably, IL-8 response 4 h post infection with CD108519 was significantly lower (at only 366.5 ± 31.0 pg/mL) than that seen with the TcdA+ isolate CD98011. Of note too, in Caco2 cells that underwent pre-treatment with 10 mg/mL plantain NSP but were not treated with *C. difficile* toxin or bacterium, it was observed that there was also a decrease in cellular IL-8 response (reduced from 206.7 ± 7.8 to 135.5 ± 25.9 pg/mL upon pre-treatment with plantain NSP (**Figure 6.4**).

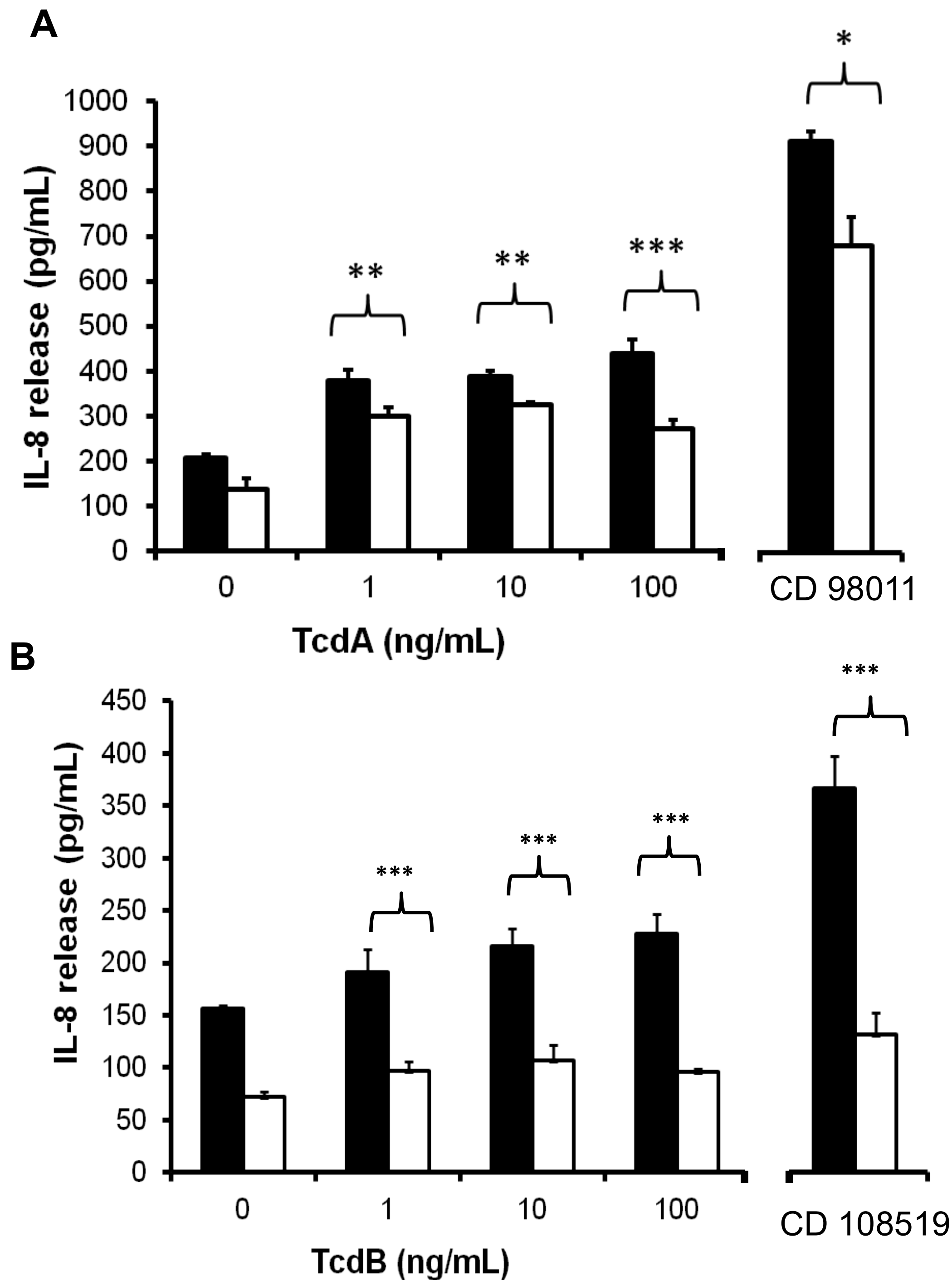


Figure 6.4. Soluble plantain NSP reduces *C. difficile* bacterium and toxin-mediated IL-8 release in intestinal epithelial cells. Caco2 cell monolayers were treated with **A)** TcdA (0-100 ng/mL; 24h) and CD98011 (MOI 100; 4h) or **B)** TcdB (0-100 ng/mL; 48 h) and CD108519 (MOI 100; 4h) in the absence (black bars) or presence of 10 mg/mL plantain NSP (white bars). Media harvested from cells was analysed for the presence of IL-8 by ELISA. (N=2, n=3; * P<0.05, ** P<0.01, *** P <0.001; Kruskal-Wallis).

6.5.4 Soluble plantain NSP reduces *C. difficile* bacterium and *C. difficile*-mediated activation of pro-apoptotic caspase-3/7 in intestinal epithelial cells

C. difficile TcdA and TcdB both elicited increased cellular caspase-3/7 activation in Caco2 cells in a dose-dependent manner, with peak response observed at 100 ng/mL for both toxins (**Figure 6.5A and B**). As per observed toxin-mediated IL-8 responses, caspase 3/7 activation was also observed to be higher in Caco2 cells that had been treated with TcdA compared to those treated with TcdB (At 100 ng/mL, TcdA-elicited caspase 3/7 activation was 2.2-fold higher than that induced by TcdB; **Figure 6.5A**). Incubation of cells with 10 mg/mL plantain NSP resulted in significant decrease in caspase-3/7 activation post-treatment with either 10 or 100 ng/mL toxin. In Caco2 cells treated with 100 ng/mL TcdA and TcdB, plantain NSP decreased caspase 3/7 activation by 63.8 ± 1.6% and 58.2 ± 10.4%, respectively (both $P < 0.05$; Kruskal-Wallis; **Figure 6.5A and B**).

Following 4 h infection of Caco2 cells with *C. difficile* isolate CD9811 (TcdA+/TcdB+), there was a 13.1 ± 3.3-fold increase in caspase-3/7 activation in comparison to untreated controls ($P < 0.001$; Kruskal-Wallis; N=2, n=3). In the presence of 10 mg/mL plantain NSP, there was a significant reduction in CD98011-mediated caspase-3/7 activation (84.1 ± 7.0% reduction compared to that seen in the absence of plantain NSP; $P < 0.001$; **Figure 6.5A**). Cellular caspase-3/7 activation was also increased (10.1 ± 1.6 fold) after infection with *C. difficile* isolate CD108519 (TcdA-/TcdB+) albeit at a lower level than that seen with CD98011 (**Figure 6.5B**). Again, pre-incubation 10 mg/mL plantain NSP resulted in a marked reduction in caspase-3/7 activation, of 87.6 ± 1.4% ($P< 0.001$; **Figure 6.5B**). As was seen with IL-8

response, there was a decrease in caspase-3 activation in cells pre-treated with plantain NSP but not treated with bacterium or toxin.

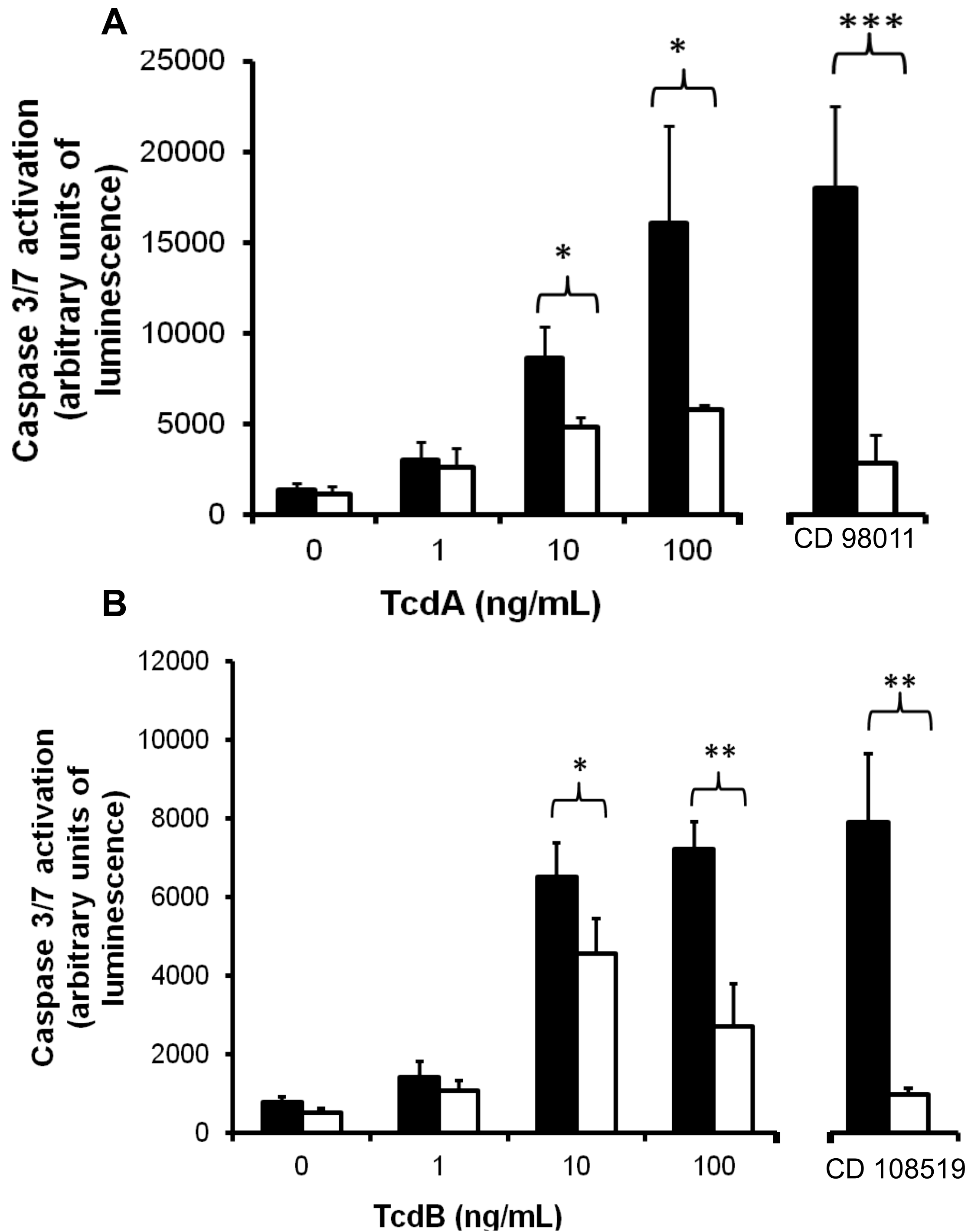


Figure 6.5. Soluble plantain NSP reduces *C. difficile* bacterium and toxin-mediated caspase-3 activation in intestinal epithelial cells. Caco2 cell monolayers were treated with **A)** TcdA (0-100 ng/mL; 24h) and CD98011 (MOI 100; 4h) or **B)** TcdB (0-100 ng/mL; 48 h) and CD108519 (MOI 100; 4h) in the absence (black bars) or presence of 10 mg/mL plantain NSP (white bars). Caspase-3 activation was measured using a Caspase-Glo® 3/7 Assay, as per manufacturer's instructions. (N=2, n=3; * P<0.05, ** P<0.01, *** P <0.001; Kruskal-Wallis).

6.5.5 Soluble plantain NSP reduces *C. difficile* bacterium-mediated and *C. difficile*-toxin-mediated adenylate kinase release from intestinal epithelial cells

As seen with other toxin-mediated effects, *C. difficile* TcdA and TcdB evoked a dose-dependent increase in cytotoxicity (as measured by release of adenylate release to the culture supernatant from Caco2 cell monolayers), with the peak response seen at 100 ng/mL for both toxins (**Figure 6.6A and B**). Again, adenylate kinase release was higher in Caco2 cells treated with TcdA (TcdA, 8.2 ± 0.9-fold increase in AK release; TcdB, 1.8 ± 0.2-fold increase; P < 0.01; Kruskal-Wallis; N=2, n=3). Pre-incubation with 10 mg/mL plantain NSP significantly decreased adenylate kinase release in 1, 10 and 100 ng/mL TcdA and TcdB treated cells (For all, P ≤ 0.01). For example, in cells treated with 100 ng/mL TcdA and TcdB, there was a significant decrease in adenylate kinase release by 63.3 ± 4.1% and 35.7 ± 9.2%, respectively (P < 0.001; **Figure 6.6A and B**).

Following 4 h infection of Caco2 cells with CD98011 (TcdA+/TcdB+), there was an 8.9 ± 0.3-fold increase in adenylate kinase release in comparison to vehicle treated controls, which was significantly decreased following pre-incubation with 10 mg/mL plantain NSP (47.3 ± 1.03%; P < 0.001; **Figure 6.6A**). Whilst adenylate kinase release increased by 2.1 ± 0.3-fold in cells infected with CD108519 (TcdA-/TcdB+), the response was lower than that seen with CD98011. Again, pre-incubation of Caco2 cells with 10 mg/mL plantain NSP resulted in a significant reduction of cellular adenylate kinase release, by 56.2 ± 1.9% in comparison to that seen in the absence of plantain NSP (P < 0.001; **Figure 6.6B**). As seen previously, basal levels of adenylate kinase release were observed to be decreased in Caco2 cells pre-treated with soluble plantain NSP alone, i.e. untreated with either bacterium or toxin (**Figure 6.6B**)

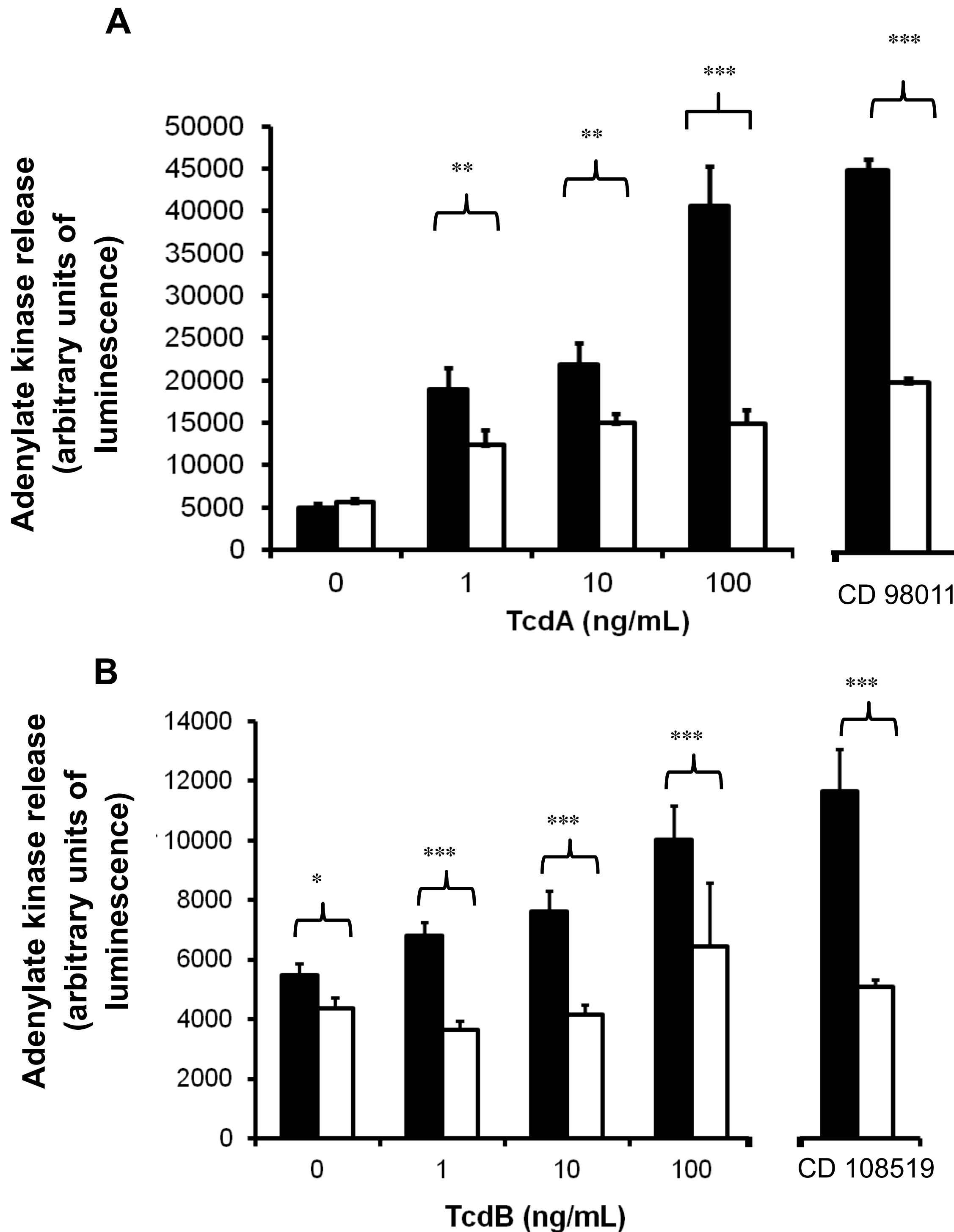


Figure 6.6. Soluble plantain NSP reduces *C. difficile* bacterium and toxin-mediated cytotoxicity response intestinal epithelial cells. Caco2 cell monolayers were treated with **A)** TcdA (0-100 ng/mL 24 h) and CD98011 (MOI 100; 4 h) or **B)** TcdB (0-100 ng/mL; 48 h) and CD108519 (MOI 100; 4h) in the absence (black bars) or presence of 10 mg/mL plantain NSP (white bars). Media harvested from cells was analysed for the presence of adenylate kinase using a ToxiLight™ bioassay kit. (N=2, n=3; * $P<0.05$, ** $P<0.01$, *** $P<0.001$; Kruskal-Wallis).

6.6 SUMMARY OF RESULTS

1. *C. difficile* TcdA induces a higher level of epithelial cell damage and inflammation than *C. difficile* TcdB

2. Soluble plantain NSP has little or no effect on *C. difficile* toxin-mediated cellular Rac1 mono-glucosylation or cell rounding

3. Soluble plantain NSP does however significantly block the pro-inflammatory, apoptotic and cytotoxicity response mediated by *C. difficile* and its toxins TcdA and TcdB

6.7 DISCUSSION

Here, we demonstrate that treatment of Caco2 cells with 10 ng/mL purified native TcdA and TcdB for 24 h and 48 h, respectively, results in a significant increase in cellular Rac1 monoglucosylation. Whilst other Rho-GTPases are targeted for mono-glucosylation by *C. difficile* TcdA and TcdB, we chose to focus on Rac1 in particular due to the fact that its inactivation has been deemed the main mechanism responsible for toxin-mediated cytopathic effects (Popoff and Geny 2011). In addition, cellular Rac1 monoglucosylation can be easily monitored using the commonly available anti-Rac1 clone 102 antibody, which exhibits impaired recognition of Rac1 following its mono-glucosylation at amino acid Thr-35 (Genth *et al.* 2006). Our findings reflect those from other studies, which have also shown TcdA and TcdB induced Rac1 mono-glucosylation in fibroblasts (Genth *et al.* 2006) and HT29 cells (Gerhard *et al.* 2008).

Other early effects of *C. difficile* toxins include the expression of pro-inflammatory cytokines and loss of cell membrane integrity, followed by programmed cell death. The induction of the pro-inflammatory response has been demonstrated previously in epithelial cells (Kim *et al.* 2006), neutrophils (Linevsky *et al.* 1997) and monocytes (Flegel *et al.* 1991). The exact mechanism of *C. difficile* toxin induced mucosal inflammation has not been fully elucidated, but it has been suggested that *C. difficile* TcdA might mediate this response via IκB kinase (IKK) activation, which subsequently phosphorylates and targets inhibitory protein IκB for ubiquitin-mediated proteolysis, resulting in the activation of NF-κB (Kim *et al.* 2006). Studies have also demonstrated that *C. difficile* TcdA and TcdB induce cytotoxic responses in intestinal epithelial cells, as well as a number of other cell types, such as neuronal cells (Stankiewicz *et al.* 2015), fibroblasts (Genth *et al.* 2006) and endothelial cells

(Hippenstiel *et al.* 2002). Similarly, in our own studies, 24 h treatment of Caco2 cells with 1-100 ng/mL TcdA or TcdB significantly increased the cellular pro-inflammatory IL-8 response, caspase-3 activation and adenylate kinase release in a dose dependent manner. Additionally, these cellular responses were also increased following infection of Caco2 cells for 4 h with *C. difficile* isolates CD98011 (TcdA+/TcdB+) and CD108519 (TcdA-/TcdB+) at MOI 100.

In our studies, it is interesting that *C. difficile* TcdA induced a more potent cellular response than *C. difficile* TcdB. Indeed, there has been great debate about the individual importance of TcdA and TcdB during the course of clinical infection, and many conflicting studies exist. Whilst our results are in agreement with studies that suggest TcdA exhibits higher potency and cytotoxicity *in vitro* (Kuehne *et al.* 2010), other contradictory studies indicate that TcdB is the more potent toxin (Riegler *et al.* 1995; Shen 2012). *In vivo* animal studies have also created a paradox over the relative importance of TcdA and TcdB; whilst TcdA+/TcdB- mutants used in a study by Keuhne and colleagues were able to induce an *in vivo* pathogenic effect (Kuehne *et al.* 2010), Lyras and colleagues determined that equivalent mutants were avirulent (Lyras *et al.* 2009). In addition, TcdA-/TcdB+ *C. difficile* stains are routinely isolated from CDI patients and are capable of causing extensive disease, whilst very few TcdA+/TcdB- strains have been reported (Sun *et al.* 2010). It is important to note that inherent variability exists between the toxins of different *C. difficile* strains, which include differences in enzymatic activity and/or host cell specificity (Kuehne *et al.* 2010), and could explain the conflicting results.

Instead, it is likely that both TcdA and TcdB play an important role in CDI pathogenesis. Indeed, the majority of CDI patients are infected with bacterial strains that express both TcdA and TcdB (Sutton *et al.* 2008). Moreover, studies have shown that hypervirulent *C. difficile* strains (such as 027/BI/NAP1) produce greater amounts of both TcdA and TcdB *in vitro* (Warny *et al.* 2005). In our own studies, we demonstrate that whilst infection of Caco2 cells with TcdA-/TcdB+ CD108519 did mount a considerable cellular response, the response was much higher when cells were treated with a TcdA+/TcdB+ CD98011, suggesting that the toxins might work together in synergy to produce an additive effect.

Our results indicate that soluble plantain fibre (plantain NSP) had no effect on toxin-mediated Rac1 monoglucosylation, with a limited effect on toxin-mediated cell rounding. In contrast, soluble plantain fibre did significantly reduce cellular pro-inflammatory IL-8 release, caspase-3 activation and adenylate kinase release induced by purified native *C. difficile* toxins and *C. difficile* bacteria. Although most of the cellular effects of *C. difficile* toxins are attributed to the monoglucosylation and subsequent inactivation of Rho proteins, numerous studies have demonstrated that toxin-mediated epithelial cell damage and inflammation can in fact occur independently of this event. Indeed, Materrese and colleagues demonstrated that TcdB causes apoptosis in human epithelial HEp-2 cells by directly acting on mitochondria, which does not require the N-terminal Rho-inhibiting activity of the toxin (Matarrese *et al.* 2007). Another study illustrated that IL-8 release from TcdA-treated enterocytes is dependent on an oxidative burst originating from mitochondria, which is transduced via the IκB- NF-κB pathway. Interestingly, early events in this signal transduction pathway were observed within 30 min of toxin exposure, occurring before detectable glucosylation activity of Rho proteins (He *et al.* 2002). These findings suggest that

plantain NSP might inhibit toxin-mediated effects that occur independently of Rho inactivation. However, it is also important to note that basal levels of IL-8, apoptosis and cell death were also reduced by plantain NSP pre-treatment in untreated control Caco2 cells. This suggests that to some extent, dietary fibre might instead have a general protective effect against the induction of epithelial damage and inflammation.

Whilst our findings are inconclusive as to whether soluble plantain fibre inhibits the specific mechanisms of action of *C. difficile* toxins within intestinal epithelial cells, its ability to reduce *C. difficile* bacterium and toxin mediated epithelial cell damage and inflammation is likely to be of significant clinical benefit. In fact, anti-inflammatory agents have been shown to reduce CDI severity in a number of animal models of infection (Anton *et al.* 2004; Chen *et al.* 2006; Cottrell *et al.* 2007; Kokkotou *et al.* 2009; Pothoulakis *et al.* 1993; Warny *et al.* 2005). Our results therefore add to the already existing evidence to suggest that dietary supplementation with soluble plantain fibre might represent an effective therapy to treat CDI. In addition, these findings suggest that soluble plantain fibre might also reduce the epithelial cell damage and inflammation associated with other intestinal inflammatory conditions, such as IBD.

Chapter 7

The Effect of Soluble Plantain Fibre on the Epithelial Damage and Inflammation Induced by Flagellin and LPS

7.1 INTRODUCTION

UC, a major form of IBD, is characterised by chronic inflammation that extends proximally from the rectum (Danese and Fiocchi 2011). UC is a cause of significant morbidity worldwide, and moreover, its incidence appears to be increasing with time (da Silva *et al.* 2014). The hallmark symptom of UC is bloody diarrhoea, with or without mucus. Depending on the severity of disease, diarrhoea can be accompanied by abdominal pain, urgency, tenesmus, fever, malaise and weight loss (Feuerstein and Cheifetz 2014). The onset of disease is typically gradual, whilst the clinical course is marked by exacerbations and remissions that may occur spontaneously in response to treatment changes or illness (Kornbluth *et al.* 2010; Danese and Fiocchi 2011).

Conventional therapies for UC (such as mesalazine, corticosteroids, thiopurines and anti-TNF agents) fail to successfully induce remission and prevent relapse, and in addition, cause various side effects (Kornbluth *et al.* 2010). Indeed, approximately 50% of patients with UC have chronically active disease, which has a hugely negative impact on quality of life (Lix *et al.* 2008). More severe cases of UC require colectomy, which is considered a curative strategy (Dignass *et al.* 2012). However, this puts a considerable economic burden on the healthcare system, and can be associated with complications (Lindsay *et al.* 2015). As such, the development of alternative treatment strategies for UC is of great interest.

In order to develop new treatment strategies for UC, it is important to understand the pathogenesis of disease. Whilst the exact pathophysiology of UC is not clearly understood, it is likely that pre-disposition to a number of genetic and environmental factors can lead to alterations in barrier function of the intestinal mucosa, which can lead to harmful interactions

between bacteria, bacterial components and the surface epithelium (Cooney and Jewell 2009).

In active UC, the normally continuous adherent colonic mucus layer is depleted or completely absent (Johansson 2014). This has been attributed to a lack of predominant mucus component, mucin glycoprotein MUC2, which is normally secreted by goblet cells (Johansson 2014). Indeed, a number of studies have shown that patients with UC exhibit reduced goblet cell numbers and depleted mucus secretion (Boltin *et al.* 2013), whilst others have documented the development of spontaneous colitis in several mouse strains with MUC2 defects (Johansson *et al.* 2014; Wenzel *et al.* 2014).

As a consequence of the loss of the protective colonic mucus layer, the surface epithelium becomes exposed to colonic bacteria and their components (Johansson *et al.* 2010; Wenzel *et al.* 2014; Johansson *et al.* 2014). In addition, impaired cell adhesion and defective regulation of tight junctions between intestinal epithelial cells leads to an increase in the permeability of the intestinal epithelium, which also brings about increased bacterial translocation through the lamina propria (Ohkusa *et al.* 2009; Hotte *et al.* 2012). Subsequently, bacterial antigens can interact with toll-like receptors (TLRs), resulting in the activation of NF-κB signalling and release of pro-inflammatory cytokines such as TNF-α, IL-12, IL-23, IL- 6 and IL-1β (Ordas *et al.* 2012), as well as potent neutrophil chemoattractant IL-8 (Danese and Fiocchi 2011; Mitsuyama *et al.* 1994), which results in the subsequent activation of innate and adaptive immune responses (Ordas *et al.* 2012).

It is becoming increasingly evident that enteric bacteria play an important role in the initiation and development of inflammation in UC. Indeed, several studies have reported an increase in adherent, invasive *E. coli* (AIEC) in the colonic mucosa of UC patients (Kotlowski *et*

al. 2007; Mylonaki *et al.* 2005; Sokol *et al.* 2006; Swidsinski *et al.* 2002). Moreover, the dysregulation of intestinal barrier function results in direct contact between bacteria, bacterial components such as flagellin and LPS and the surface epithelium, with major potential for interaction with TLRs and consequent release of pro-inflammatory cytokines. Hence, the ability to prevent these harmful bacteria-epithelial interactions is an attractive therapeutic strategy.

In Chapter 6 of this thesis, it was shown that soluble plantain fibre could significantly reduce the epithelial damage and inflammation mediated by *C. difficile* and its toxins, suggesting that it could be developed as potential prophylaxis or treatment for CDI. These results suggest that soluble plantain fibre might also have the ability to reduce the epithelial cell damage and inflammation induced by other bacterial components, such as *E. coli* flagellin and LPS, and therefore might represent an alternative therapy to treat UC.

7.2 HYPOTHESIS

Soluble plantain fibre can reduce the epithelial cell damage and inflammation mediated by bacterial flagellin and LPS

7.3 AIMS

1. To determine if soluble plantain fibre can reduce the cytotoxicity, apoptotic and pro-inflammatory response mediated by five different mucosally-associated UC *E. coli* isolates, as well as their pooled, bacteria-free filtrate of culture media supernatant (containing OMVs and flagellin)

2. To determine if soluble plantain fibre can reduce the cytotoxicity, apoptotic and pro-inflammatory response mediated by purified bacterial flagellin

3. To determine if soluble plantain fibre can reduce the cytotoxicity, apoptotic and pro-inflammatory response mediated by bacterial LPS

7.4 METHODS

The mucosally-associated *E. coli* strains (HM250, HM295, HM378, HM380 and HM387; *Appendix 3*) used in this study were previously isolated from colonic biopsies of five patients with UC, as described elsewhere (Martin *et al.* 2004; Subramanian *et al.* 2008). UC *E. coli* isolates were grown as described in *Section 4.4.1*, and then pooled to generate a bacterial suspension of OD 0.125 at 550_{nm}. The culture suspension was then sterile filtered using a 0.2 µm filter (Corning/Costar) to generate a bacteria-free filtrate, confirmed bacteria-free by overnight culture on LB agar. This preparation has previously been shown to contain OMVs and associated flagellae (Subramanian *et al.* 2008). HT29 cell monolayers, which had undergone pre-treatment with or without 10 mg/mL plantain NSP, were then treated with 50 µL of the bacteria-free filtrate for 4h. In parallel, HT29 cell monolayers were infected with each individual UC *E. coli* isolate at an MOI of 10, or treated with 1 µg/mL flagellin purified from *S*. Typhimurium LT2 (Sigma-Aldrich) each for 4 h at 37°C (*Section 4.9*) (Subramanian *et al.* 2008). HT29 cells were used in these studies as this particular cell line expresses TLR5 on the apical membrane of intestinal epithelial cells. This is important as *E. coli* flagellin acts in a TLR5-dependent manner to induce cellular IL-8 release (Subramanian *et al.* 2008).

To assess the effect of plantain NSP on the epithelial cell damage and inflammation induced by bacterial LPS, Caco2 cell monolayers were also pre-incubated with or without 10 mg/mL plantain NSP prior to treatment with 1, 100 or 1000 µg/mL LPS purified from *E. coli* 0111: B4 for 24 h (*Section 4.10*).

To assess the pro-inflammatory response induced by flagellin and LPS, harvested media from HT29 cell monolayers was measured for the presence of released pro-inflammatory cytokine IL-8 using a human IL-8 ELISA (*Section 4.15*). To assess the apoptotic response, the

cellular activation of caspase-3/7 was measured using a commercial Caspase-Glo® 3/7 assay, as per manufacturer instructions (*Section 4.16*). Finally, to assess for any evidence of cellular cytotoxicity, harvested cell culture media was analysed for the presence of adenylate kinase using a Toxilight bioassay (Lonza) (*Section 4.17*).

7.5 RESULTS

7.5.1 Soluble plantain fibre reduces the epithelial cell damage and inflammation induced by mucosally-associated UC *E. coli* isolates

As observed previously by Subramanian and colleagues (Subramanian *et al.* 2008), infection of HT29 cells with each of the five individual UC *E. coli* isolates (at an MOI of 10) evoked comparable levels of pro-inflammatory IL-8 release (median of 1009.1 pg/mL; range of 849.1 ± 82.3 to 1068 ± 105.4 pg/mL in comparison to the basal level of 258.2 ± 18.7 pg/mL) (**Figure 7.1**). Pre-treatment of HT29 cells with 10 mg/mL plantain NSP for 30 min prior to infection, reduced four out of five pro-inflammatory responses mediated by UC *E. coli* isolate (HM250, HM295, HM378, HM387), but with significant inhibition observed for only one isolate, HM250. Plantain NSP significantly reduced HM250 mediated IL-8 response by 32.5 ± 6.2% in comparison to the untreated control ($P < 0.05$; **Figure 7.1**), whilst inhibition of the IL-8 response to the other four isolates ranged from 23.0 ± 1.8% to 31.7 ± 17.5%. Conversely, IL-8 release in response to UC *E. coli* isolate HM380 was not reduced following 30 min pre-treatment with 10 mg/mL plantain NSP (**Figure 7.1**).

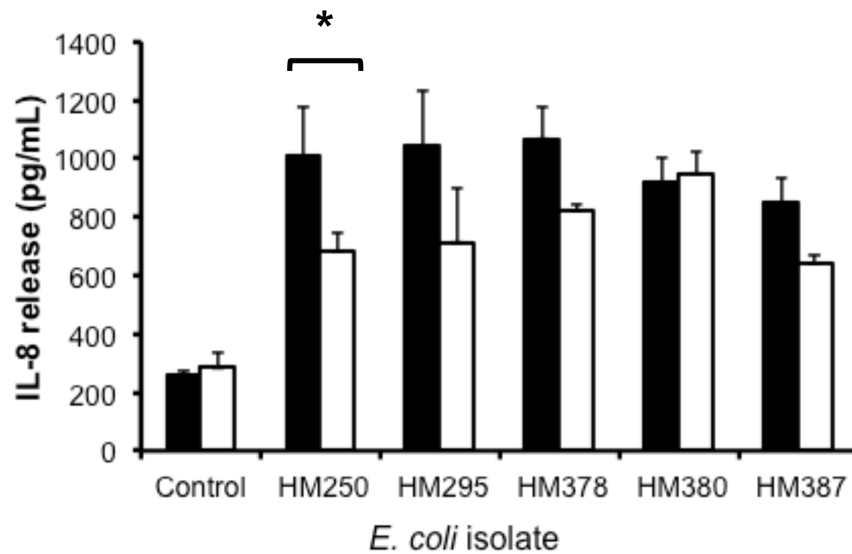


Figure 7.1 Soluble plantain fibre reduces the release of pro-inflammatory IL-8 induced by mucosally associated UC *E. coli* from HT29 colonocytes. HT29 cell monolayers were pre-incubated with (white bars) or without (black bars) 10 mg/mL plantain NSP for 30 min and infected with five different UC *E. coli* isolates (MOI 10; 4 h). Cell culture media harvested from cells was analysed for the presence of pro-inflammatory cytokine IL-8 using an IL-8 human ELISA kit (N=2, n=3; * $P < 0.05$; Kruskal-Wallis).

Cellular cytotoxicity induced in response to infection of HT29 cells with each individual UC *E. coli* isolate was observed to be highly variable. *E. coli* isolates HM295 and HM378 induced much higher cytotoxicity responses than other *UC* isolates tested (13.1 ± 2.7 and 16.8 ± 3.3-fold increase, respectively). Despite this, pre-treatment of HT29 cells with 10 mg/mL plantain NSP reduced the cytotoxicity response induced by all five UC *E. coli* isolates (**Figure 7.2**). Interestingly, whilst plantain NSP had no effect on the cellular IL-8 response induced by *E. coli* HM380, it significantly reduced release of cellular adenylate kinase to the culture media by 18.6 ± 2.2% in comparison to the untreated control cells ($P < 0.05$; **Figure 7.2**). Whilst plantain NSP did not significantly inhibit adenylate kinase release in response to all other UC *E. coli* isolates tested, smaller reductions in the cytotoxicity response were still observed, which ranged from 9.2 ± 4.5% to 32.5 ± 6.7% (**Figure 7.2**).

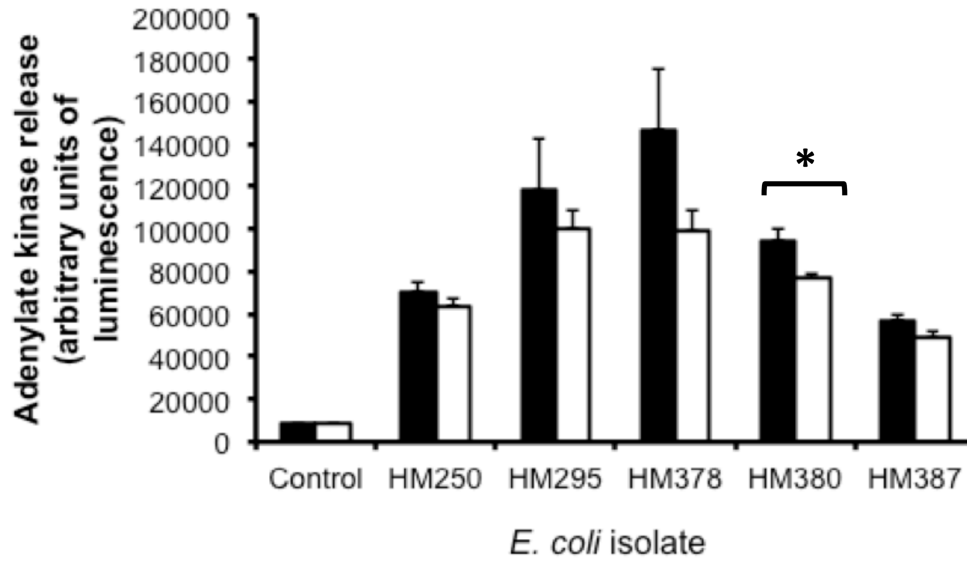


Figure 7.2 Soluble plantain fibre reduces the cellular cytotoxicity response induced by mucosally-associated UC *E. coli*. HT29 cell monolayers were pre-incubated with (white bars) or without (black bars) 10 mg/mL plantain NSP and infected with five UC *E. coli* isolates (MOI 10; 4 h). Cell culture media harvested from cell monolayers was analysed for the presence of adenylate kinase using a Toxilight™ bioassay kit (N=2, n=3; * $P < 0.05$; Kruskal-Wallis).

7.5.2 Soluble plantain fibre reduces the epithelial cell damage and inflammation induced by mucosally-associated UC *E. coli* flagellin

Treatment of HT29 cells with 50 µL of 0.2 µm-filtered pooled UC *E. coli* culture supernatant increased the pro-inflammatory IL-8 response from 258.2 ± 18.7 pg/mL to 1060.7 ± 95.7 pg/mL (**Figure 7.3**), which was a similar level to the IL-8 response evoked by individual mucosal UC *E. coli* isolates (**Figure 7.1**), as has been observed previously (Subramanian et al. 2008). However, 10 mg/mL plantain NSP exhibited a more significant effect against the IL-8 response evoked by the pooled UC *E. coli* supernatant, which was reduced by 66.0 ± 7.2% ($P < 0.05$; **Figure 7.3**). Similarly, plantain NSP also had a more significant effect against the epithelial cell damage mediated by the pooled UC *E. coli* supernatant, significantly reducing release of cellular adenylate kinase to the culture medium by 39.7 ± 0.5% ($P < 0.01$; **Figure 7.4**) and intracellular caspase-3/7 activation by 50.3 ± 7.1% ($P < 0.05$; **Figure 7.5**)

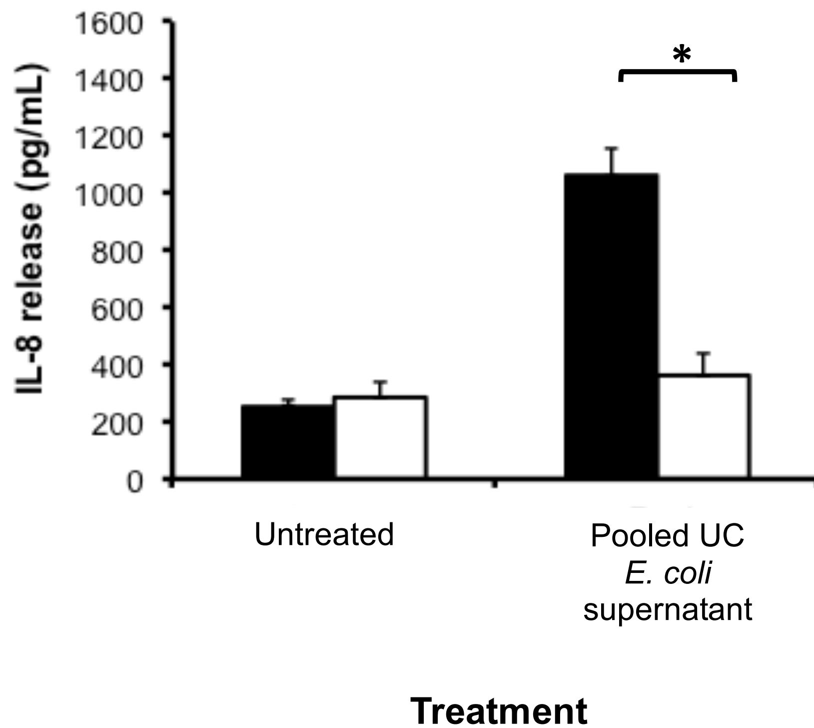


Figure 7.3 Soluble plantain fibre reduces the cellular IL-8 response induced by the pooled supernatant of five UC *E. coli* isolates. HT29 cell monolayers were pre-incubated with (white bars) or without (black bars) 10 mg/mL plantain NSP and treated with 50 µL 0.2 µm-filtered culture supernatant pooled from five mucosal UC *E. coli* isolates (HM250, HM295, HM378, HM380, HM387) (50 µL; 4 h). Cell culture media harvested from cells was analysed for the presence of pro-inflammatory cytokine IL-8 using an IL-8 Human ELISA kit (N=2, n=3; * $P < 0.05$; Kruskal-Wallis).

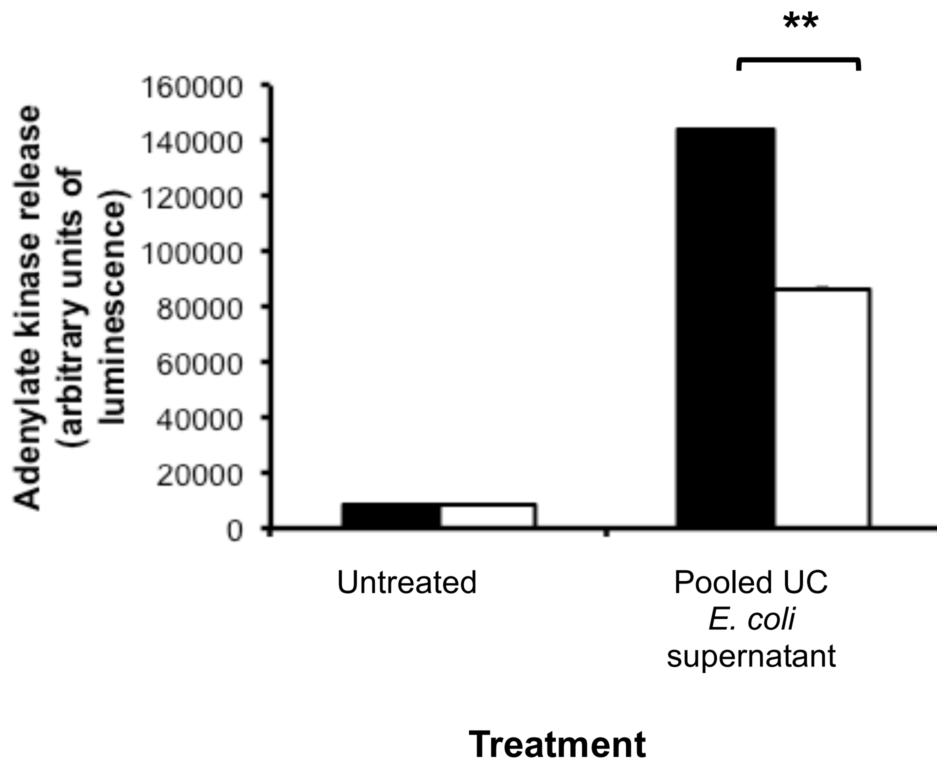


Figure 7.4 Soluble plantain fibre reduces cellular adenylate kinase release induced by the pooled supernatant of five UC *E. coli* isolates. HT29 cell monolayers were pre-incubated with (white bars) or without (black bars) 10 mg/mL plantain NSP and treated with 50 µL 0.2 µm-filtered culture supernatant pooled from five mucosal UC *E. coli* isolates (HM250, HM295, HM378, HM380, HM387) (50 µL; 4 h). Cell culture media harvested from cells was analysed for the presence of adenylate kinase using a Toxilight™ bioassay kit (N=2, n=3; ** P < 0.01; Kruskal-Wallis).

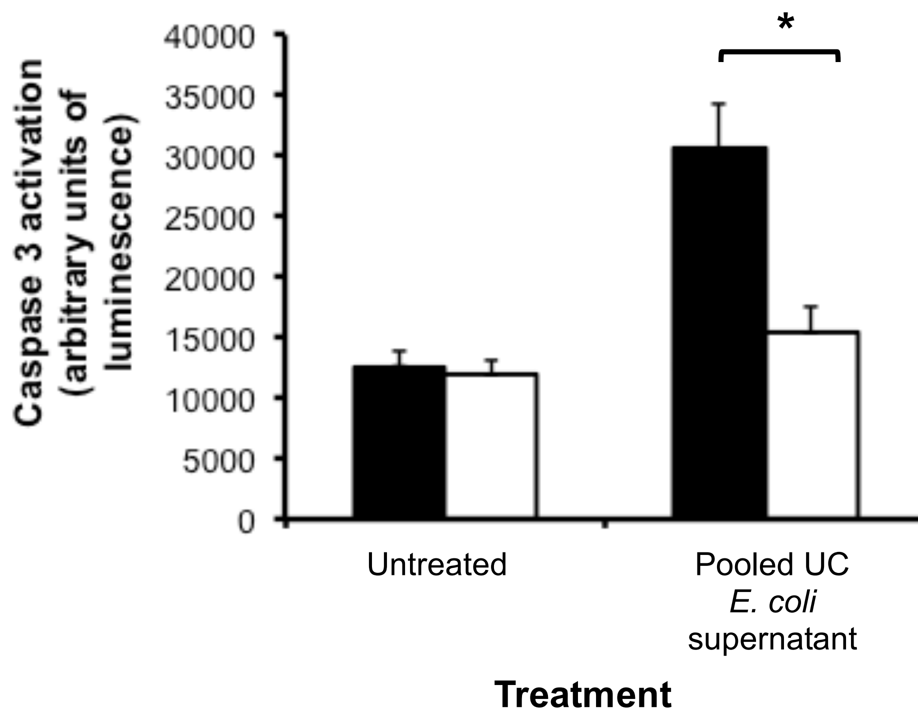


Figure 7.5 Soluble plantain fibre reduces cellular caspase-3/7 activation induced by the pooled supernatant of five UC *E. coli* isolates. HT29 cell monolayers were pre-incubated with (white bars) or without (black bars) 10 mg/mL plantain NSP and treated with 50 µL 0.2 µm-filtered culture supernatant pooled from five mucosal UC *E. coli* isolates (HM250, HM295, HM378, HM380, HM387) (50 µL; 4 h). Caspase-3/7 activation was measured using a Caspase-Glo® 3/7 Assay, as per manufacturer's instructions (N=1, n=3; * $P < 0.05$; Kruskal-Wallis).

7.5.3 Soluble plantain fibre reduces the epithelial cell damage and inflammation induced by purified flagellin

Treatment of HT29 cells with 1 µg/mL purified bacterial flagellin for 24 h increased release of pro-inflammatory IL-8 and adenylate kinase by 5.3 ± 0.5 fold (**Figure 7.6**) and 38.5 ± 0.3 fold (**Figure 7.7**) respectively, in comparison to basal levels. Again, plantain NSP exhibited a more significant effect against the epithelial inflammation and cell damage induced by purified flagellin than compared with that seen with the individual UC *E. coli* isolates. In comparison to the vehicle-treated controls, pre-treatment with 10 mg/mL plantain NSP significantly reduced flagellin-mediated release of both IL-8 and adenylate kinase by 69.1 ± 3.6% ($P < 0.001$; **Figure 7.6**) and 76.0 ± 0.5% ($P < 0.001$; **Figure 7.7**) respectively.

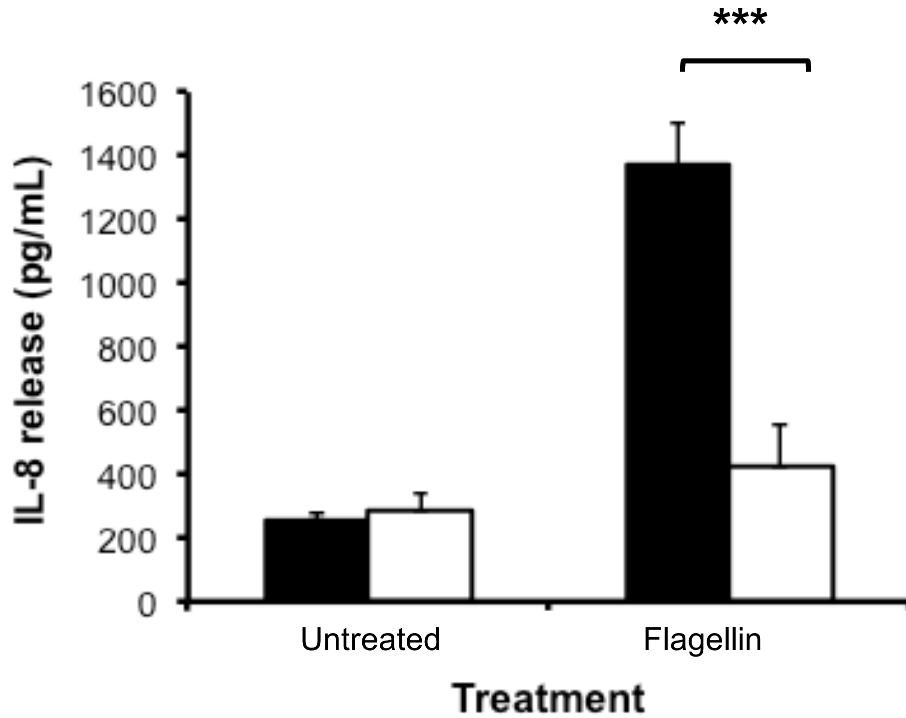


Figure 7.6 Soluble plantain fibre reduces cellular IL-8 response induced by purified bacterial flagellin. HT29 cell monolayers were pre-incubated with (white bars) or without (black bars) 10 mg/mL plantain NSP and treated with purified flagellin (1µg/mL; 4 h). Cell culture media harvested from cells was analysed for the presence of pro-inflammatory cytokine IL-8 using an IL-8 Human ELISA kit (N=2, n=3; *** $P < 0.001$; Kruskal-Wallis).

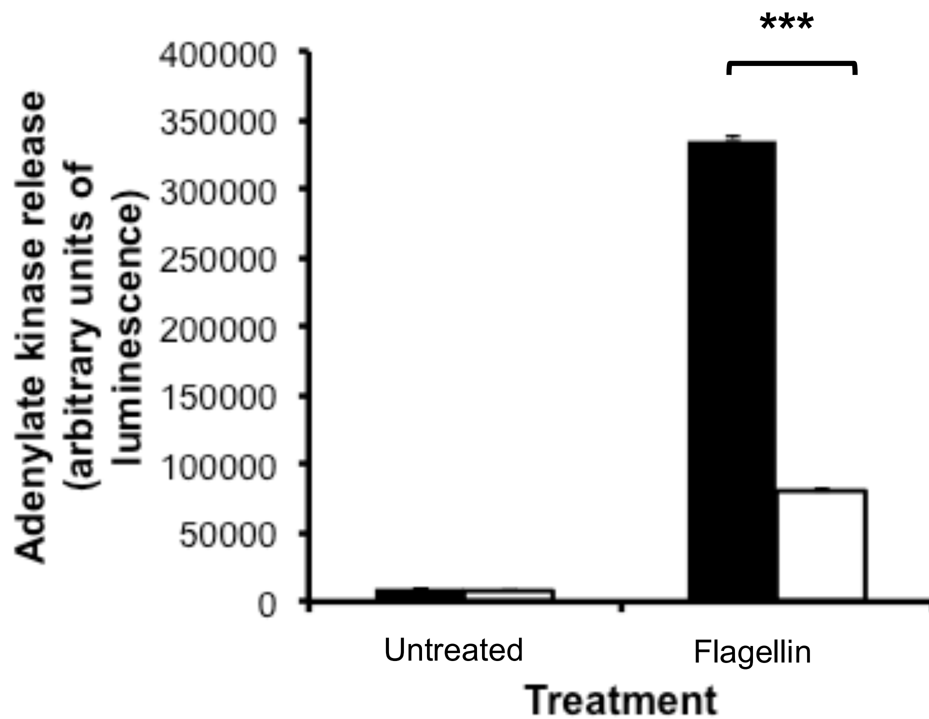


Figure 7.7 Soluble plantain fibre reduces cellular adenylate kinase release induced by purified bacterial flagellin. HT29 cell monolayers were pre-incubated with (white bars) or without (black bars) 10 mg/mL plantain NSP and treated with purified flagellin (1µg/mL; 4 h). Cell culture media harvested from cells was analysed for the presence of adenylate kinase using a Toxilight™ bioassay kit (N=2, n=3; *** $P < 0.001$; Kruskal-Wallis).

7.5.4 Soluble plantain fibre reduces the epithelial cell damage and inflammation induced by bacterial LPS

Treatment of intestinal Caco2 cells with 1 – 1000 µg/mL LPS isolated from *E. coli* for 24 h resulted in a dose-dependent increase in the pro-inflammatory IL-8 response, which was reduced in the presence of 10 mg/mL plantain NSP (**Figure 7.8**). Treatment of Caco2 cells with 1000 µg/mL LPS increased IL-8 release from the observed basal level of 629.4 ± 159.3 pg/mL to 1242.0 ± 361.3 pg/mL which was reduced by 69.4 ± 21.9% following pre-treatment with plantain NSP (**Figure 7.8**). Similarly, LPS mediated a dose-dependent increase in cellular cytotoxicity and apoptosis, which were both significantly inhibited by 30 min pre-treatment with 10 mg/mL plantain NSP; LPS, at 1000 µg/mL, increased adenylate kinase release from 3642.7 ± 685.7 to 35589.3 ± 7873 (arbitrary units of luminescence, AUL) which was markedly reduced by 93.5 ± 0.3% in the presence of 10mg/mL plantain NSP ($P < 0.001$; **Figure 7.9**), and increased cellular caspase-3/7 activation by 2.7 ± 0.12 fold in comparison to basal levels, which was reduced by 72.5 ± 2.0% in the presence of 10 mg/mL plantain NSP ($P < 0.001$; **Figure 7.10**).

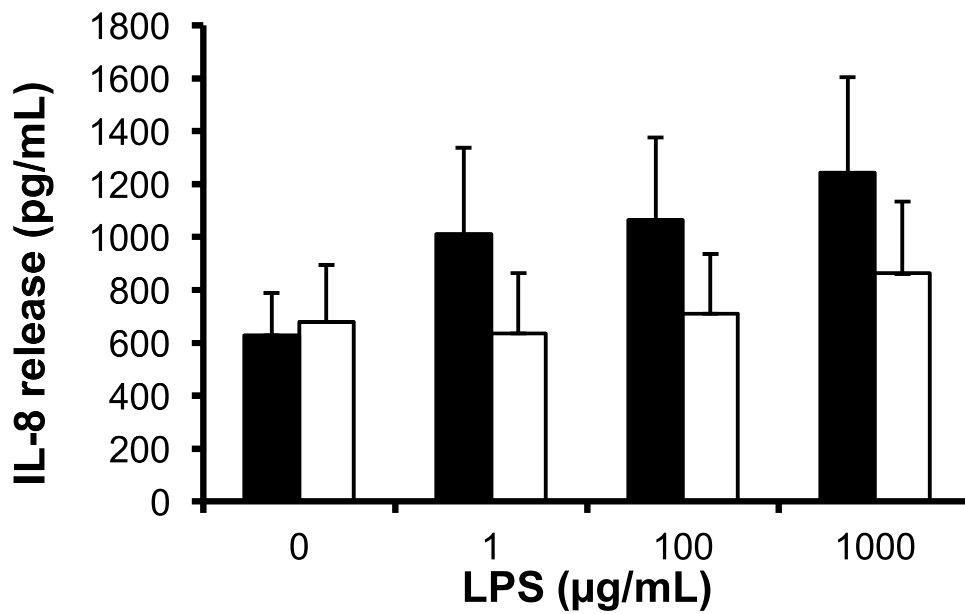


Figure 7.8 Soluble plantain fibre reduces cellular IL-8 response induced by bacterial LPS. Caco2 cell monolayers were pre-incubated with (white bars) or without (black bars) 10 mg/mL plantain NSP and treated with LPS purified from *E. coli* (1 – 1000 µg/mL; 24 h). Cell culture media harvested from cells was analysed for the presence of pro-inflammatory cytokine IL-8 using an IL-8 Human ELISA kit (N=2, n=3).

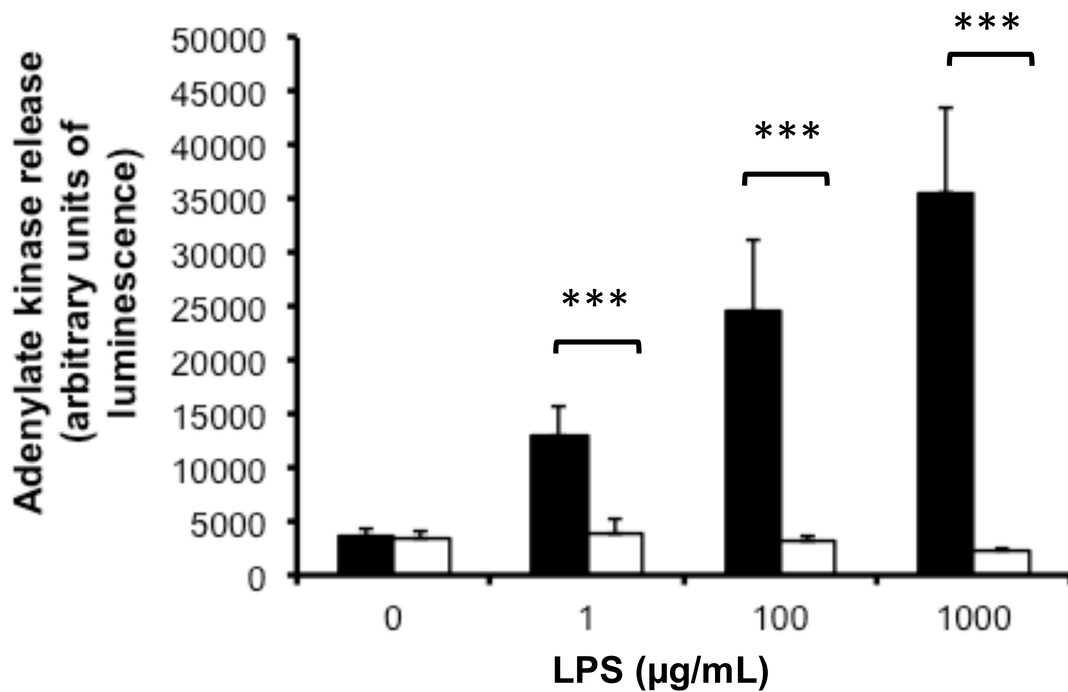


Figure 7.9 Soluble plantain fibre reduces cellular adenylate kinase release induced by bacterial LPS. Caco2 cell monolayers were pre-incubated with (white bars) or without (black bars) 10 mg/mL plantain NSP and treated with purified *E. coli* LPS (1 – 1000 µg/mL; 24 h). Cell culture media harvested from cells was analysed for the presence of adenylate kinase using a Toxilight™ bioassay kit (N=2, n=3; *** P < 0.001; Kruskal-Wallis).

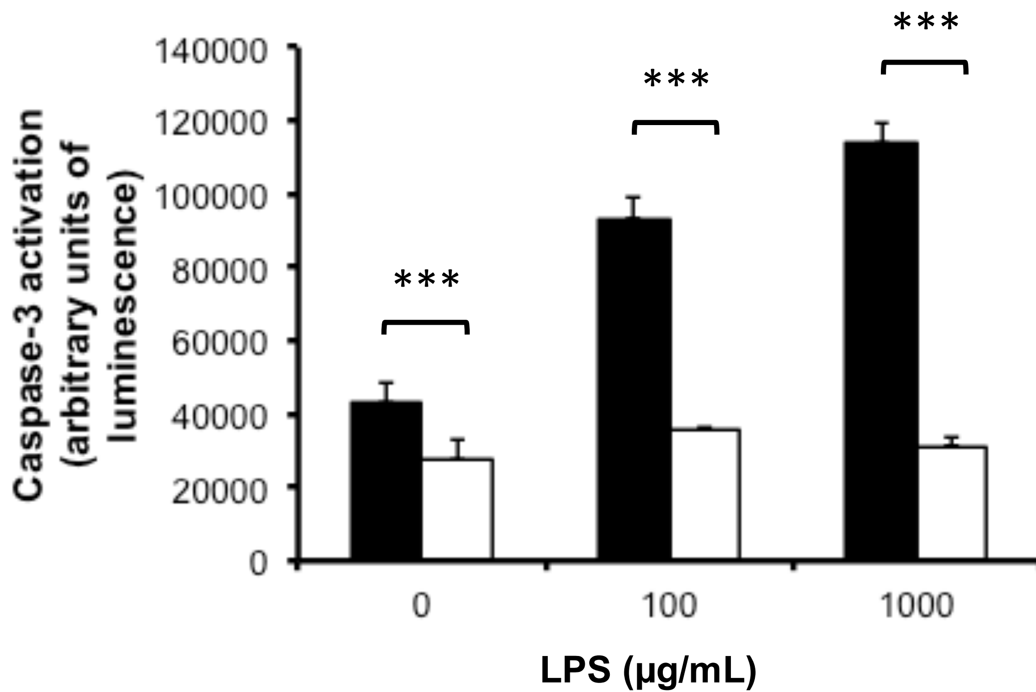


Figure 7.10 Soluble plantain fibre reduces cellular caspase-3/7 activation induced by bacterial LPS. Caco2 cell monolayers were pre-incubated with (white bars) or without (black bars) 10 mg/mL plantain NSP and treated with purified *E. coli* LPS (100 – 1000 µg/mL; 24 h). Caspase-3/7 activation was measured using a Caspase-Glo® 3/7 Assay, as per manufacturer's instructions (N=1, n=3; *** P < 0.001; Kruskal-Wallis).

7.6 SUMMARY OF RESULTS

1. Soluble plantain fibre reduces the cellular pro-inflammatory and cytotoxic response induced by five individual mucosally-associated UC *E. coli* isolates

2. Soluble plantain fibre reduces the pro-inflammatory, cytotoxic and apoptotic response induced by bacteria-free pooled UC *E. coli* culture supernatant (containing OMVs and flagellae)

3. Soluble plantain fibre reduces the pro-inflammatory and cytotoxic response induced by purified bacterial flagellin

4. Soluble plantain fibre reduces the cellular pro-inflammatory, cytotoxic and apoptotic response induced by purified LPS isolated from *E. coli*

7.7 DISCUSSION

Intestinal inflammation in IBD is initiated as a consequence of an aberrant immune response to the commensal microbiota (Cooney and Jewell 2009). Therefore, it is plausible that dietary components with the ability to modify the intestinal microflora or disrupt bacterial interaction with the intestinal epithelium might have a protective effect (Simpson and Campbell 2014).

The traditional rationale for the use of dietary fibre in managing IBD relates to its beneficial effects on gastrointestinal function (Lattimer and Haub 2010), SCFA production (Macfarlane *et al.* 2006) and subsequent prebiotic effect (Hamer *et al.* 2008). Indeed, several studies have reported that some prebiotics exert protective effects against colitis in humans. For example, number of *in vivo* human studies have demonstrated the role of germinated barley foodstuff (GBF) in the maintenance of UC remission (Kanauchi *et al.* 2002), as well as inducing remission in patients with mild-moderate active UC (Bamba *et al.* 2002; Kanauchi *et al.* 2002; Scaldaferri *et al.* 2013). Inulin supplementation has also showed some promise with respect to acute UC; a randomised, placebo controlled trial using oligofructose-enriched inulin was associated with lowered faecal calprotectin, a marker of intestinal inflammation (Casellas *et al.* 2007). Some benefit was also accrued to the use of pysillium (Isphaghula husk fibre) in maintaining remission in UC patients (Kanauchi *et al.* 2013), where one trial reported no significant difference between the fibre and conventional antibiotic mesalazine (Fernandez-Banares *et al.* 1999).

Previous work performed by our group has shown that, unlike other dietary fibre components, soluble plantain fibre possesses limited prebiotic activity (Backman 2009). Instead, we propose that soluble plantain NSP can inhibit potentially harmful interactions

between bacteria, bacterial components and the gut epithelium via a 'contrabiotic' effect (Simpson and Campbell 2015; Simpson *et al.* 2014). Indeed, we have previously shown that soluble plantain fibre can inhibit the epithelial interactions of a range of different bacteria, such as Crohn's mucosa-associated AIEC, *Salmonella* Typhimurium, *Shigella sonnei*, *C. difficile* and ETEC (Parsons *et al.* 2014; Roberts *et al.* 2010; Roberts *et al.* 2013), as well as down-regulate the epithelial inflammatory response to bacterial components, such as *C. difficile* toxins (Chapter 6 of this thesis). Importantly, inhibition of these cellular responses occurred at soluble fibre concentrations readily achievable within the human distal colon following dietary supplementation (Parsons *et al.* 2014; Roberts *et al.* 2013), suggesting that soluble dietary fibre might have a protective effect against intestinal pathogens implicated in a range of different diarrhoeal diseases.

Here, we wanted to assess if soluble plantain fibre would have the ability to down-regulate the epithelial inflammation and damage induced by other bacterial components, such as mucosally-associated flagellin and LPS, which are implicated in the pathogenesis of UC. In active UC disease, the normally continuous adherent colonic mucus is depleted or completely absent (Johansson 2014). Consequently, the surface epithelium becomes exposed to colonic bacteria and their components, which leads to excessive TLR stimulation, secretion of pro-inflammatory cytokines and subsequent activation of innate and adaptive immune responses (Ordas *et al.* 2012). Indeed, it has previously been demonstrated that mucosal-associated *E. coli*, which are markedly increased in UC (Kotlowski *et al.* 2007; Mylonaki *et al.* 2005; Sokol *et al.* 2006; Swidsinski *et al.* 2002), mediate cellular pro-inflammatory IL-8 release via an interaction between *E. coli* flagellin and TLR5 (Subramanian *et al.* 2008). LPS, an integral component of Gram-negative bacteria such as *E. coli*, is known to activate the innate immune

response via an interaction with TLR4 (Beutler *et al.* 2003). Promisingly, we illustrate here that soluble plantain fibre can in fact reduce the cellular pro-inflammatory, cytotoxicity and apoptotic response mediated by mucosally-associated UC *E. coli*, as well as mucosal interacting flagellin and LPS, suggesting a possible role for soluble plantain fibre in the treatment of UC.

It is interesting that soluble plantain NSP exhibited a more significant effect against the epithelial cell damage and inflammation induced by the pooled UC *E. coli* supernatant and purified flagellin than compared to individual UC *E. coli* isolates. As already mentioned, the 0.2um filtered pooled UC *E. coli* culture supernatant is known to contain shed OMVs and associated flagellae/flagellin (Subramanian *et al.* 2008). OMVs are known to express proteins relevant to pathogenicity (Davis *et al.* 2006), however, previous work has suggested that the IL-8 response to the bacteria-free supernatant is largely due to the presence of flagellin, which has been shown to evoke IL-8 release in a TLR5 dependent manner (Subramanian *et al.* 2008). Conversely, only two out of the five UC *E. coli* isolates used in this study elicited IL-8 release in a TLR5-dependent manner (Subramanian *et al.* 2008). As all five isolates evoked a comparable cellular pro-inflammatory IL-8 response (median of 1009.1 pg/mL IL-8), this indicates that other TLR5-independent mechanisms are involved in the mediation of the pro-inflammatory IL-8 response. Indeed, plantain NSP might lack the ability to effectively inhibit IL-8 release mediated by TLR-5 independent mechanisms, explaining the less significant results observed with respect to mucosal-associated UC *E. coli* isolates.

Due to the promising *in vitro* results observed in these studies, it would next be beneficial to assess if supplementation with plantain NSP might have a protective effect against an *in vivo* colitis model. One such experimental model includes the TNBS-induced

colitis model, in which green banana flour (Musa spp. AAA) has been previously shown to prevent colonic inflammation (Scarminio *et al.* 2012) The impact of plantain supplementation on colonic inflammation could also be examined in mice with acute DSS-induced colitis (Valatas *et al.* 2015), as well as in Muc2$^{-/-}$ mice (Wenzel *et al.* 2014).

It would also be interesting to determine efficacy of plantain NSP *in vivo* on epithelial cell apoptosis and shedding observed in a model of acute LPS-induced murine gut injury, described by Williams and colleagues (Williams *et al.* 2015; Williams *et al.* 2013). However, as LPS is administered systemically in this particular model (Williams *et al.* 2013), it is possible that plantain NSP might not confer a protective effect in this instance.

Chapter 8

The Characterisation of the Polysaccharide Component Present in Soluble Plantain Fibre That Confers its Inhibitory Activity Against Gut Pathogens

8.1 INTRODUCTION

In the previous chapters of this thesis, it has been shown that soluble plantain fibre can inhibit the epithelial interactions of *C. difficile* and ETEC bacteria, as well as *C. difficile* spores (**Chapter 5**). Furthermore, soluble plantain fibre also reduces the cellular damage and inflammatory response induced by bacterial components, such as *C. difficile* toxin TcdA and TcdB (**Chapter 6**), as well as bacterial flagellin and LPS (**Chapter 7**). These results provide considerable evidence to suggest that soluble plantain fibre can disrupt potentially harmful bacterial-epithelial interactions. To further elucidate the exact nature of this interaction, it would be beneficial to characterise the polysaccharide component of soluble plantain NSP that confers its bioactivity.

The main source of dietary fibre is from the primary plant cell wall, which is composed of three main polysaccharides: cellulose, hemicellulose and pectin (Blackwood *et al.* 2000). Plant cells are surrounded by layers of long cellulose fibrils that are composed of 30 – 36 chains of β-1,4-linked glucose, which are hydrogen bonded to form an insoluble and inelastic structural framework (Vorwerk *et al.* 2004). Structural cellulose microfibrils are also interconnected via hydrogen bonds with high molecular weight polysaccharide hemicellulose, which is thought to be embedded in a matrix of pectin (Carpita and McCann 2000; Somerville *et al.* 2004). Pectins are galacturonate (GalA) rich acidic polysaccharides, which include homogalacturonans (HG), as well as rhamnogalacturonans (RG) I and II (Carpita and McCann 2000; Fry 2004). Conversely, hemicelluloses are GalA-free, neutral or slightly acidic polysaccharides (Carpita and McCann 2000; Fry 2004), which include xylans (such as arabinoxylans, glucuronoarabinoxylans), xyloglucans (composed mainly of glucose, fucose, galactose, xylose) and mannans (Scheller and Ulvskov 2010).

The structure and composition of plant cell walls exhibit significant intra-species variation. Significant differences can also occur within species according to a number of factors including cultivar, stage of ripeness and environmental conditions (Carpita and McCann 2000; Knox 2008; Popper 2008). As such, it was essential that the plantain NSP used in these studies originated from a consistently reproducible source of soluble fibre. Our preferred source of soluble NSP was from green plantain (ripeness stage 1) flour produced in Ecuador from locally grown cultivars *Musa* AAB (Horn) variation Dominico (Roberts *et al.* 2010). The use of green plantain ensures that water-soluble components of the cell wall, in particular pectins, have a wider range of structural characteristics and a higher molecular weight than that obtained from ripe plantain fruits (Shiga *et al.* 2011). The yield of soluble non-starch fibre from green plantain is 6-7% dry matter, with a ratio of acidic to neutral polysaccharides of approximately 9:1 (*See Appendix 2*). In addition, the soluble plantain fibre used in these studies contains 45% by weight of plantain-derived maltodextrin, which was added as part of the bulk manufacturing process to aid solubilisation. Maltodextrin is a polysaccharide that is commonly added to processed foods, cosmetics, and medications as a filler, thickener, texturiser or coating agent (Nickerson *et al.* 2014). The complex carbohydrate is created through chemical and enzymatic processing of a variety of starches to produce chains of up to 20 glucose molecules, which are linked by α(1-4) and α(1-6) glycosidic bonds (Chronakis 1998).

Preliminary work has previously been performed by our research group to begin to characterise the polysaccharide component of plantain NSP that is responsible for its inhibitory activity (Parsons *et al.* 2014). As part of this work, soluble plantain fibre was fractionated into acidic and neutral polysaccharide containing fractions by small-scale,

analytical strong anion-exchange chromatography (**Figure 8.1A**). These purified fractions were then assessed for their efficacy at inhibiting the *in vitro* adhesion of *Salmonella enterica* serovar Typhimurium to porcine intestinal epithelial B1OX1 cells. *S.* Typhimurium is one of the most frequent causes of food-borne gastroenteritis in humans, and is also an important pathogen in food-producing animals, including pigs and chickens (Ibarra and Steele-Mortimer 2009). Pre-treatment of cell monolayers with 5 mg/mL acidic fraction of plantain NSP resulted in significant blockade of *S*. Typhimurium adhesion by up to 50%, which was a similar level of inhibition to that seen with soluble plantain NSP (**Figure 8.1B**). These preliminary results suggested that the active component conferring inhibitory activity against *S*. Typhimurium to the host epithelium lies within the acidic fraction of soluble plantain NSP.

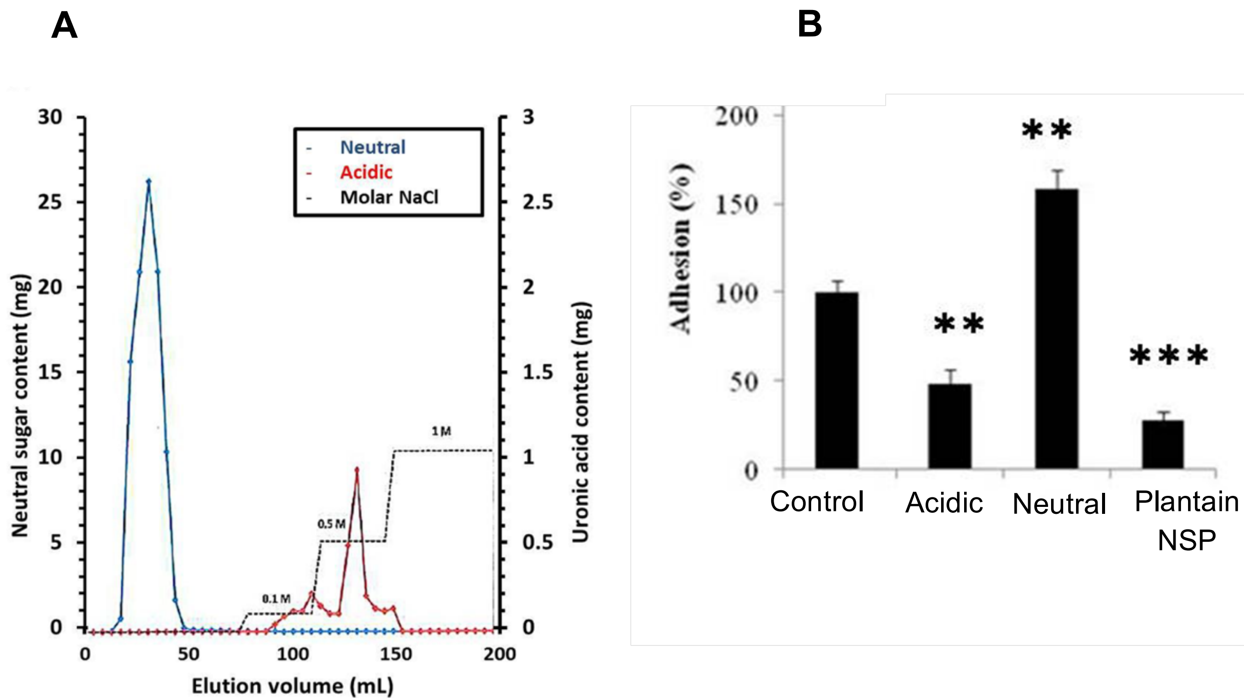


Figure 8.1 Purified analytical acidic polysaccharide fractions of plantain NSP significantly inhibit *Salmonella* adhesion to intestinal epithelial cells. **A)** High performance on exchange fractionation of soluble plantain NSP was performed on an analytical scale using a HiPrepTM Q Sepharose FF 16/10 column on the AKTA Prime Plus chromatography system. Column unbound (neutral fraction) was retained and bound material (acid fraction) was eluted step-wise with 50 mM Tris-HCl, pH 7.4 containing 0.1, 0.5 and 1M NaCl) at 5 mL/min. The neutral and acid fractions were subsequently desalted on PD-MidiTrap G-10 columns. This work was performed by Dr D O'Brien, Provexis plc, Windsor; 2011). **B)** Acidic fractions of plantain NSP blocked adhesion of *S*. Typhimurium to B10XI intestinal epithelial cells; N=4, n=4. All fractions tested at 5 mg/ml. *, P<0.05, ***, P<0.001 Kruskal-Wallis, compared to untreated control (i.e. in the absence of plantain NSP [set as 100%]). This work was performed by Dr B. Parsons (Parsons *et al.* 2014).

To confirm previous findings, it was necessary to perform strong-anion exchange chromatography on a larger scale to produce a bulk preparation of acidic and neutral plantain NSP fractions. These purified fractions could then be evaluated for their ability to disrupt a range of bacterial-host epithelium interactions. It was also important to assess the inhibitory effect of maltodextrin against bacterial-epithelial interactions.

8.2 HYPOTHESIS

The inhibitory activity of plantain NSP against pathogen-host epithelium carbohydrate interaction lies in its acidic polysaccharide fraction

8.3 AIMS

1. Produce a bulk preparation of acidic and neutral polysaccharide fractions of plantain NSP via scaled up, preparative scale strong anion exchange chromatography

2. Assess the inhibitory activity of polysaccharide fractions against the epithelial adhesion of diarrhoeal pathogens *C. difficile*, ETEC and *Salmonella*

3. Perform composition analysis on acidic and neutral polysaccharide fractions of plantain NSP

4. Assess the inhibitory effect of maltodextrin against pathogen-host carbohydrate interactions

8.4 METHODS

According to the results from the small-scale analytical fractionation of plantain NSP performed previously by Provexis Plc (Parsons *et al.* 2014), a bulk preparation of purified neutral and acidic fractions of soluble plantain fibre was carried out. This was also performed using strong-anion exchange chromatography, but in the absence of a column (Clark 1976). Detailed methods of strong-anion exchange fractionation can be found in *Section 4.3.2.2*.

Neutral and acidic fractions were desalted using PD MidiTrap G-10 gravity columns (GE Healthcare Sciences), as per the manufacturer's instructions. Columns were pre-calibrated with small molecular size marker phenol red (354 Da) (*Section 4.3.2.3*). To determine column fractionation elution profiles, carbohydrate content was measured using a phenol-sulphuric acid total hexose assay (*Section 4.3.2.4*)

Following their lyophilisation (*Section 4.3.4.5*), neutral and acidic plantain NSP fractions were evaluated for their ability to inhibit the epithelial adhesion of *C. difficile*, ETEC and *S.* Typhimurium. Inhibitory activity was assessed using bacterial adhesion assays, in which Caco2 cell monolayers were pre-incubated for 30 min with 5 mg/mL neutral or acidic plantain NSP fractions prior to bacterial infection (MOI 100). In parallel, cells were also pre-incubated for 30 min with 5 mg/mL whole plantain NSP or 5 mg/mL maltodextrin. Full methods for bacterial culture and bacterial adhesion assays can be found in *Section 4.6* and *Section 4.5*, respectively.

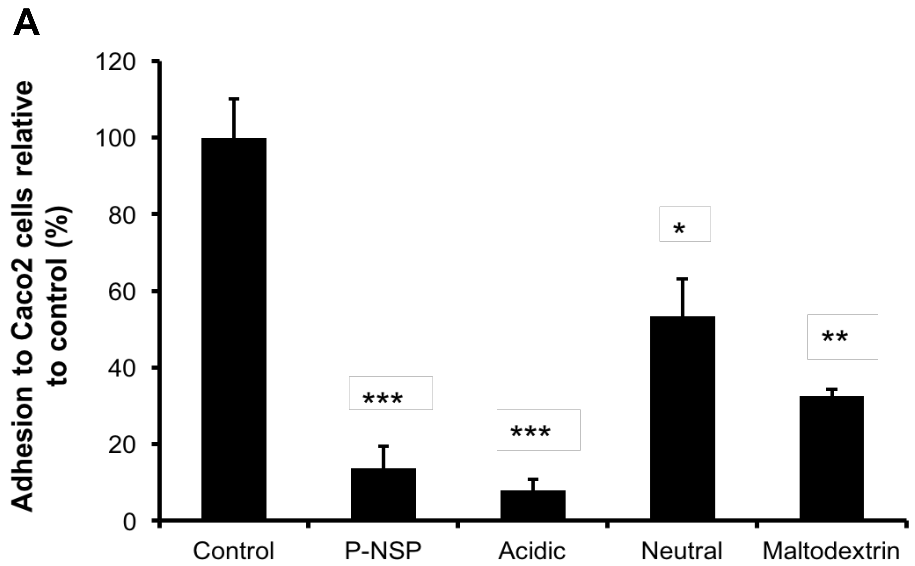
To further assess the inhibitory effect of maltodextrin on bacterial-epithelial interaction, HT29 colonocytes were pre-incubated with 5 or 10 mg/mL maltodextrin prior to treatment with 50 µL pooled supernatant of 5 UC mucosally-associated isolates (HM250, HM295, HM378, HM380 and HM387) for 4h. As a positive control, cells were also pre-incubated with 5 or 10 mg/mL plantain NSP prior to treatment. To assess the the effect of maltodextrin on the pro-inflammatory response mediated by UC *E. coli* flagellin, harvested media from cells was measured for the presence of pro-inflammatory cytokine IL-8 using a human IL-8 ELISA kit (*Section 4.15*). To assess the level of apoptosis, cellular activation of caspase-3/7 was measured using a commercial Caspase-Glo® 3/7 Assay, as per the manufacturer's instructions (*Section 4.16*). To assess cell cytotoxicity, harvested cell culture media was also assessed for the presence of adenylate kinase using a Toxilight Bioassay Kit (Lonza) (*Section 4.17*).

8.5 RESULTS

8.5.1 The inhibitory activity of plantain NSP against the epithelial adhesion of diarrhoeal pathogens lies in its acidic polysaccharide fraction

When human intestinal Caco2 cells were pre-treated with 5 mg/mL Q-Sepharose-purified acidic polysaccharide fraction of plantain NSP, there was significant blockade of diarrhoeal pathogen adhesion (**Figure 8.2**). In comparison to the untreated control, the acidic polysaccharide fraction of plantain NSP reduced the epithelial adhesion of *C. difficile* by 52.3 ± 7.3% (**Figure 8.2A**; $P < 0.01$), whilst ETEC and *S*. Typhimurium adhesion was reduced by 86.2 ± 5.7% (**Figure 8.2B**; $P < 0.001$) and 92.0 ± 2.9% (**Figure 8.2C**; $P < 0.001$), respectively. Conversely, pre-treatment of Caco2 cells with 5 mg/mL neutral polysaccharide fraction of plantain NSP (that was unbound to Q-Sepharose) exhibited a significantly reduced ability to inhibit bacterial adhesion (**Figure 8.2**). These results suggest that the activity conferring soluble plantain fibre's ability to block pathogen-host intestinal epithelium interactions lies predominantly in the acidic (pectic) component of plantain fibre.

Interestingly, maltodextrin also exhibited significant inhibitory activity against the epithelial adhesion of gut pathogens. However, with *C. difficile* as the only exception, the inhibitory activity of maltodextrin was less than that observed with respect to whole plantain NSP or its acidic fraction. In comparison to the untreated control, 5 mg/mL maltodextrin significantly reduced the epithelial adhesion of *C. difficile* by 61.8 ± 5.5% (**Figure 8.2A**; $P < 0.01$), ETEC by 54.5 ± 8.1% (**Figure 8.2B**; $P < 0.05$) and *S*. Typhimurium by 67.4 ± 1.8% (**Figure 8.2C**; $P < 0.01$).


A
Adhesion to Caco2 cells relative to control (%)
120
100
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60
40
20
0
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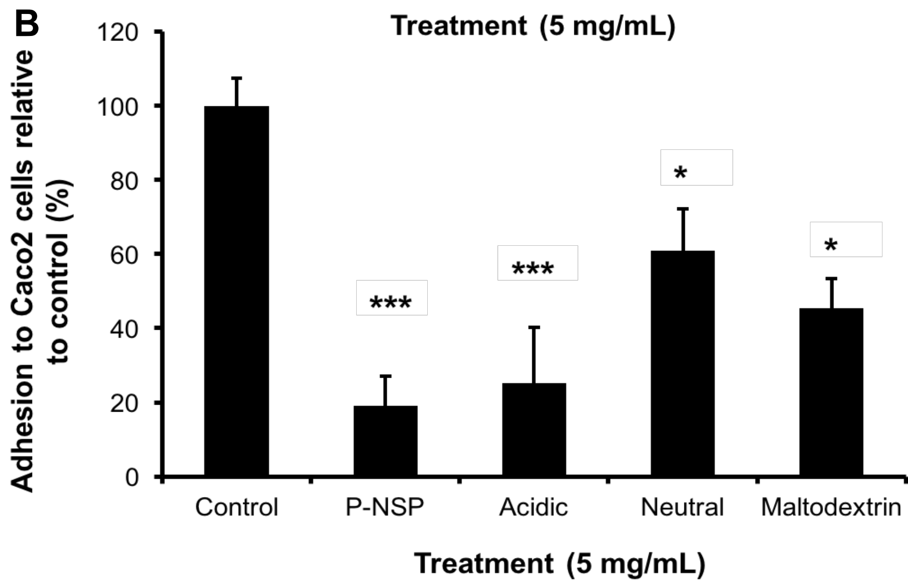
Control
P-NSP
Acidic
Neutral
Maltodextrin
Treatment (5 mg/mL)


B
Adhesion to Caco2 cells relative to control (%)
120
100
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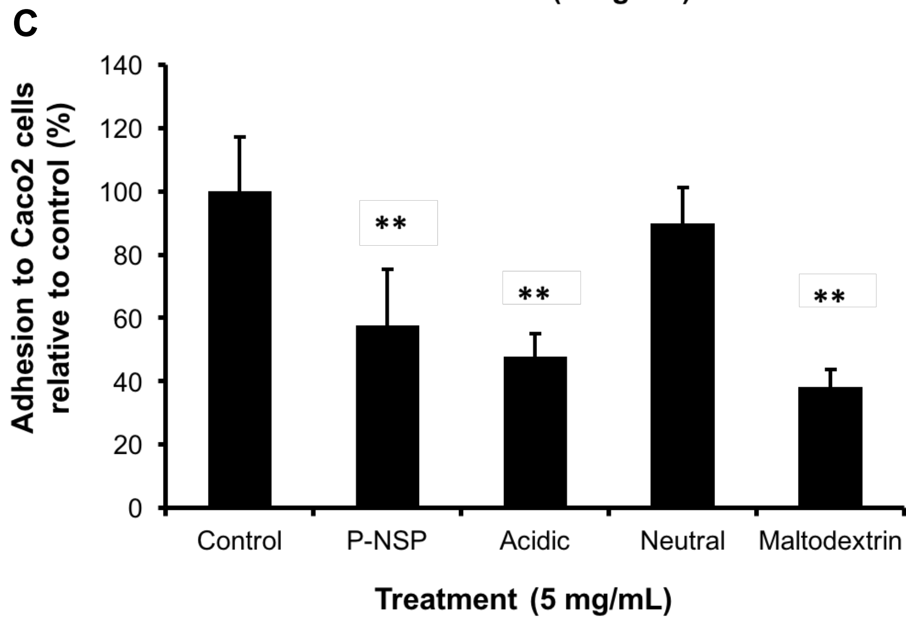
Treatment (5 mg/mL)


C
Adhesion to Caco2 cells relative to control (%)
140
120
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Treatment (5 mg/mL)

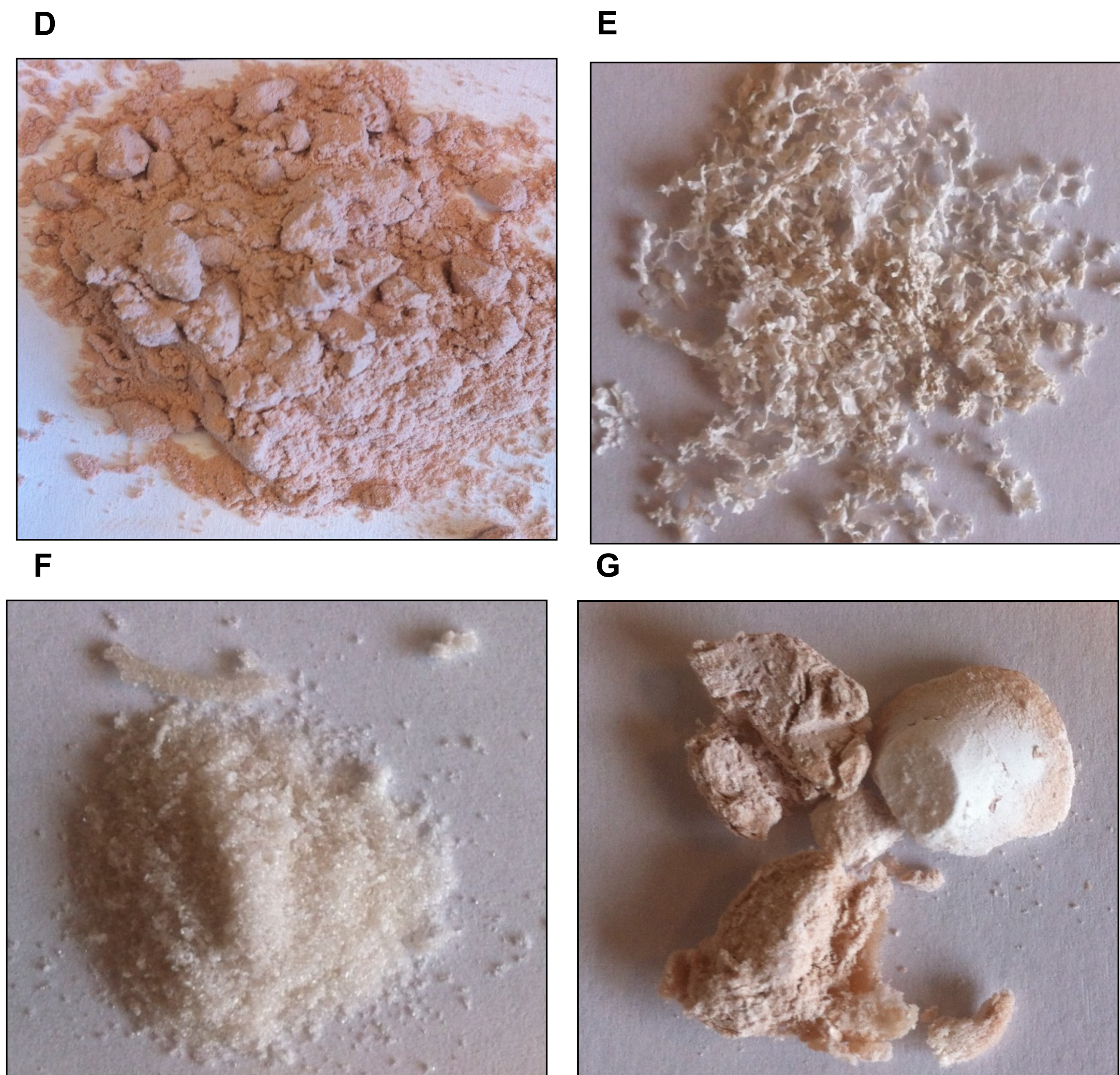

Figure 8.2 The action of soluble plantain NSP to block gut pathogen-host intestinal epithelium interaction lies within its acidic polysaccharide fraction. Pre-incubation of intestinal epithelial cells with 5 mg/mL acidic polysaccharide fraction of plantain NSP significantly blocked the adhesion of gut pathogens **A)** *C. difficile*, **B)** ETEC and **C)** *S*. Typhimurium. The neutral polysaccharide fraction of plantain NSP and maltodextrin (both 5mg/mL) exhibited reduced ability to block bacterial adhesion. Photographs are also included showing **D)** whole plantain NSP, **E)** acidic fraction of plantain NSP, **F)** neutral fraction of plantain NSP and **G)** maltodextrin. Bacterial adhesion is expressed relative to CFU/mL found in the absence of any fibre (set as 100%) (N=2, n=3; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; Kruskal-Wallis).

8.5.2 Maltodextrin significantly reduces the epithelial adhesion of *C. difficile*, but has no significant effect on the epithelial cell damage and inflammation mediated by flagellin

As it was shown that 5 mg/mL maltodextrin exhibited inhibitory activity against the epithelial adhesion of diarrhoeal pathogens (**Figure 8.2**), its ability to inhibit bacterial-epithelial interactions was further assessed in dose-related studies, using soluble plantain NSP as a positive control.

Maltodextrin was first further evaluated for its ability to inhibit the epithelial adhesion of diarrhoeal pathogens. As part of this work, Caco2 cells were pre-incubated with or without maltodextrin or plantain NSP (1, 5 or 10 mg/mL) for 30 minutes prior to infection with *C. difficile* (**Figure 8.3**). Pre-incubation of Caco2 cells with 1, 5 and 10 mg/mL maltodextrin significantly reduced the epithelial adhesion of *C. difficile* ($P \leq 0.01$) in a dose-dependent manner, with the optimum concentration occurring at 10 mg/mL (69.7 ± 11.1%; $P < 0.001$). However, it was also observed that maltodextrin exhibited a much lower efficacy than compared with plantain NSP, which at 10 mg/mL, reduced *C. difficile* adhesion by 92.6 ± 2.5% ($P < 0.001$) (**Figure 8.3**).

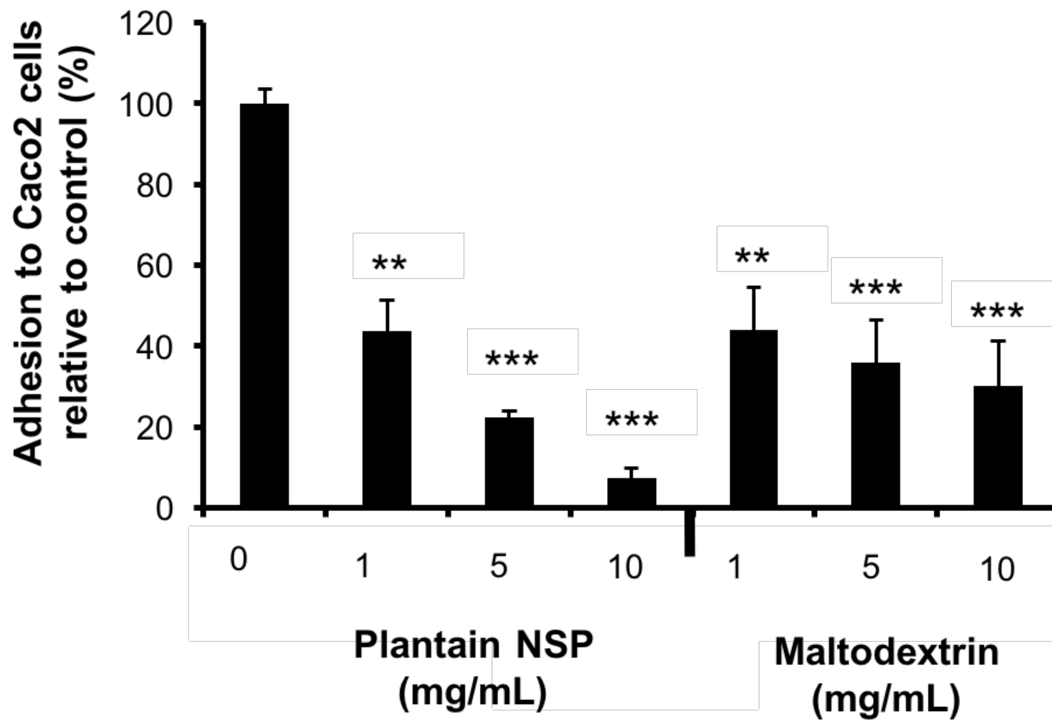

Figure 8.3 Soluble plantain fibre and maltodextrin both significantly inhibit *C. difficile* epithelial adhesion in a dose dependent manner. Caco2 cells were pre-incubated with 1, 5 or 10 mg/mL maltodextrin or plantain NSP (used as a positive control) for 30 min prior to infection with *C. difficile* (MOI 100) for 2h. Adhesion is expressed relative to CFU/mL found in the untreated control (set as 100%) (N=3, n=3; ** $P < 0.01$, *** $P < 0.001$; Kruskal-Wallis).

Maltodextrin was next assessed for its ability to reduce the epithelial cell damage and inflammation mediated by *E. coli* flagellin. Similarly, HT29 cells were pre-incubated with or without 5 and 10 mg/mL maltodextrin or plantain NSP prior to treatment with pooled UC *E. coli* supernatant, which contained flagellin and OMVs.

Pre-incubation of cells with 5 or 10 mg/mL plantain NSP resulted in considerable inhibition of the flagellin-mediated pro-inflammatory response, reducing IL-8 release by 33.6 ± 5.3% and 46.4 ± 7.4%, respectively. Oppositely, pre-treatment of cells with 5 or 10 mg/mL maltodextrin had little effect on the flagellin-mediated pro-inflammatory response, resulting only in small inhibitory levels of IL-8 release (7.4 ± 19.2% and 8.5 ± 12.0%, respectively) (**Figure 8.4**).

Similarly, 5 and 10 mg/mL plantain NSP significantly reduced flagellin-mediated cytotoxicity (P-NSP at 10 mg/mL reduced adenylate kinase release by 86.0 ± 3.4%; $P < 0.001$; **Figure 8.5**) and reduced cellular apoptosis (P-NSP at 10 mg/mL reduced caspase-3/7 activation by 49.7 ± 7.3%; $P < 0.001$; **Figure 8.6**). Maltodextrin, however, exhibited limited inhibitory activity (10 mg/mL reduced adenylate kinase release and caspase-3/7 activation in response to flagellin by only 19.4 ± 14.3% and 18.8 ± 7.9%, respectively; **Figure 8.5, Figure 8.6**).

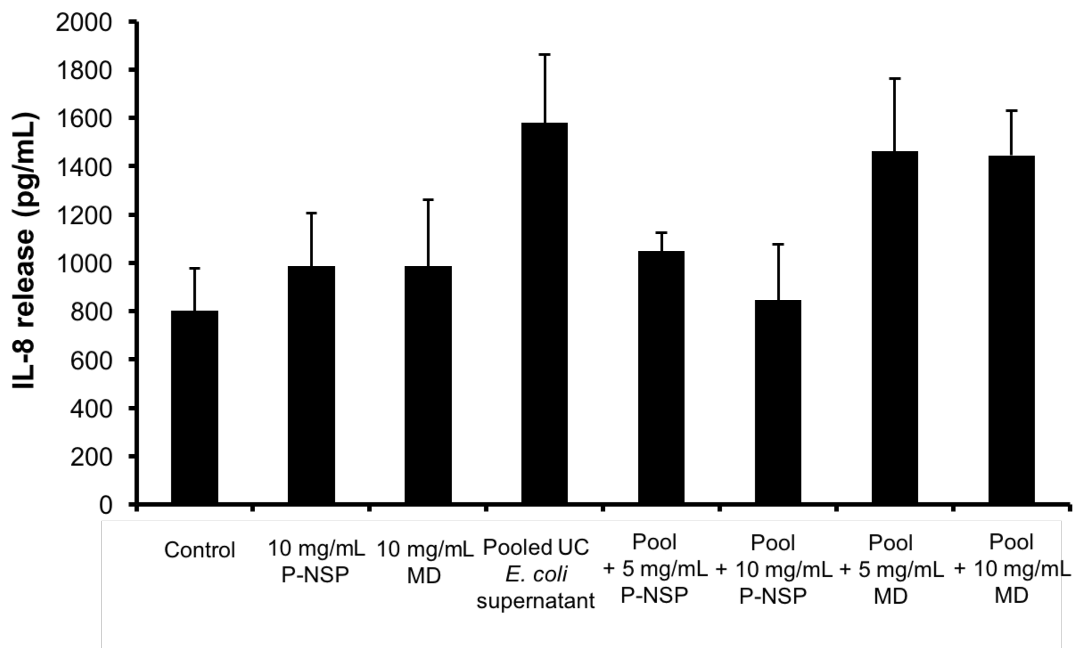

Figure 8.4 Soluble plantain fibre reduces the cellular IL-8 response induced by the pooled UC *E. coli* supernatant of five UC *E. coli* isolates, but maltodextrin has no effect. HT29 cell monolayers were pre-incubated with or without maltodextrin (5 or 10 mg/mL) for 30 min prior to treatment with 50 µL 0.2 µm-filtered culture supernatant pooled from five mucosal UC *E. coli* isolates (HM250, HM295, HM378, HM380, HM387) (50 µL; 4 h). As a positive control, cells were pre-incubated in parallel with or without plantain NSP (5 or 10 mg/mL) prior to treatment. Cell culture media harvested from cells was analysed for the presence of pro-inflammatory cytokine IL-8 using an IL-8 Human ELISA kit (N=3, n=3; One Way ANOVA).

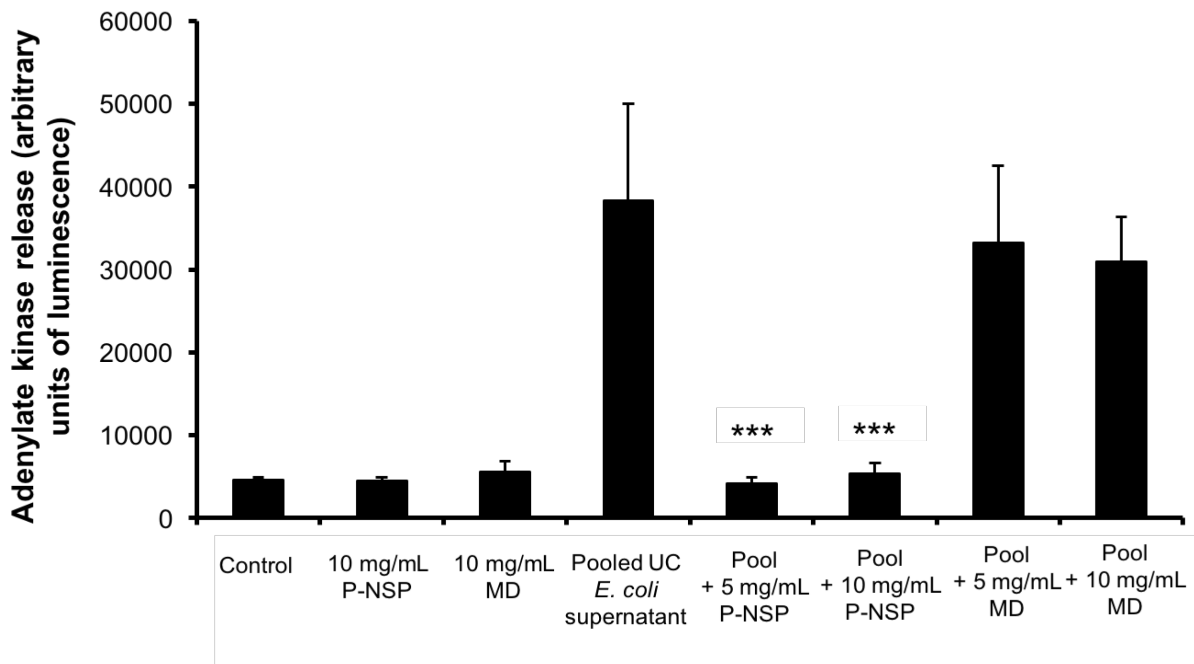

Figure 8.5 Soluble plantain fibre significantly reduces the cellular adenylate kinase release induced by the pooled supernatant of five UC *E. coli* isolates, **but maltodextrin has no effect.** HT29 cell monolayers were pre-incubated with or without maltodextrin (5 or 10 mg/mL) for 30 min prior to treatment with 50 µL 0.2 µm-filtered culture supernatant pooled from five mucosal UC *E. coli* isolates (HM250, HM295, HM378, HM380, HM387) (50 µL; 4 h). As a positive control, cells were pre-incubated in parallel with or without plantain NSP (5 or 10 mg/mL) prior to treatment. Cell culture media harvested from cells was analysed for the presence of adenylate kinase using a Toxilight™ bioassay kit (N=3, n=3; *** $P < 0.001$; Kruskal-Wallis).

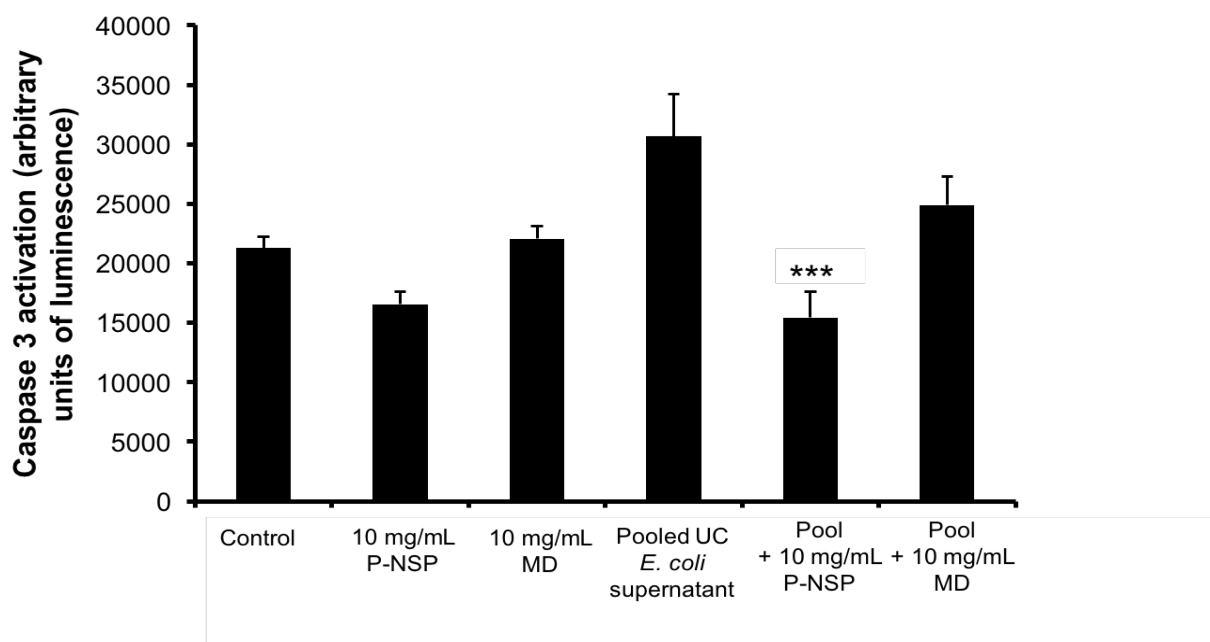

Figure 8.6 Soluble plantain fibre significantly reduces the cellular caspase-3/7 activation induced by the pooled supernatant of five UC *E. coli* isolates, but maltodextrin has no effect. HT29 cell monolayers were pre-incubated with or without 10 mg/mL maltodextrin or 10 mg/mL plantain NSP (used as a positive control) for 30 min prior to treatment with 50 µL 0.2 µm-filtered culture supernatant pooled from five mucosal UC *E. coli* isolates (HM250, HM295, HM378, HM380, HM387) (50 µL; 4 h). Caspase-3/7 activation was measured using a Caspase-Glo® 3/7 Assay, as per manufacturer's instructions (N=2, n=3; *** $P < 0.001$; Kruskal-Wallis).

8.6 SUMMARY OF MAIN RESULTS

1. The acidic, pectic polysaccharide fraction of plantain NSP is responsible for its inhibitory activity against the epithelial adhesion of diarrhoeal pathogens *C. difficile*, ETEC and *Salmonella*

2. Maltodextrin also exhibits dose-dependent inhibitory activity against the epithelial adhesion of diarrhoeal pathogens, but has no significant effect on the epithelial cell damage and inflammation mediated by *E. coli* flagellin

8.7 DISCUSSION

In these studies, we wanted to further characterise the polysaccharide or structure present in plantain NSP that is responsible for bioactivity, which was achieved by fractionation of plantain NSP into its neutral and acidic fractions. Results from bacterial adhesion assays demonstrated that pre-treatment of intestinal epithelial cells with the acidic fraction of plantain NSP significantly inhibited the epithelial adhesion of *C. difficile*, ETEC and *S*. Typhimurium, which was similar to the level of inhibitory activity exhibited by whole plantain NSP. Conversely, the neutral polysaccharide fraction of soluble plantain NSP exhibited a decreased inhibitive effect on bacterial adhesion. Overall, these results suggest that the inhibitory activity of soluble plantain NSP against bacteria-epithelial interaction is largely due to the acidic, pectic fraction of plantain NSP. These findings reflect the preliminary results previously shown by our group, which also demonstrated that pre-treatment of porcine B1OX1 cells with the acidic, pectin fraction of plantain NSP resulted in significant inhibition of S. Typhimurium adhesion (Parsons *et al.* 2014).

Pectins comprise approximately one-third the dry weight of primary cell walls in non-poalean monocots such as plantain, playing key roles in cell-wall structure and function (Dick-Perez *et al.* 2012; Mohnen 2008). Its three distinct domains are covalently linked, with RGs containing large proportions of neutral sugars, especially L-rhamnose, D-galactose and L-arabinose, in addition to D-galacturonic acid (Fry 2011). Some pectins also carry methyl and acetyl ester groups (Perrone *et al.* 2002). Pectic polysaccharides interlink to each other, e.g. homogalacturonan domains via Ca^{2+} bridges (Mohnen 2008), and RG-II domains via borate bridges (ONeill *et al.* 1997). Composition analysis performed previously by our group demonstrated that the acidic fraction contained approximately 25% by weight of pectic

material, which was mainly or only homogalacturonan. In this study, negligible rhamnose, galactose or arabinose was formed following acid hydrolysis or Driselase digestion, which indicated the absence of RG-I and RG-II. Composition analysis also revealed the presence of pectic material in the whole plantain NSP fraction, whilst the neutral fraction, which showed much reduced blockade of *C. difficile*, ETEC and *S*. Typhimurium here in these studies, contained no detectable pectic material (Parsons *et al.* 2014).

These findings reflect those of other studies which indicate that bacterial adherence to epithelial cells can often be blocked by monosaccharides or oligosaccharides present in dietary constituents. Human milk, rich in oligosaccharides, has been known for some time to be potentially beneficial in preventing certain bacterial infections, inhibiting the adhesion of a range of bacteria such as *Campylobacter jejuni* (Ofek *et al.* 2003), diffusely adherent *E. coli* (DEAC) (de Araujo and Giugliano 2000) and enteroaggregative *E. coli* (EAEC) *in vitro* (de Araujo and Giugliano 2000). More specifically, pectic oligosaccharides have also been shown to inhibit *C. jejuni* intestinal epithelial adherence *in vitro* (Ganan *et al.* 2010). In addition, pectin from ginseng has also been shown to possess anti-adhesive activity against other gut pathogens such as *Helicobacter pylori*, and some ability to inhibit haemagglutination by bacteria, including that caused by *Staphylococcus aureus* and *Propionibacterium acnes*, but not that affected by *E. coli* and *Lactobacillus acidophilus* (Lee *et al.* 2006a).

Interestingly, it was also illustrated in these studies that maltodextrin, which was added to soluble plantain fibre during its manufacturing process, exhibited inhibitory activity against the epithelial adhesion of *C. difficile*, ETEC and *S*. Typhimurium. However, the inhibitory activity of maltodextrin was generally less than that observed with respect to whole plantain NSP or its acidic fraction. Furthermore, whilst whole plantain NSP was shown to

significantly reduce the epithelial cell damage and inflammation mediated by *E. coli* flagellin, pre-incubation of cells with maltodextrin had no significant effect. Composition analysis also demonstrated that maltodextrin was present in whole, acidic and neutral fractions, however, this did not correlate with the level of inhibitory activity that was observed. Instead, the inhibitory activity of each fraction correlated with the presence of pectic material. Collectively, these results suggest that maltodextrin only contributes slightly towards the inhibitory activity of plantain NSP against bacterial-epithelial interactions, and indeed, they add to the evidence to suggest that the acidic, pectic polysaccharide fraction is largely responsible for bioactivity.

Our findings presented here are in contrast to those reported by other groups. Indeed, previous studies have suggested that maltodextrin alters the intestinal microbiota namely through its ability to enhance bacterial survival and mucosal adhesion. Using an *in vivo* mouse model, one study reported that maltodextrin enhanced *Salmonella* survival and mucosal colonisation (Nickerson and McDonald 2012), whilst another study suggested that maltodextrin promoted AIEC biofilm production and adhesion to intestinal epithelial HT29 cell monolayers (Nickerson *et al.* 2014). Maltodextrins are classified according to glucose chain length, known as dextrose equivalent (DE), which varies from 3 – 20 (Chronakis 1998). Thus, the contrasting results might be explained by the difference in DE between studies.

It is also important to note that starches such as maltodextrin are hydrolysed *in vivo* by oral and ileal amylases (Quezada-Calvillo *et al.* 2007a), before undergoing further degradation by epithelial cell membrane-bound maltases (Quezada-Calvillo *et al.* 2007b). Therefore, it is likely that maltodextrin would undergo almost complete fermentation during transit through the gastrointestinal tract. As such, a high dosage of maltodextrin would be

needed to achieve effective luminal concentrations in the distal colon and thus have an effect on bacterial-epithelial interaction *in vivo*. Conversely, it has been shown that the inhibitory concentrations exhibited by plantain NSP *in vitro* are readily achievable *in vivo* by twice daily supplementation with 5 g soluble fibre (Roberts *et al.* 2010).

Chapter 9

The Characterisation of the Molecular Mechanism of the Anti-inflammatory and Anti-Bacterial Actions of Soluble Plantain Fibre

9.1 INTRODUCTION

The work performed by our group to date has confirmed that soluble plantain fibre, particularly its pectin fraction, can significantly reduce bacterial epithelial adhesion and translocation, as well as down-regulate the epithelial cell damage and inflammatory response to bacterial components. However, the molecular mechanism underlying the inhibitory activity of plantain NSP is currently unclear.

There are a number of potential mechanisms by which plantain NSP could mediate its inhibitory effect against the epithelial interactions of diarrhoeal pathogens. It is likely that soluble plantain fibre exhibits its effect through one of two ways; either through direct interaction with the bacteria or via interaction with intestinal epithelial cells. Interestingly, there is evidence to support both of these hypotheses.

We originally hypothesised that plantain NSP inhibited bacterial adhesion via direct contact with bacteria, whereby the diverse array of partially or non-digested oligosaccharides present in soluble fibre might competitively bind to bacterial adhesins, thus inhibiting bacterial interaction with the intestinal epithelium. We have shown in Chapter 5 of this thesis that soluble plantain NSP does indeed interact with diarrhoeal pathogens *C. difficile* and ETEC, and in fact, provides an energy source for their growth in the absence of cells.

In contrast, previous studies by our group and others offer substantial evidence to suggest that plantain NSP might mediate its inhibitory effect via interaction with the intestinal epithelium. We have previously shown that pre-treatment with 5 mg/mL plantain NSP significantly inhibited the translocation of EGFP-expressing S. Typhimurium LT2 across isolated human ileal FAE mounted in Ussing chambers (Roberts *et al.* 2013). Interestingly, we

also reported that pre-treatment of human ileal FAE with plantain NSP was associated with a marked increase in transmucosal short circuit current (I_{sc}) (Parsons *et al.* 2014). This suggests that the inhibitory effect of plantain NSP could be mediated by increased epithelial chloride secretion, which as a physiological effect will cause efflux of ions and water transport, potentially impeding bacterial-epithelial interaction (Strober and McGhee 2005). Indeed, a study by Keeley and colleagues showed that secretagogue enhanced epithelial Cl- secretion and water transport reduced *Salmonella* internalisation to, and translocation across T84 colonocytes by over 70% (Keely *et al.* 2012).

The observed mucosally protective and anti-inflammatory effects of soluble plantain fibre in many ways mimic the dense continuous mucus layer lining the healthy colon, which shields the underlying intestinal epithelium from bacterial interaction (Turner 2009). The protective mucus barrier is organised around the highly *O*-glycosylated MUC2 mucin, which is secreted by goblet cells to form a large net-like polymer (Johansson *et al.* 2011). Recently, it has been shown that MUC2, via an action dependent on its numerous *O*-glycans, has an important anti-inflammatory effect on the mucosa that is mediated by interaction with cell-surface galectin-3 and subsequent recruitment of Dectin-1. This complex interaction leads to the activation and nuclear translocation of β-catenin, subsequent inactivation of NF-κβ, consequent suppression of a range of pro-inflammatory cytokine genes and increase in anti-inflammatory IL-10 (Shan *et al.* 2013). Indeed, preliminary results from our research group have shown that soluble plantain fibre, like MUC2 glycans, also interacts with galectin-3 (Shapanis A, Gastroenterology Research Unit, Department of Cellular & Molecular Physiology, University of Liverpool; unpublished data). Therefore, it is possible that the inhibitory effect

of plantain NSP might lie in its ability to mimic MUC2 glycans and interact with cell-surface galectin-3, with a consequent ‘contrabiotic’ effect (Simpson and Campbell 2015).

9.2 HYPOTHESES/AIMS

To address the hypotheses that:

1. Soluble plantain NSP inhibits bacterial adhesion via an interaction with the intestinal epithelium or through direct interaction with bacteria

2. The inhibitory activity of plantain NSP is mediated by increased epithelial chloride secretion

3. Soluble plantain NSP can induce the nuclear translocation of β-catenin

4. The inhibitory activity of plantain NSP is mediated by cell-surface galectin-3 expression

9.3 METHODS

To determine whether the inhibitory action of plantain NSP against diarrhoeal pathogens occurs via an interaction with the epithelial cell monolayer or, alternatively, through direct interaction with bacteria, modifications were made to the bacterial adhesion assays described in *Section 4.6* (Parsons *et al.* 2014). To evaluate if soluble plantain NSP exhibits its inhibitory effect via interaction with the intestinal epithelium, plantain NSP was added to cell monolayers 30 min prior to infection as earlier described, but then removed by three washes with pre-warmed sterile PBS (1 min each, 37°C). Monolayers were then provided with fresh antibiotic-free DMEM and infected with *C. difficile*, ETEC or *Salmonella* as normal. To test if soluble plantain NSP exhibits its inhibitory effect via a direct interaction with bacteria, plantain NSP was pre-incubated with *C. difficile*, ETEC or *Salmonella* for 30 min, followed by centrifugation, resuspension of bacteria in antibiotic-free DMEM and inoculation of epithelial cell monolayers. The remainder of the bacterial adhesion assays were performed according to the methods described fully in *Section 4.6*.

Ussing chamber experiments that were carried out previously by our group suggested that plantain NSP might mediate increased epithelial chloride secretion (Parsons *et al.* 2014). Therefore, plantain NSP was further assessed *in vitro* for its ability to induce cellular chloride channel activation, and importantly, if this mediated its inhibitory action. The first part of this work was performed using a functional chloride channel activity assay, which measures cellular iodide efflux as a surrogate for monitoring cellular chloride channel activation (Tang and Wildey 2004) (*Section 4.12*). First, dose-response studies were used to determine if soluble plantain NSP (0 – 20 mg/mL) could activate cellular chloride channel activity in Caco2 cells compared with known chloride channel activators forskolin and RP107 (0 – 200 µM for

both), which were used as positive controls (*Section 4.12.1*). Secondly, dose-response studies were performed using chloride channel antagonists CFTR-inh-172 (0 – 200 μM) and NPPB (0 – 800 μM) to assess if cellular chloride channel activity could be knocked down (*Section 4.12.2*). To determine if the inhibitory activity of plantain NSP was mediated by chloride secretion, the chloride channel modulators (at optimum concentrations determined in dose-response studies) were then used alongside plantain NSP in bacterial adhesion assays (*Section 4.12.3)*.

To assess whether the inhibitory activity of plantain NSP is mediated by the expression of galectin-3, the mucosally protective and anti-inflammatory effect of plantain NSP were compared between stable galectin-3 knockdown SW620 colonic epithelial cells ($SW620^{Gal3-}$) and galectin-3 expressing colonic epithelial cells ($SW620^{Gal3+}$). SW620 cells were transfected by Paulina Sindrewicz (Gastroenterology Research Unit, Department of Cellular & Molecular Physiology, University of Liverpool) according to the methods described in *Section 4.2.2*. Stable transfected $SW620^{Gal3-}$ cells showed an 84% reduction of galectin-3 expression in comparison to negatively transfected $SW620^{Gal3+}$ cells, as confirmed by immunoblotting and immunohistochemistry performed by Duckworth and colleagues (Duckworth *et al.* 2015).

To assess whether the inhibitory effect of plantain NSP against bacterial adhesion is mediated by cell-surface galectin-3 expression, $SW620^{Gal3-}$ and $SW620^{Gal3+}$ cells were pre-treated with or without 10 mg/mL plantain NSP for 30 min prior to infection with *C. difficile* (MOI 100; 2 h). The bacterial adhesion assay was then performed as previously (*Section 4.6*). To assess whether the anti-inflammatory effect of plantain NSP is mediated by interaction with cell-surface galectin-3, $SW620^{Gal3-}$ and $SW620^{Gal3+}$ cells were pre-treated with 10 mg/mL plantain NSP for 30 min prior to treatment with 50 μl pooled 0.2 um filtered UC *E. coli* culture

supernatant (containing *E. coli* flagellae and OMVs) for 2 h (*Section 4.9*). Cell media was then harvested and assessed for the presence of pro-inflammatory IL-8 using a solid phase sandwich ELISA (*Section 4.15*).

The intracellular localisation of β-catenin was visualised by immunocytofluorescence microscopy, in which fixed SW480 colon cancer cells were incubated with primary mouse monoclonal IgG anti-human β-catenin antibody (1:1000; 1 h) followed by secondary FITC-conjugated rabbit polyclonal anti-mouse Ig antibody (1:2000; 1 h). Cell nuclei were then counterstained with DAPI (*Section 4.13.2*). SW480 cells were used in this part of the study purely for practical reasons, which was due to a lack of cultured Caco2 cells and time restraints within the project.

9.4 RESULTS

9.4.1 Soluble plantain fibre inhibits the epithelial adhesion of diarrhoeal pathogens via an effect on the intestinal epithelium

When Caco2 cells were pre-incubated for 30 min with 0 – 10 mg/mL plantain NSP that was removed by three washes with sterile PBS prior to bacterial inoculation, levels of adherent bacteria were observed to be significantly reduced. In comparison to the untreated control, 10 mg/mL plantain significantly reduced *C. difficile*, ETEC and *S*. Typhimurium by 78.5 ± 7.0% (**Figure 9.1B**), 39.4 ± 1.1% (**Figure 9.2B**) and 75.1 ± 1.2% (**Figure 9.3B**), respectively (All $P < 0.001$; Kruskal-Wallis). Whilst there was significant residual inhibition following the removal of plantain NSP, there was a higher level of inhibition observed in experiments where plantain NSP was added to cells for 30 min without removal before bacterial infection (in comparison to the untreated control, 10 mg/mL plantain NSP reduced *C. difficile*; ETEC and *S*. Typhimurium by 94.9 ± 0.9 [**Figure 9.1A**], 64.4 ± 5.7 [**Figure 9.2A**] and 92.9 ± 2.0% [**Figure 9.3A**], respectively; All $P < 0.001$, Kruskal-Wallis). In contrast, pre-incubation of plantain NSP with bacteria for 30 min, followed by removal of soluble fibre by centrifugation, resulted in no significant inhibition of *C. difficile*, ETEC and *S*. Typhimurium compared with untreated controls (**Figure 9.1C, Figure 9.2C and Figure 9.3C**, respectively). These results suggest that soluble plantain NSP exhibits its inhibitory effect via interaction with the intestinal epithelium rather than by direct interaction with bacteria.

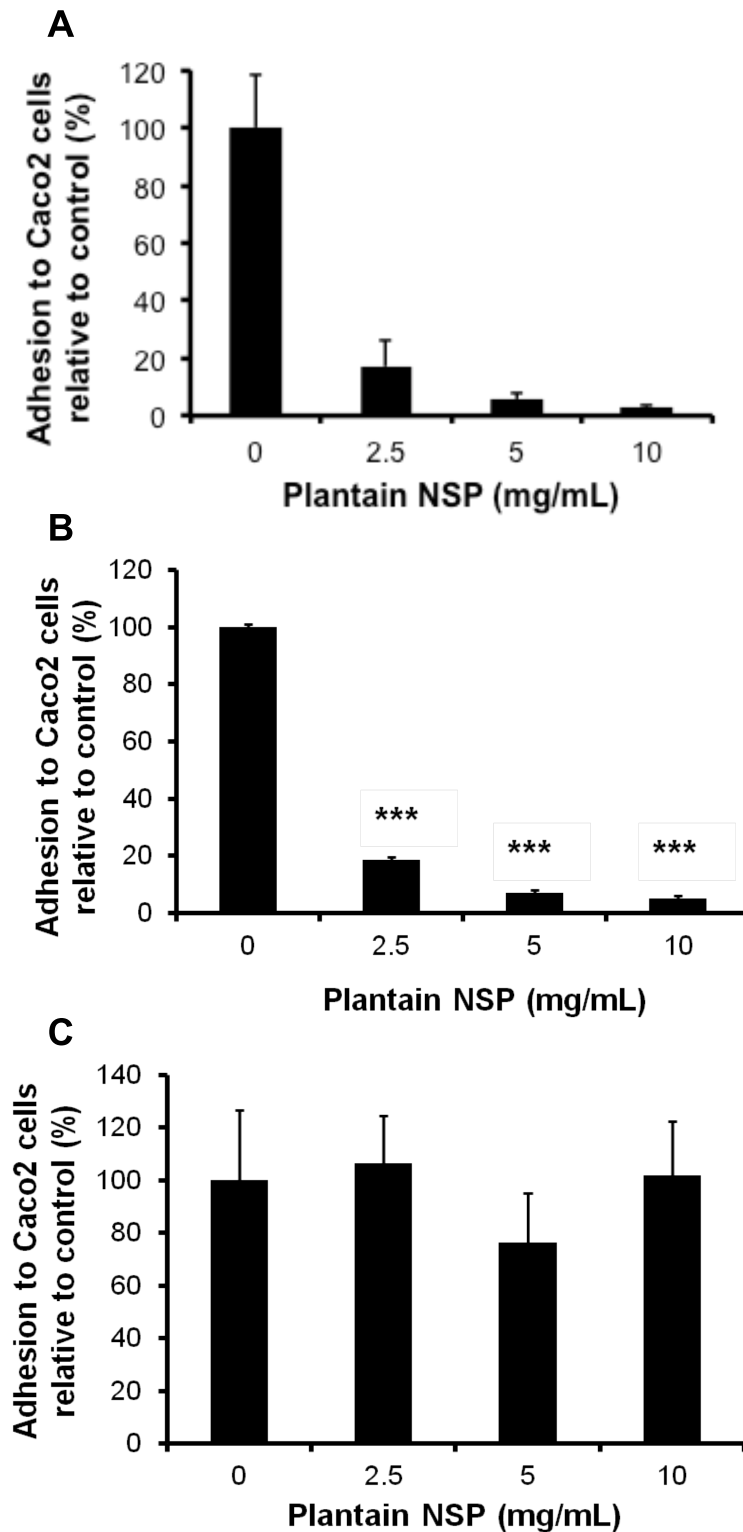

Figure 9.1 Soluble plantain NSP acts on the epithelium to inhibit the interaction of *C. difficile*. Plantain NSP blockade of *C. difficile* adhesion to Caco2 cells was assessed under different pre-treatment conditions. **A)** Standard pre-treatment of cell monolayers with plantain NSP (30 min), followed by infection for 2 h. **B)** Pre-treatment of cell monolayers with soluble plantain NSP (30 min), followed by removal from monolayers with three sterile PBS washes prior to infection for 2 h. **C)** Pre-treatment of *C. difficile* with plantain NSP (30 min), followed by centrifugation, resuspension of bacteria in antibiotic-free media and infection for 2 h. Adhesion is expressed relative to CFU/mL found in untreated control (set as 100%) (N ≥1, n=3; ** $P < 0.01$, *** $P < 0.001$; Kruskal-Wallis).

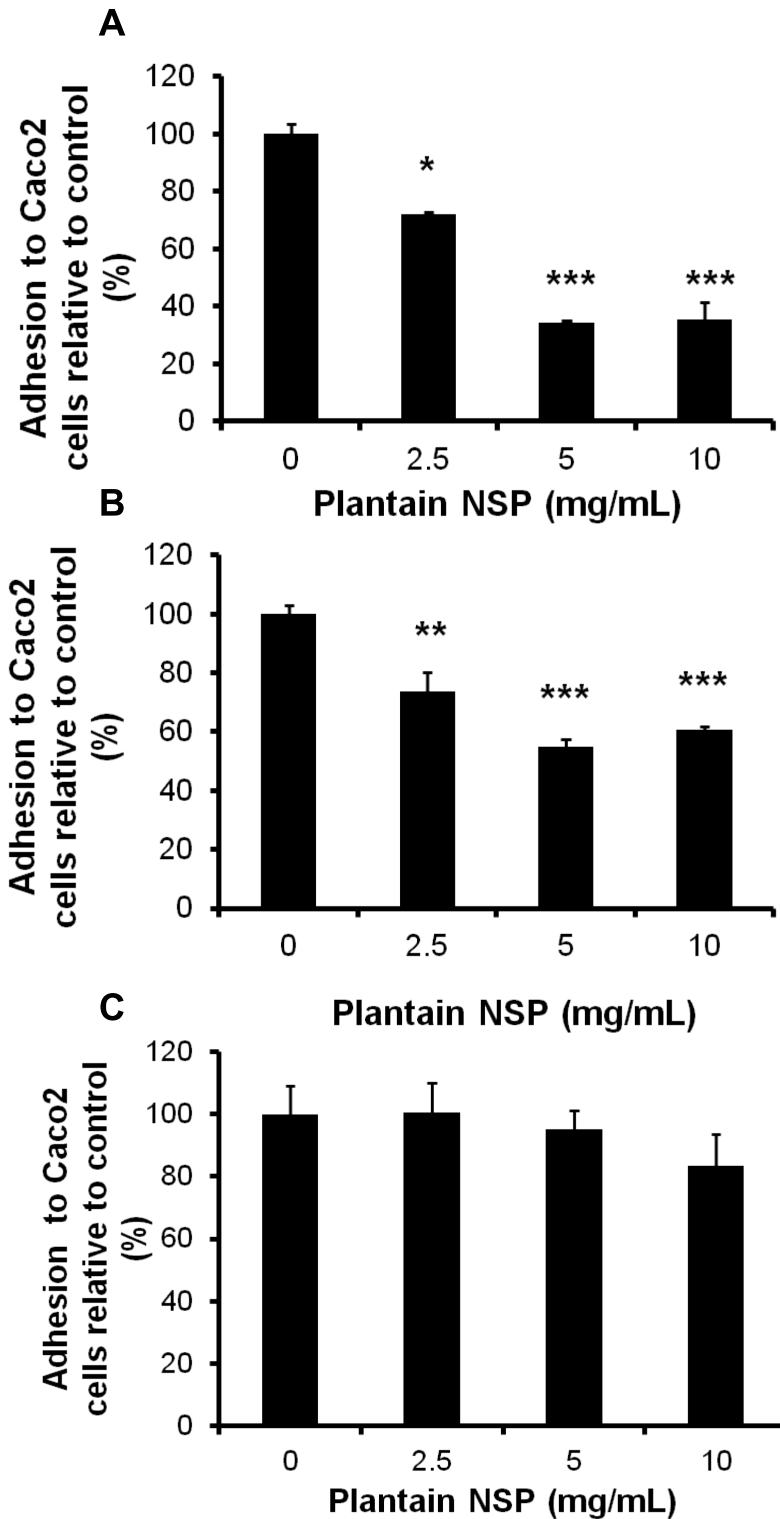

Figure 9.2 Soluble plantain NSP acts on the epithelium to inhibit the interaction of ETEC. Plantain NSP blockade of ETEC adhesion to Caco2 cells was assessed under different pre-treatment conditions. **A)** Standard pre-treatment of cell monolayers with plantain NSP (30 min), followed by infection for 1.5 h. **B)** Pre-treatment of cell monolayers with soluble plantain NSP (30 min), followed by removal from monolayers with three sterile PBS washes prior to infection for 1.5 h. **C)** Pre-treatment of ETEC with plantain NSP (30 min), followed by centrifugation, resuspension of bacteria in antibiotic-free media and infection for 1.5 h. Adhesion is expressed relative to CFU/mL found in untreated control (set as 100%) (N ≥1, n=3; ** P < 0.01, *** P< 0.001; Kruskal-Wallis).

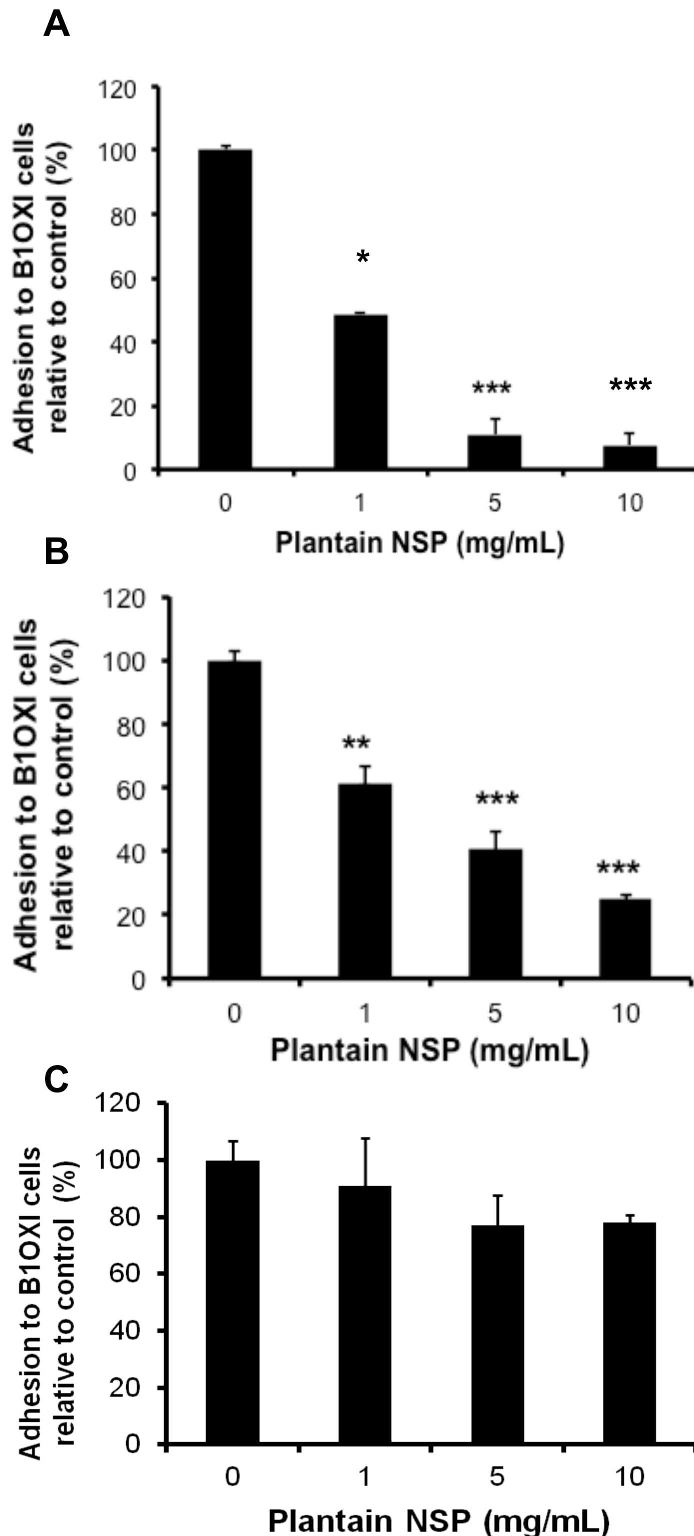

Figure 9.3 Soluble plantain NSP acts on the epithelium to inhibit the interaction of S. Typhimurium LT2. Plantain NSP blockade of *S.* Typhimurium LT2 adhesion to B1OXI porcine epithelial cells was assessed under different pre-treatment conditions. **A)** Standard pre-treatment of cell monolayers with plantain NSP (30 min), followed by infection for 1.5 h. **B)** Pre-treatment of cell monolayers with soluble plantain NSP (30 min), followed by removal from monolayers with three sterile PBS washes prior to infection for 1.5 h. **C)** Pre-treatment of *S.* Typhimurium with plantain NSP (30 min), followed by centrifugation, resuspension of bacteria in antibiotic-free media and infection for 1.5 h. Adhesion is expressed relative to CFU/mL found in untreated control (set as 100%) (N ≥1, n=3; ** P < 0.01, *** P< 0.001; Kruskal-Wallis).

9.4.2 Soluble plantain fibre mediates increased cellular chloride channel activity *in vitro*

Our group has previously shown that 5 mg/mL plantain NSP significantly reduces the translocation of EGFP-expressing *S*. Typhimurium LT2 across isolated human ileal FAE mounted in Ussing chambers (Roberts *et al.* 2013). Interestingly, we also reported that this was associated with a significant increase in transmucosal $I_{sc,}$ which was likely due to increased epithelial chloride secretion (Parsons *et al.* 2014). As such, further experiments were performed to assess if plantain NSP could induce cellular chloride channel activation *in vitro.* This was monitored using a functional chloride channel activity assay.

As a positive control, Caco2 cells were first treated with 0 – 200 µM of known chloride channel agonists forskolin and RP107, which significantly increased cellular chloride channel activity in a dose-dependent manner. Treatment of Caco2 cells with 200 µM forskolin and 100 µM RP107 resulted peak levels of activation, increasing chloride channel activity by 5.4 ± 0.5 fold and 8.2 ± 1.0 fold, respectively, in comparison to the untreated vehicle control (both $P < 0.001$, Kruskal-Wallis, **Figure 9.4A and B**).

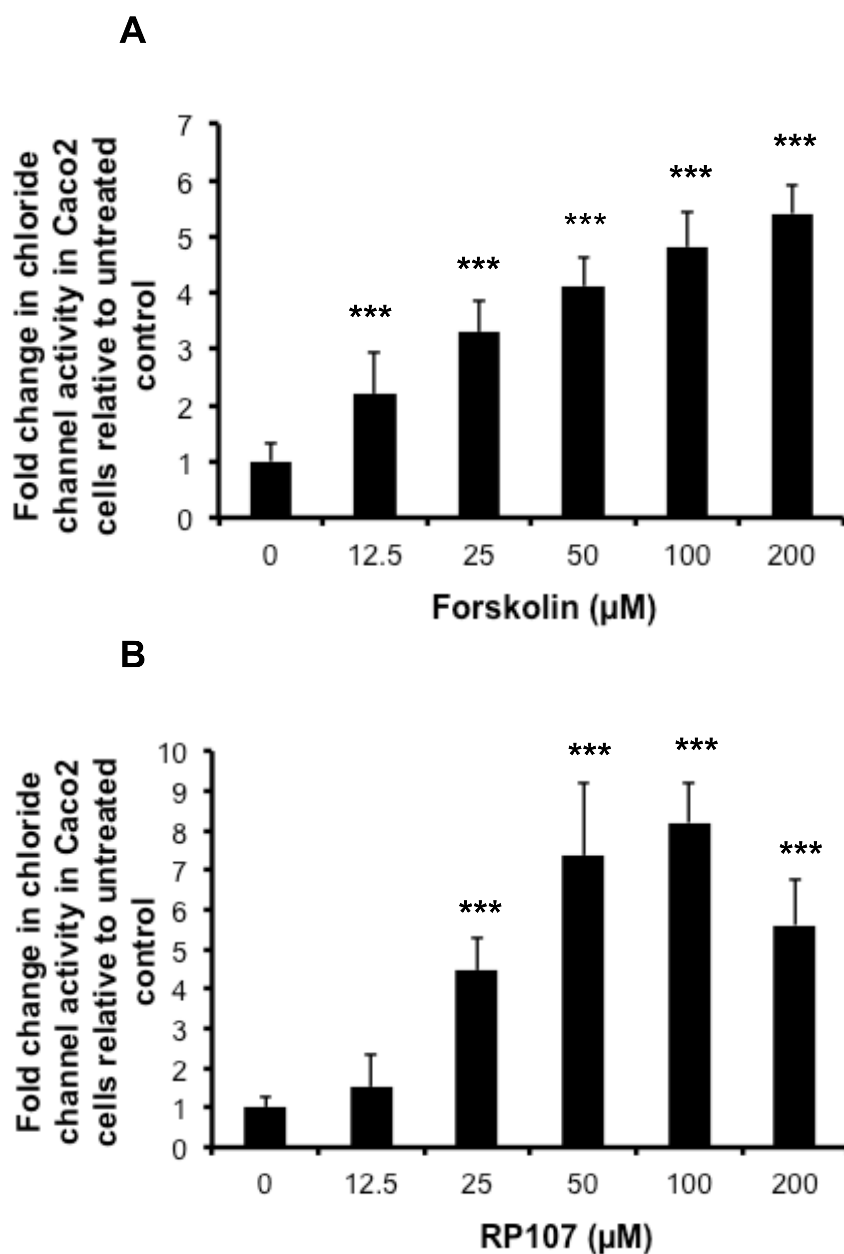

Figure 9.4. **Chloride channel agonists forskolin and RP107 induce increased cellular chloride channel activation in a dose-dependent manner.** Chloride channel activity was measured by monitoring cellular iodide efflux. Caco2 cell monolayers were loaded with iodine-loading buffer for 4 h, and then incubated with chloride channel activator **A)** forskolin (0 – 200 µM) or **B)** RP107 (0 – 200 µM) for 30 min. Following cell lysis, the Modified Sandell-Kolthoff (SK) reaction was used to measure cellular iodide concentration. Chloride channel activity was expressed as fold change in comparison to Caco2 cells treated with the vehicle-control (N=3, n=6; *** $P < 0.001$; Kruskal-Wallis).

Treatment of Caco2 cells with 2.5 – 20 mg/mL plantain NSP also resulted in a dose-dependent increase in cellular chloride channel activity, where 10 and 20 mg/mL plantain NSP resulted in 5.3 ± 0.5 fold and 5.4 ± 0.4 fold increase in chloride channel activity in comparison to the untreated control (both $P < 0.001$, Kruskal-Wallis, **Figure 9.5**). Other NSP fibres that have previously shown no inhibitory effect against bacterial adhesion were also evaluated for their ability to induce chloride channel activation. These fibres exhibited less efficacy; oat and bean NSP increased chloride channel activation by 2.2 ± 0.6 fold and 2.0 ± 0.3 fold, respectively ($P < 0.001$, Kruskal-Wallis), whilst NSP from neither tomato, pear, pepper nor apple reduced chloride channel activity in comparison to the untreated control (**Figure 9.5**).

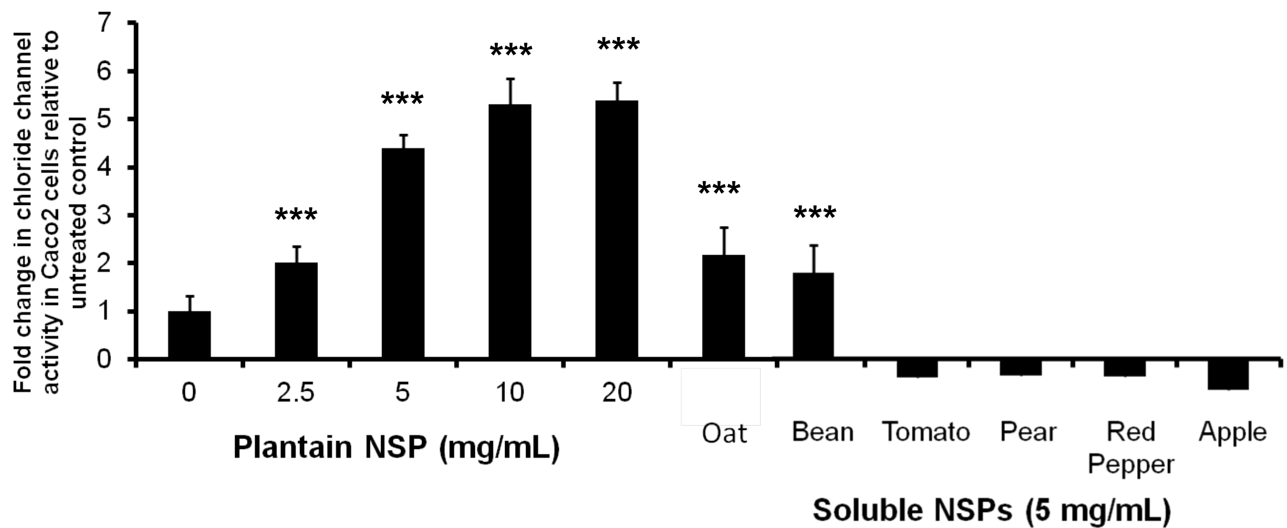

Figure 9.5 Plantain NSP induces cellular chloride channel activity in a dose-dependent manner. Chloride channel activity was measured by monitoring cellular iodide efflux. Caco2 cell monolayers were loaded with iodine-loading buffer for 4 h, and then incubated with soluble plantain NSP (0-20 mg/mL) or other soluble NSP (5 mg/mL) for 30 min. Following cell lysis, cellular iodide concentration was measured using the Modified Sandell-Kolthoff (SK) reaction. 2.5 – 20 mg/mL plantain NSP significantly induced chloride channel activity in a dose-dependent manner. 5 mg/mL oat and bean NSP also significantly increased chloride channel activity, whilst pear, pepper and apple NSP exhibited no significant effect. Chloride channel activity was expressed as fold change in comparison to the vehicle treated control (N=3, n ≥ 3; *** P < 0.001; Kruskal-Wallis)

Next, it was assessed if cellular chloride channel activity could be knocked down in the presence of known chloride channel antagonists NPPB and CFTR-inh-172. In Caco2 cells pre-treated with 10 mg/mL plantain NSP, chloride channel activity was inhibited by NPPB and CFTR-inh-172 in dose-dependent manner (**Figure 9.6**). Treatment of Caco2 cells with 800 µM NPPB and 200 µM CFTR-inh-172 resulted in the optimum level of inhibition, reducing chloride channel activity by 94.7 ± 10.0% ($P < 0.001$; **Figure 9.6A**) and 86.9 ± 8.5% ($P < 0.001$; **Figure 9.6B**), respectively, in comparison to the untreated control.

Similarly, NPPB and CFTR-inh-172 also dose-dependently reduced the cellular chloride channel activity evoked by 200 µM forskolin (**Figure 9.7**) and 100 µM RP107 (**Figure 9.8**). NPPB, at 800 µM, resulted in peak levels of inhibition, reducing forskolin- and RP107-mediated chloride channel activity by 103.3 ± 3.2% (**Figure 9.7A**) and 86.7 ± 8.9% (**Figure 9.8A**), respectively ($P < 0.001$ for both; Kruskal-Wallis). CFTR-inh-172, at 200 µM, also resulted in peak levels of inhibition, reducing forskolin- and RP107-mediated chloride channel activity by 99.6 ± 5.0 (**Figure 9.7B**) and 105.7 ± 3.3% (**Figure 9.8B**), respectively, in comparison to the untreated control ($P < 0.001$ for both; Kruskal-Wallis).

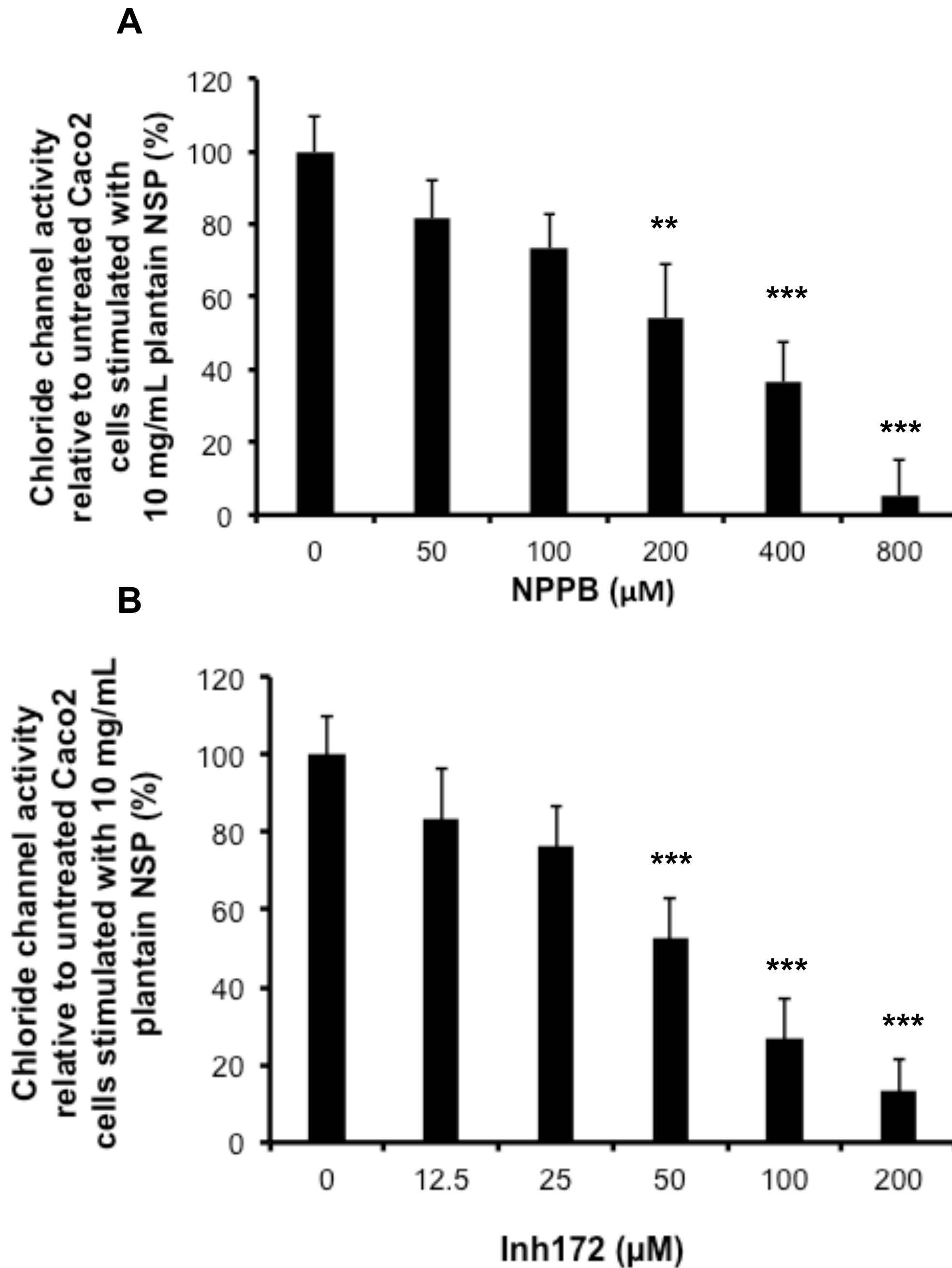

Figure 9.6 Soluble plantain fibre-mediated cellular chloride channel activity is significantly reduced in the presence of chloride channel antagonists A) NPPB and B) CFTR. Chloride channel activity was measured by monitoring cellular iodide efflux. Caco2 cells were loaded with iodine loading buffer containing **A)** NPPB (0 – 800 µM) or **B)** Inh172 (0 – 200 µM), and then treated with 10 mg/mL plantain NSP for 30 min. Following cell lysis, cellular iodide concentration was measured using the Modified Sandell-Kolthoff (SK) reaction. Chloride channel activity is expressed relative to the untreated vehicle control (set as 100%) (N=3, n ≥ 3; *** $P < 0.001$, ** $P < 0.01$; Kruskal-Wallis).

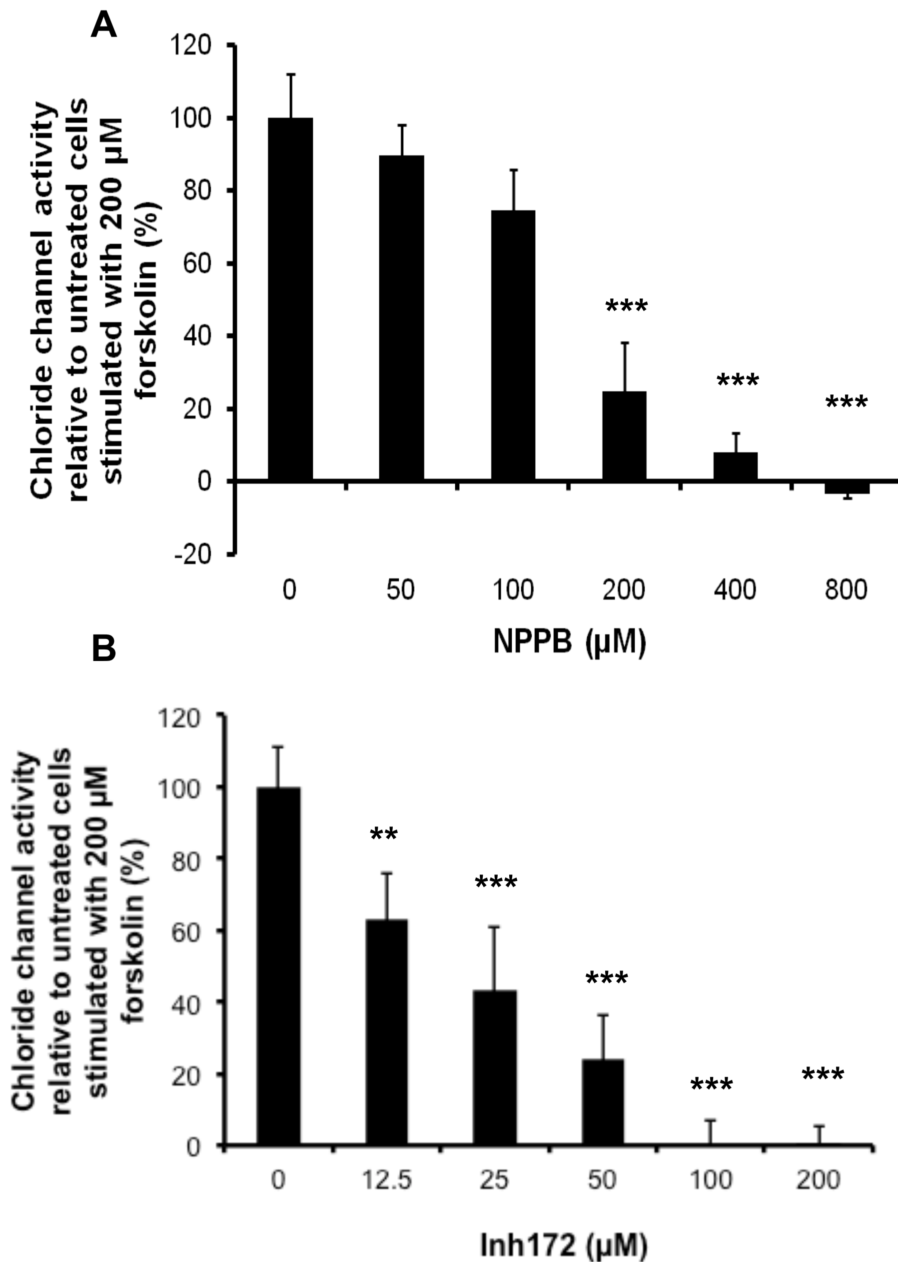

Figure 9.7 Forskolin-mediated cellular chloride channel activity is significantly reduced in the presence of chloride channel antagonists NPPB and Inh172. Chloride channel activity was measured by monitoring cellular iodide efflux. Caco2 cells were loaded with iodine loading buffer containing **A)** NPPB (0 – 800 µM) or **B)** Inh172 (0 – 200 µM), and then treated with 200 µM RP107 for 30 min. Following cell lysis, cellular iodide concentration was measured using the Modified Sandell-Kolthoff (SK) reaction. Chloride channel activity is expressed relative to the untreated vehicle control (set as 100%) (N=3, n ≥ 3; *** $P < 0.001$, ** $P < 0.01$; Kruskal-Wallis)

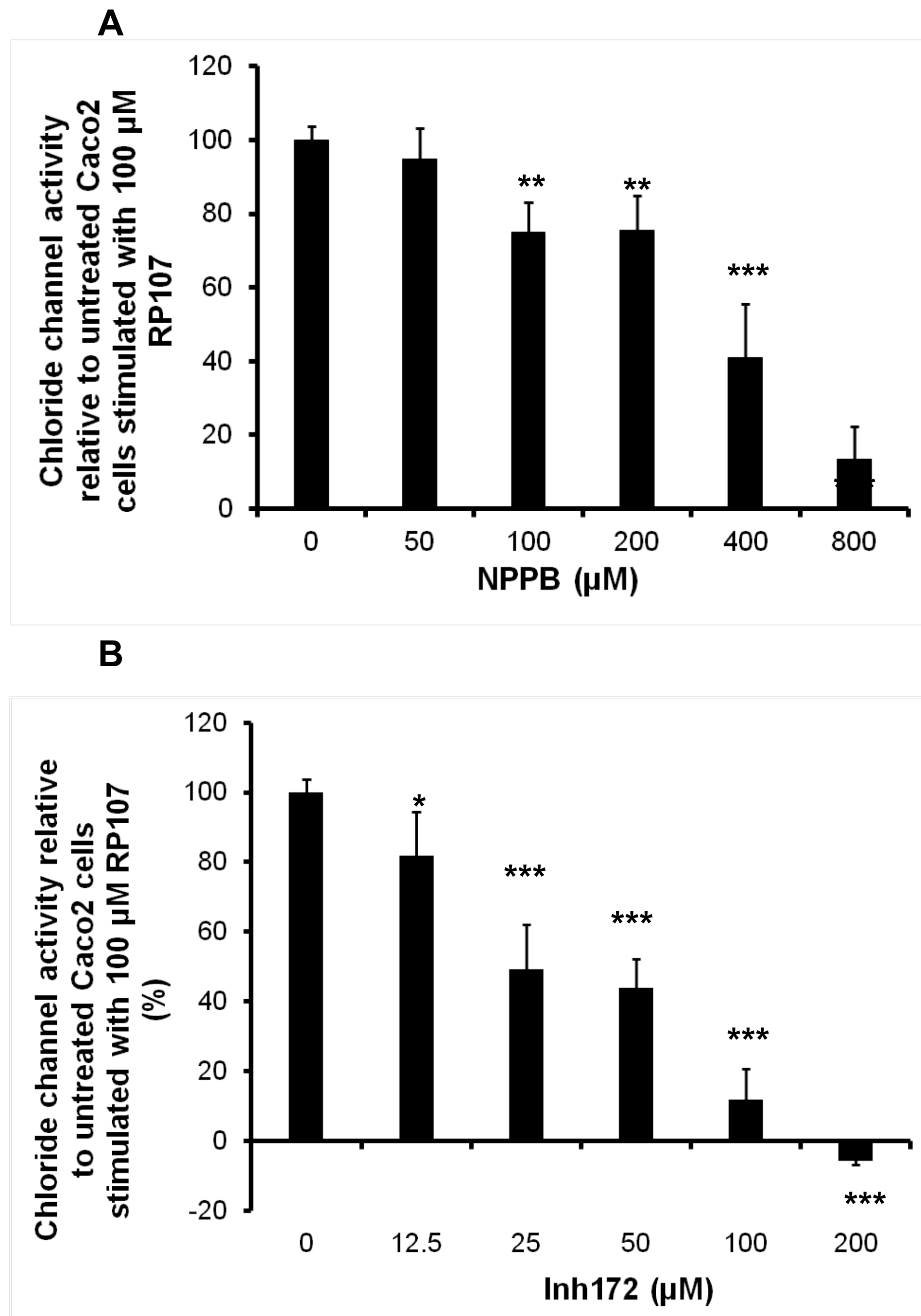

Figure 9.8 RP107-mediated cellular chloride channel activity is significantly reduced in the presence of chloride channel antagonists NPPB and CFTR. Chloride channel activity was measured by monitoring cellular iodide efflux. Caco2 cells were loaded with iodine loading buffer containing **A)** NPPB (0 – 800 µM) or **B)** Inh172 (0 – 200 µM), and then treated with 200 µM RP107 for 30 min. Following cell lysis, cellular iodide concentration was measured using the Modified Sandell-Kolthoff (SK) reaction. Chloride channel activity is expressed relative to the untreated vehicle control (set as 100%) (N=3, n ≥ 3; *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$; Kruskal-Wallis).

9.4.3 The inhibitory activity of plantain NSP is not mediated by increased cellular chloride channel activity

The chloride channel activity assays confirmed that soluble plantain NSP could induce increased chloride channel activation, which was significantly knocked down in the presence of chloride channel antagonists NPPB and CFTR-inh-172. If the inhibitory activity of plantain NSP was mediated by increased chloride secretion, then plantain NSP would also lose its ability to inhibit bacterial adhesion in this instance. However, bacterial adhesion assays indicated that even in the presence of 800 µM NPPB or 200 µM CFTR-inh-172, pre-treatment of Caco2 cells with 10 mg/mL plantain NSP significantly reduced both *C. difficile* (74.9 ± 3.6% and 76.8 ± 3.4% reduction, respectively; **Figure 9.9A**) and ETEC adhesion (91.8 ± 2.7 and 95.0 ± 2.1, respectively; **Figure 9.9B**). Indeed, similar levels of bacterial adhesion were observed when Caco2 cells were pre-treated with plantain NSP alone (in comparison to the untreated control, *C. difficile* and ETEC adhesion was reduced by 80.9 ± 2.5% and 84.7 ± 3.7%, respectively; **Figure 9.9A and B**).

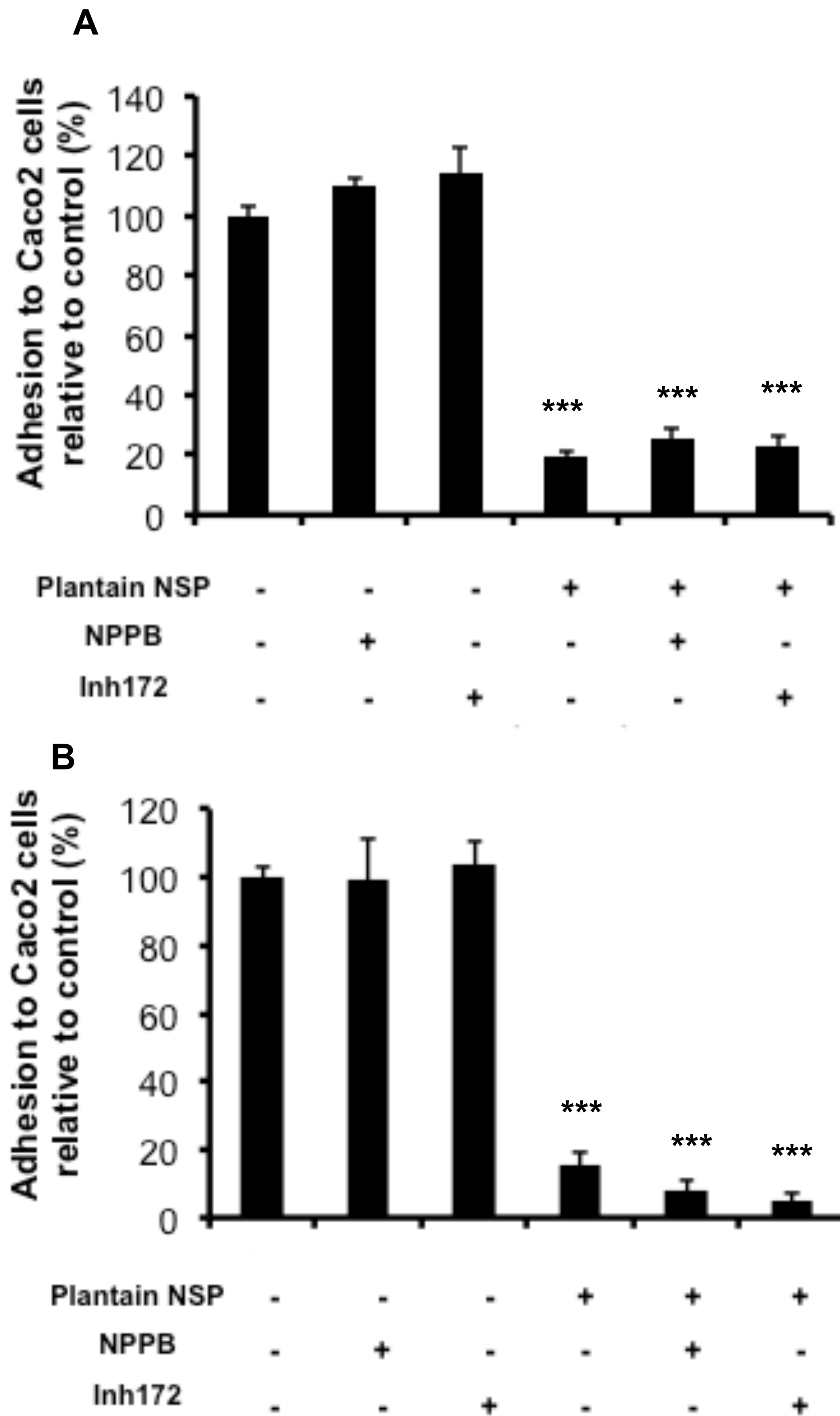

Figure 9.9 The effect of plantain NSP on *C. difficile* and ETEC epithelial adhesion in the presence of chloride channel antagonists NPPB and CFTR-inh-172. A) *C. difficile* and **B)** ETEC adhesion was assessed following pre-treatment of Caco2 cells with or without 800 µM NPPB or 200 µM Inh172 (1 h), followed by treatment with or without 10 mg/mL plantain NSP (30 min). Bacterial adhesion is expressed relative to the CFU/mL found in the untreated control (set as 100%) (N=3, n=3; *** P < 0.001, Kruskal-Wallis).

The chloride channel activity assays also indicated, that as expected, chloride channel agonists forskolin and RP107 mediated an increase in chloride channel activity. Similarly, if the inhibition of bacterial adhesion was mediated by increased chloride secretion, then treatment of Caco2 cells with chloride channel agonists would also inhibit bacterial adhesion. When Caco2 cells were treated with 100 μM RP107, *C. difficile* adhesion significantly decreased by 23.7 ± 9.0% in comparison to the untreated control ($P < 0.01$; Kruskal-Wallis; **Figure 9.10A**). This was reversed in the presence of chloride channel antagonists NPPB and CFTR-inh-172, causing bacterial adhesion to increase by 7.9 ± 8.1% and 4.5 ± 4.1%, respectively, in comparison to the untreated control (**Figure 9.10A**).

However, whilst 100 μM RP107 also significantly reduced ETEC epithelial adhesion by 19.5 ± 7.8% in comparison to the untreated control ($P < 0.05$, Kruskal-Wallis), ETEC inhibition was similar or only slightly reversed in the presence of NPPB and CFTR-inh-172 (**Figure 9.10B**). Similarly, a small level of bacterial inhibition was also seen in cells treated with 200 μM forskolin, which reduced *C. difficile* and ETEC epithelial adhesion by 8.4 ± 10.7% (**Figure 9.11A**) and 23.1 ± 5.7 ($P < 0.01$; **Figure 9.11B**), respectively, however, bacterial inhibition was again similar or only partly reversed in the presence of chloride channel antagonists (**Figure 9.11A and B**). Collectively, these results suggest that increased cellular chloride secretion plays little or no role in the inhibition of bacterial adhesion.

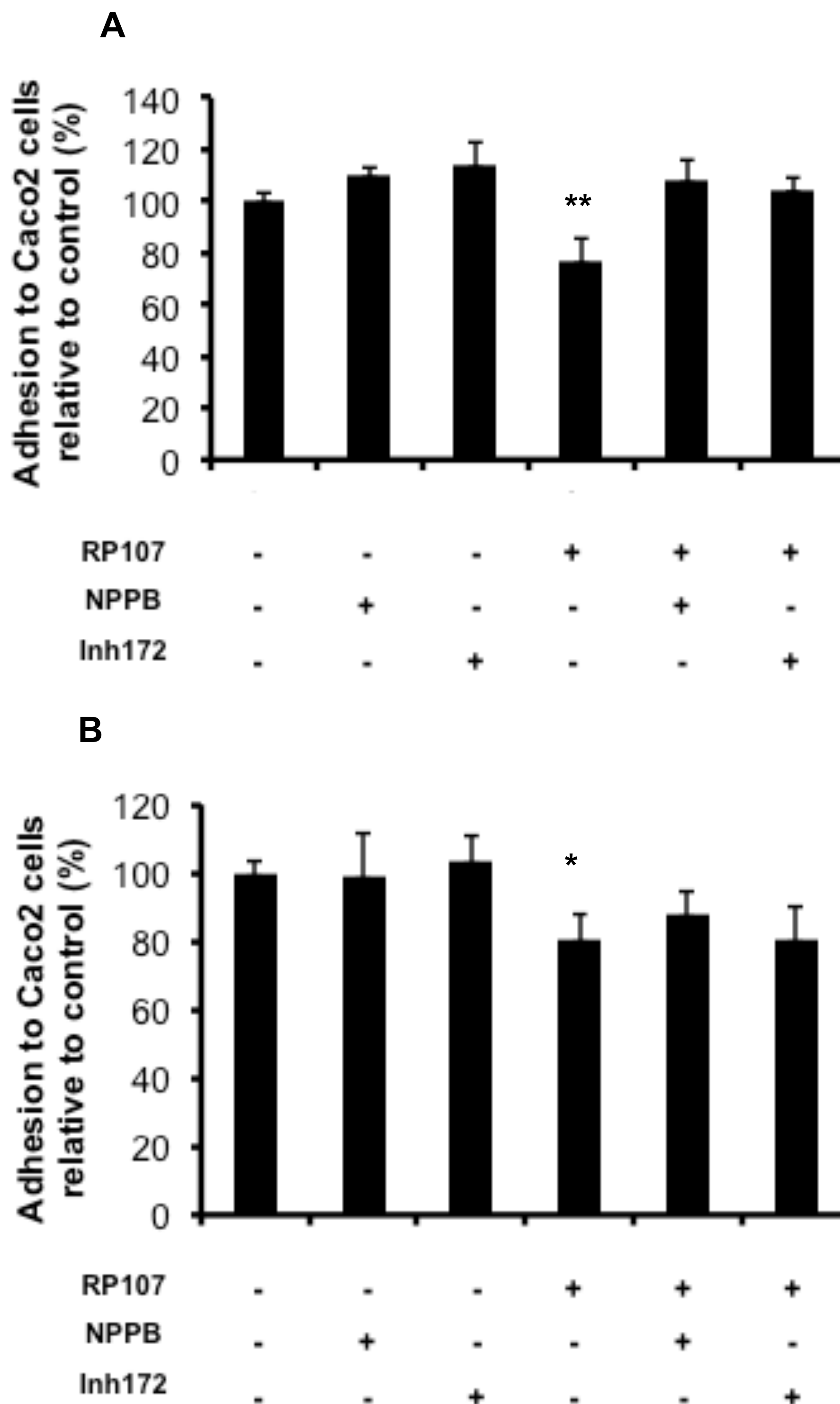

Figure 9.10. The effect of RP107 on *C. difficile* and ETEC epithelial adhesion, both alone and in the presence of chloride channel antagonists NPPB and CFTR-inh-172. A) *C. difficile* and **B)** ETEC adhesion was assessed following pre-treatment of Caco2 cells with or without 800 µM NPPB or 200 µM Inh172 (1 h), followed by treatment with or without 100 µM RP107 (30 min). Bacterial adhesion is expressed relative to the CFU/mL found in the untreated control (set as 100%) (N=2, n=3; * P < 0.05, ** P < 0.01; Kruskal-Wallis).

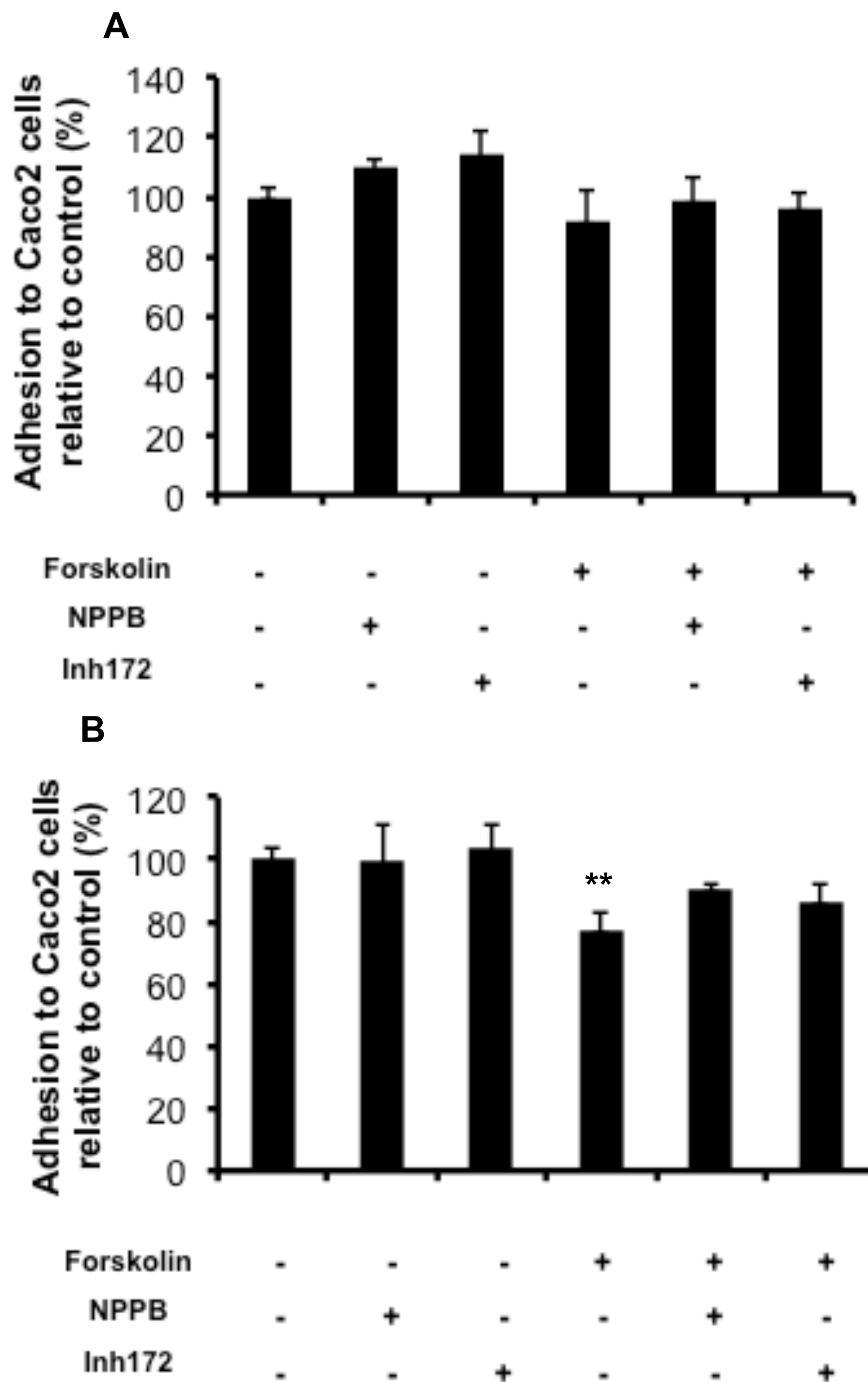

Figure 9.11 The effect of forskolin on *C. difficile* and ETEC epithelial adhesion in the presence of chloride channel antagonists NPPB and CFTR-inh-172. A) *C. difficile* and **B)** ETEC adhesion was assessed following pre-treatment of Caco2 cells with or without 800 µM NPPB or 200 µM Inh172 (1 h), followed by treatment with or without 200 µM forskolin (30 min). Bacterial adhesion is expressed relative to the CFU/mL found in the untreated control (set as 100%) (N=2, n=3; ** $P < 0.01$, Kruskal-Wallis).

9.4.4 Plantain NSP induces the nuclear translocation of β-catenin

As revealed by immunocytofluorescent staining, pre-treatment of intestinal epithelial cells with 10 mg/mL plantain NSP for 30 min markedly induced the activation and subsequent nuclear translocation of β-catenin, which was a similar level to that induced by 1 μM PGE_2 (used as a positive control) (**Figure 9.12**).

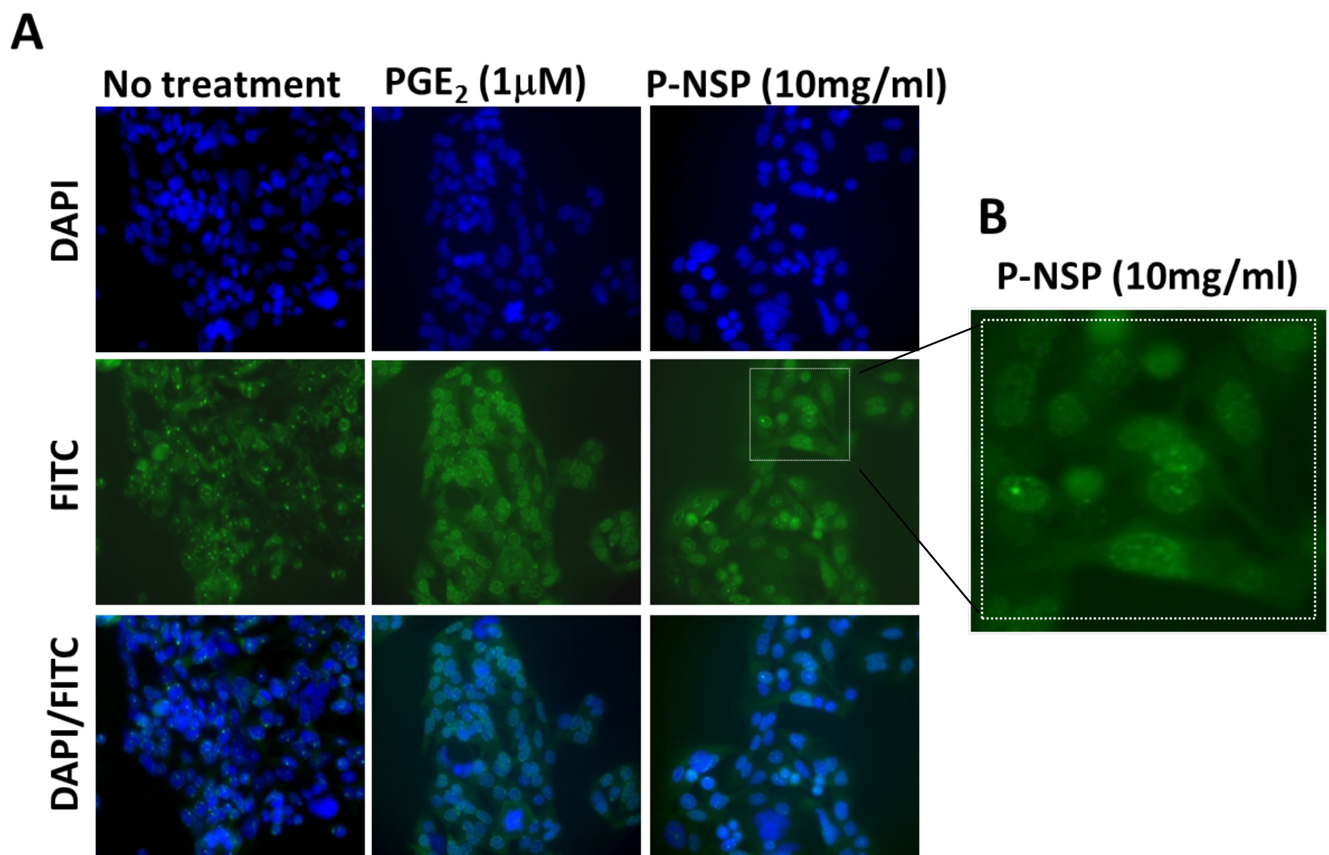

Figure 9.12 Plantain NSP induces the nuclear translocation of β-catenin. Colonic SW480 cells were treated with 10 mg/mL plantain NSP or 1 μM PGE2 (as a positive control), then fixed and stained with primary mouse monoclonal IgG anti-β-catenin antibody (1:1000; 1 h) followed by secondary FITC-conjugated rabbit polyclonal anti-mouse antibody (1:2000; 2 h). **A)** Cell nuclei were then counterstained with DAPI and cells visualised by fluorescent microscopy. **B)** The nuclear translocation of β-catenin is also apparent in the expanded image of FITC stained SW480 cells treated with 10 mg/mL plantain NSP.

Previous unexplained, unpublished observations from our Department, undertaken by Dr Paul Collins (Department of Gastroenterology, University of Liverpool), had also shown that plantain NSP could affect the nuclear translocation of β-catenin in colonocytes. Here, DLD-1 colon cancer cells treated with 5 mg/mL plantain NSP (containing approximately 0.85 mg/mL pectin) for 30 min showed increased nuclear translocation of β-catenin above that induced by 1μM PGE_2, compared to untreated controls ($P < 0.01$; $P < 0.001$ respectively; **Figure 9.13**).

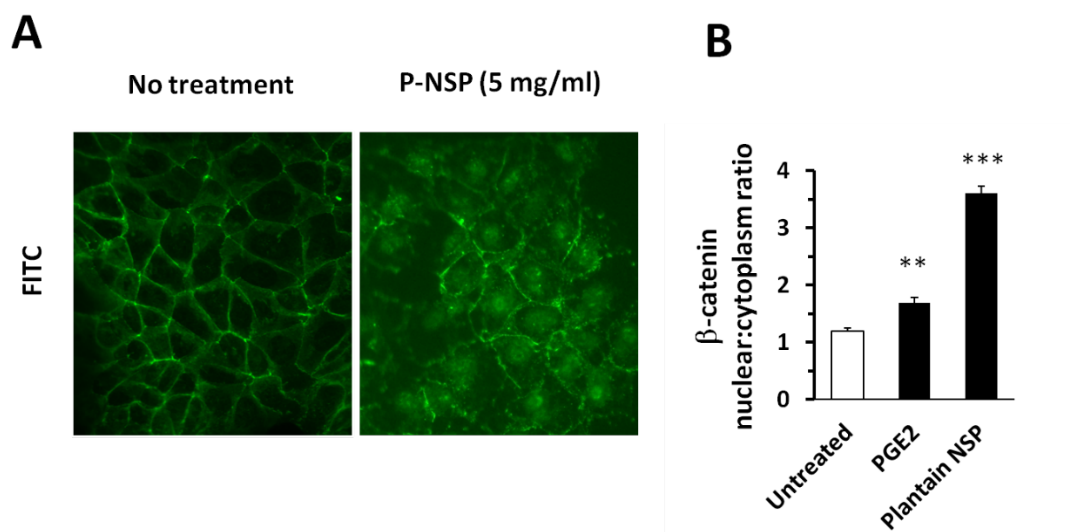

Figure 9.13 Plantain NSP induces the nuclear translocation of β-catenin in DLD-1 colonocytes. DLD-1 cells were treated with 5 mg/mL plantain NSP (containing approximately 0.85 mg/mL pectin) or 1 μM PGE2 (as a positive control), then fixed and stained with primary mouse monoclonal IgG anti-β-catenin antibody (1:1000; 1 h) followed by secondary FITC-conjugated rabbit polyclonal anti-mouse antibody (1:2000; 2 h). **A)** Cells were visualised by fluorescent microscopy and **B)** nuclear:cytoplasmic localisation quantified using Image J software (** $P < 0.01$; *** $P < 0.001$). This work was performed by Dr Paul Collins, Department of Gastroenterology, University of Liverpool *(unpublished data)*.

9.4.5 The inhibitory effect of plantain NSP is not mediated by cellular galectin-3 expression

To assess if the inhibitory activity of soluble plantain fibre was mediated by the cellular expression of galectin-3, SW620^{Gal3-} cells and SW620^{Gal3+} cells were pre-treated for 30 min with 10 mg/mL plantain NSP and subsequently infected with either *C. difficile* at an MOI of 100 (**Figure 9.14**) or 50 µL pooled UC *E. coli* supernatant (**Figure 9.15**).

Pre-treatment of SW620^{Gal3-} cells with 10 mg/mL plantain NSP resulted in a similar level of bacterial adhesion to that seen in SW620^{Gal3+} cells, reducing *C. difficile* adhesion by 64.0 ± 0.9% and 69.2 ± 3.0%, respectively, compared to the untreated control (**Figure 9.14**). Whilst 10 mg/mL plantain NSP also reduced the IL-8 response to bacterial flagellin in SW620^{Gal3-} cells (reduction of 36.5 ± 5.8% in comparison to the untreated control; **Figure 9.15**), the level of inhibition higher was higher when SW620^{Gal3+} cells were pre-incubated with 10 mg/mL plantain NSP prior to flagellin treatment (reduction of 62.7 ± 1.6% in comparison to untreated control; **Figure 9.15**).

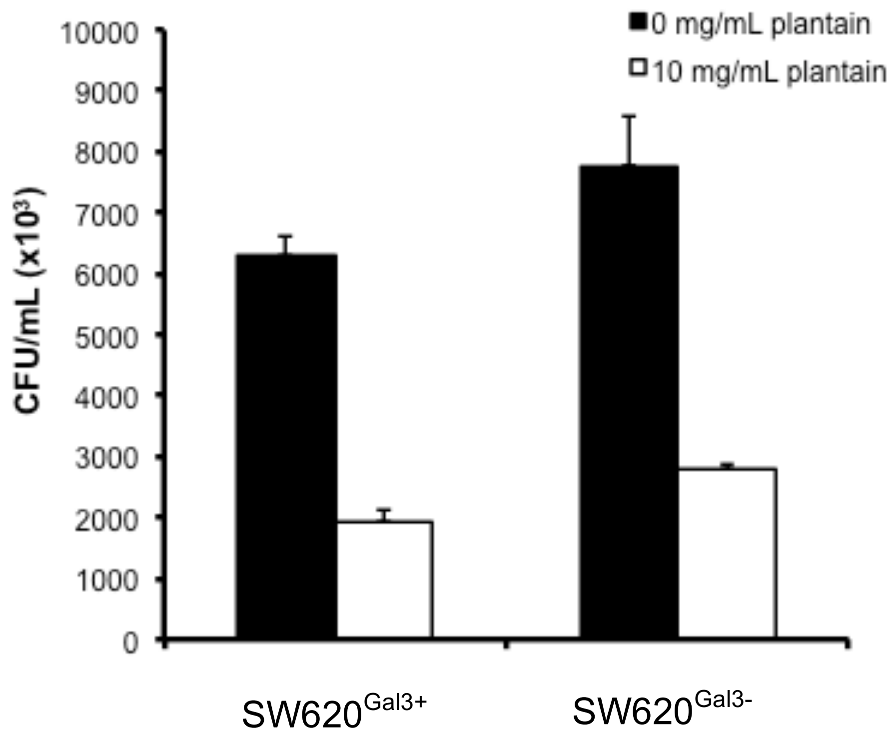

Figure 9.14. The ability of plantain NSP to inhibit *C. difficile* epithelial adhesion is not affected by cell-surface galectin-3 expression. Pre-treatment of SW620Gal^{3+} (galectin-3 expressing) and SW620Gal^{3-} (galectin-3 knockdown) cells with 10 mg/mL prior to infection with *C. difficile* (MOI 100; 2h) significantly reduced bacterial adhesion.

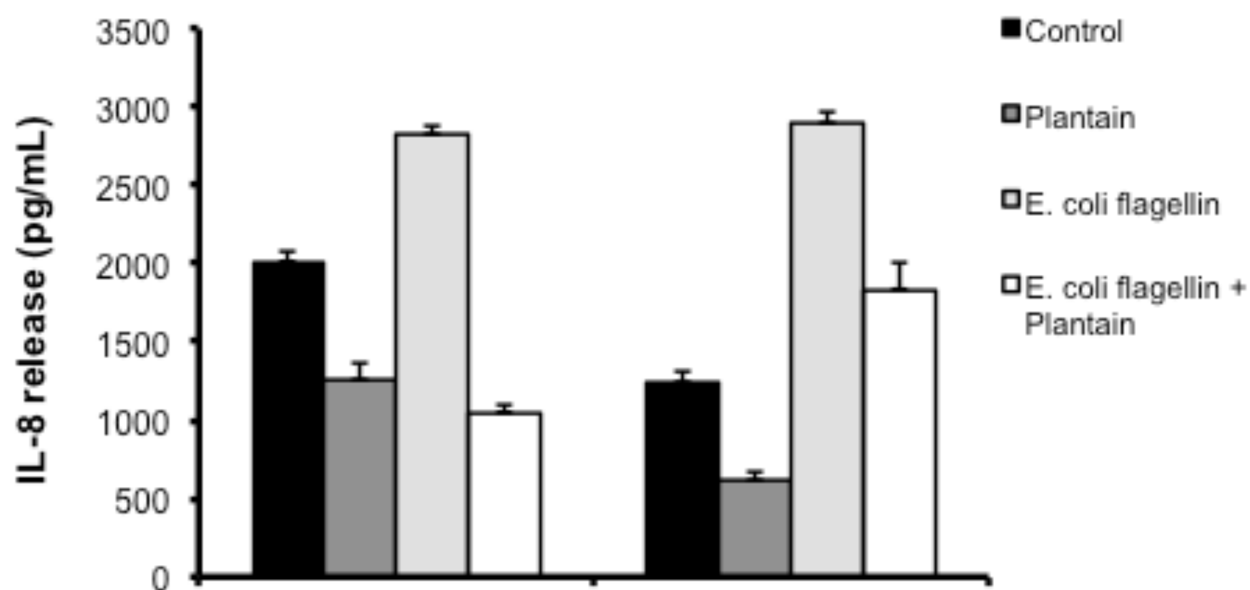

Figure 9.15. The ability of plantain NSP to significantly reduce IL-8 response to *E. coli* filtrate is not affected by the expression of cell-surface galectin-3. Pre-treatment of SW620^{Gal3+} (galectin-3 expressing) and SW620^{Gal3-} (galectin-3 knockdown) cells with 10 mg/mL prior to treatment with 0.2µm filtered pooled UC *E. coli* culture supernatant (50 µL; 4 h) significantly reduced pro-inflammatory IL-8 response. Harvested media was measured for the presence of IL-8 using an IL-8 Human Elipair kit.

9.5 SUMMARY OF RESULTS

1. Soluble plantain fibre inhibits bacterial adhesion via an interaction with the intestinal epithelium, rather than through direct interaction with bacteria

2. Soluble plantain fibre significantly increases cellular chloride channel activity, but this has little or no effect on its inhibitory activity against bacterial adhesion

3. Soluble plantain fibre induces the nuclear translocation of β-catenin in intestinal epithelial cells suggesting that, like MUC2 glycans, its inhibitory effect might be mediated by interacting with cell-surface galectin-3

4. The ability of soluble plantain fibre to inhibit bacterial adhesion and the IL-8 response to bacterial flagellin is probably not mediated by cell-surface galectin-3 expression

9.6 DISCUSSION

Here, a further insight into the mechanism of the inhibitory action of soluble plantain fibre is provided. Interestingly, soluble plantain fibre was shown to exert its inhibitory effect against diarrhoeal pathogens *C. difficile*, ETEC and *S*. Typhimurium via an interaction with intestinal epithelial cells, rather than through interaction with bacterial adhesins. Whilst we have previously shown that plantain NSP interacts with and provides a fuel source for diarrhoeal pathogens (*Chapter 5 of this thesis*), the results presented here suggest that this interaction is likely unrelated to the underlying mechanism of its inhibitory effect.

Previous studies by our research group demonstrated that pre-treatment of isolated human FAE, mounted in Ussing chambers, with 5 mg/mL soluble plantain fibre resulted in significantly increased transmucosal I_{sc} (Parsons *et al.* 2014; Roberts *et al.* 2013). We therefore postulated that soluble plantain NSP might inhibit bacterial epithelial adhesion via increased chloride secretion, which is associated with increased ion efflux and water transport (Parsons *et al.* 2014). Indeed, epithelial secretion of fluid is already recognised as a form of innate immunity, thought to serve as a non-specific method of clearing bacteria and their toxins from the intestinal mucosa (Strober and McGhee 2005).

Functional chloride channel activity assays illustrated that pre-incubation of Caco2 cells with 2.5 – 20 mg/mL plantain NSP significantly increased cellular chloride channel activation, thus confirming the findings from Ussing chamber experiments performed previously (Parsons *et al.* 2014). Importantly, it was also shown that other soluble plant fibres, previously shown not to exhibit an inhibitory effect on bacterial adhesion (in Chapter 5 of this thesis), either completely lacked the ability to induce chloride channel activation, or induced activity at a significantly lower level than that seen with plantain NSP.

Whilst we showed that cellular chloride channel activation induced by 10 mg/mL plantain NSP was significantly knocked down in the presence of chloride channel antagonists NPPB and CFTR-inh-172 (Linsdell 2014), this had no significant effect on *C. difficile* or ETEC epithelial adhesion. Conversely, chloride channel agonists, forskolin and RP107 (Noel *et al.* 2006), significantly reduced bacterial epithelial adhesion in most cases, but bacterial inhibition was similar or only slightly reversed in the presence of NPPB and CFTR-inh-172. Furthermore, despite similar levels of evoked cellular chloride channel activity (forskolin and RP107; 5.4 ± 0.5 and 8.2 ± 1.0-fold increase vs. plantain; 5.3 ± 0.5 fold increase), the level of inhibition caused by forskolin and RP107 was much less than that observed with 10 mg/mL plantain NSP. Collectively, these results suggest that whilst plantain NSP mediates increased epithelial chloride secretion, this has little or no effect on the inhibition of bacterial adhesion. It is therefore likely that soluble plantain fibre inhibits bacterial adhesion via another mechanism. It is worth noting that the chloride channel experiments are limited as they have only been performed *in vitro*. Indeed, it is possible that chloride secretion in such static conditions might not have the same physiological effect as would be observed *in vivo,* thus resulting in a limited effect on bacterial adhesion. Therefore, whilst these results suggest that soluble plantain fibre inhibits bacterial adhesion via another mechanism, the role of chloride secretion should not be cast aside without further investigation in an *in vivo* setting where chloride channel secretion is associated with more significant water transport.

Next, we hypothesised that the inhibitory activity of soluble plantain NSP might lie in its ability to mimic MUC2 glycans, which have been shown recently to interact with cell-surface galectin-3 with consequent anti-inflammatory and mucosally protective effects (Shan *et al.* 2013). These effects, as described by Shan and colleagues, focussed predominantly on

dendritic cells, but included preliminary data relating to intestinal epithelial cells (Shan *et al.* 2013). Our own preliminary studies have shown that plantain NSP also interacts with galectin-3 at a similar concentration to that which has an anti-inflammatory, anti-bacterial effect. As discussed in Chapter 8 of this thesis, the inhibitory activity of plantain NSP was shown to be largely due to its acidic or pectin fraction. Indeed, various pectins have also previously been shown to interact with galectin-3, including citrus and ginseng pectins (Gao *et al.* 2013; Gunning *et al.* 2009; Sathisha *et al.* 2007).

Here, we demonstrated that treatment of colonic epithelial cells with soluble plantain fibre induced a marked nuclear translocation of β-catenin, which has also been implicated in the MUC2-galectin3-Dectin 1 signalling pathway characterised previously (Shan *et al.* 2013). Conversely, our studies indicated that cell-surface galectin-3 expression did not have an effect on the inhibitory activity of soluble plantain NSP. Indeed, soluble plantain fibre retained its ability to significantly inhibit *C. difficile* epithelial adhesion, as well as reduce the IL-8 response to bacterial flagellin, in SW620^{Gal3-} knockdown cells. However, particularly with respect to the IL-8 response to flagellin, it was apparent that soluble plantain fibre exhibited a lower level of inhibitory activity in SW620^{Gal3-} cells in comparison to SW620^{Gal3+} cells (% reduction in IL-8 response; 36.4 ± 5.8% vs. 62.7 ± 1.6%).

It is important to note that galectin-3 expression was only knocked down by 82% in SW620^{Gal3-} cells (Duckworth *et al.* 2015). Therefore, it is likely that an interaction between plantain NSP and cell-surface galectin-3 may still have occurred. If the mucosally protective and anti-inflammatory effects of plantain NSP were mediated by this interaction, this would explain why plantain NSP retained some inhibitory activity in SW620^{Gal3-} cells. Alternatively, it is possible that soluble plantain fibre does not mimic MUC2 glycans and its inhibitory activity

is therefore not mediated by interaction with galectin-3. If this were the case, soluble plantain fibre would activate β-catenin by an independent mechanism.

In conclusion, the role of cell-surface galectin-3 expression on the inhibitory activity of plantain NSP needs further investigation, and therefore, it is currently unclear whether soluble plantain NSP mimics MUC2 glycans via galectin-3 interaction. $SW620^{Gal3-}$ experiments are limited as they were only performed once (with n=6 replicates), and therefore, they need to be repeated before any sound inferences are to be made. In addition, it might be beneficial to also assess if treatment of intestinal epithelial cells with an anti-galectin-3 antibody or siRNA targeting the *GAL3* gene has an effect on the inhibitory effect of plantain NSP, or to repeat these experiments using a cell line that is completely devoid of cell-surface galectin-3 expression.

Chapter 10

Summary of Key Findings

10.1 SUMMARY OF KEY FINDINGS

1. A range of soluble dietary fibres are shown to significantly inhibit the epithelial adhesion of toxin-mediated diarrhoeal pathogens *C. difficile* and ETEC, of which plantain NSP exhibits the highest efficacy

2. Soluble plantain fibre inhibits the epithelial adhesion of a range of clinically relevant *C. difficile* isolates, irrespective of their toxin or ribotype status

3. Soluble plantain fibre inhibits the epithelial interactions of *C. difficile* spores

4. Soluble plantain fibre can also inhibit the epithelial interactions of other bacterial components involved in the pathogenesis of inflammatory intestinal disease, including *C. difficile* TcdA and TcdB, as well as mucosa-associated *E. coli* flagellin and LPS

5. Soluble plantain NSP has no effect on TcdA and TcdB-mediated Rac1 monoglucosylation, but it reduces the pro-inflammatory, cytotoxic and apoptotic response induced by *C. difficile* and its toxins, suggesting a possible role in the therapy of CDI

6. Soluble plantain fibre down-regulates the cellular pro-inflammatory, cytotoxicity and apoptotic response mediated by mucosally-associated UC *E. coli*, as well as other

bacterial components such as mucosal flagellin and LPS, suggesting a possible role for plantain NSP in the treatment of UC

7. The inhibitory effects of soluble plantain fibre against bacteria-epithelial interactions are shown to be largely a consequence of its acidic (pectic) component

8. Soluble plantain NSP inhibits the epithelial adhesion of *C. difficile*, ETEC and *S*. Typhimurium via an interaction with intestinal epithelial cells, rather than through interaction with bacterial cell-surface components, such as adhesins

9. Whilst plantain NSP induces increased cellular chloride secretion, this has little or no effect on its inhibitory activity against bacteria-epithelial interaction, suggesting that the latter is mediated by an alternative mechanism

10. Pre-treatment of intestinal epithelial cells with plantain NSP induces the activation and nuclear translocation of β-catenin, which has been shown by others to mediate the anti-inflammatory effects of secreted mucus (MUC2) glycans

Chapter 11

Discussion

11.1 KEY FINDINGS OF THIS WORK

11.1.1 Soluble plantain fibre inhibits the *in vitro* epithelial interactions of diarrhoeal pathogens *C. difficile* and ETEC

Previous findings from our group have demonstrated that soluble plantain fibre has the ability to inhibit the epithelial interactions of a range of gut pathogens, including the *in vitro* epithelial adhesion, invasion and M-cell translocation of AIEC, *S*. Typhimurium and *S. sonnei*. In *ex-vivo* studies performed previously, we have also shown that plantain NSP reduces AIEC and *S*. Typhimurium translocation across isolated human ileal FAE and VE mounted in Ussing chambers (Roberts *et al.* 2010; Roberts *et al.* 2013). Furthermore, we have previously illustrated that a diet supplemented with plantain NSP significantly reduces Salmonellosis in an *in vivo* chicken model (Parsons *et al.* 2014).

The data presented here in this thesis provides further evidence to support our hypothesis that soluble plantain fibre might have a beneficial impact on intestinal health via its ability to inhibit potentially harmful interactions between bacteria and the gut epithelium. In Chapter 5, we demonstrate that whilst a range of soluble dietary fibres exhibit inhibitory activity against the epithelial adhesion of toxin-mediated diarrhoeal pathogens *C. difficile* and ETEC, plantain NSP exhibits the highest efficacy. Furthermore, we show that soluble plantain fibre significantly inhibits the epithelial adhesion of a range of clinically relevant *C. difficile* isolates, irrespective of their toxin or ribotype status. These results suggest that soluble plantain NSP could be a promising candidate for development as a potential prophylaxis or treatment for CDI and ETEC infection. Of significant note, we also show that soluble plantain NSP can inhibit the epithelial interactions of *C. difficile* spores. As this dormant form of *C. difficile* is also associated with high CDI re-infection rates (Barbut *et al.* 2000; Oka *et al.* 2012),

we suggest that dietary supplementation with soluble plantain NSP might also help maintain remission from CDI.

11.1.2 Soluble plantain fibre down-regulates the epithelial cell damage and inflammatory response to bacterial components

The results described in Chapter 5 provide convincing evidence to suggest that soluble plantain fibre can disrupt the epithelial interactions of *C. difficile* and ETEC bacteria. In addition, our findings in Chapter 6 and Chapter 7 demonstrate that soluble plantain fibre can also inhibit the epithelial interactions of other bacterial components involved in the pathogenesis of inflammatory intestinal disease, including *C. difficile* toxins TcdA and TcdB, as well as *E. coli* mucosal associated flagellin and LPS.

In Chapter 6, we show that whilst soluble plantain NSP has no effect on TcdA and TcdB-mediated Rac1 monoglucosylation, it does significantly reduce the pro-inflammatory, cytotoxicity and apoptotic response induced by *C. difficile* and its toxins. Rac1 monoglucosylation is postulated to be the main pathway through which *C. difficile* TcdA and TcdB exert their cellular effects (Popoff and Geny 2011), however our findings conclude that soluble plantain fibre is unlikely to inhibit this specific intracellular mechanism of action of *C. difficile* toxins. As such, further work is required to determine whether plantain NSP can abrogate *C. difficile* toxin-mediated activation of alternative signalling pathways occurring independently of Rho protein monoglucosylation (He *et al.* 2002; Matarrese *et al.* 2007), such as the IκB-NFκB pathway, which has been implicated in TcdA-mediated IL-8 release from enterocytes (He *et al.* 2002). It is important to note, however, that whilst plantain NSP appears

to have no effect on toxin-mediated Rac1 monoglucosylation, its marked ability to reduce the epithelial cell damage and inflammatory response to *C. difficile* and its toxins is still likely to be of great clinical benefit. Previously, a number of animal models have demonstrated that anti-inflammatory agents have a protective effect against CDI (Anton *et al.* 2004; Chen *et al.* 2006; Cottrell *et al.* 2007; Kokkotou *et al.* 2009; Pothoulakis *et al.* 1996; Warny *et al.* 2005). As such, these findings provide further evidence to support our hypothesis that dietary supplementation with plantain NSP might represent an effective therapy to treat CDI.

In Chapter 7, we also demonstrate that soluble plantain fibre has the ability to down-regulate the cellular pro-inflammatory, cytotoxicity and apoptotic response mediated by mucosally-associated UC *E. coli*, as well as other bacterial components, such as mucosal flagellin and LPS. Taken together, these findings also suggest a possible role of soluble plantain fibre in the treatment of UC.

11.1.3 The inhibitory activity of plantain NSP is mediated by its acidic, pectic polysaccharide fraction

The results presented in Chapter 8 of this thesis demonstrate that neutral and acidic fractions of plantain NSP, isolated by strong-anion exchange chromatography, exhibit differential inhibitory activity against bacteria-epithelial interactions. We show that pre-treatment of intestinal epithelial cells with the acidic fraction of plantain NSP significantly inhibits the epithelial adhesion of *C. difficile*, ETEC and *S*. Typhimurium, whilst the neutral fraction of plantain NSP exhibits a decreased level of inhibitory activity. In addition, composition analysis performed previously by our group has shown that the acidic

polysaccharide fraction of plantain NSP is mainly composed of pectic material, which is completely absent in the neutral plantain NSP fraction. Taken together, these results suggest that the inhibitory activity of soluble plantain NSP against bacteria-epithelial interactions is largely due to its acidic, pectic polysaccharide fraction. These findings are in agreement with preliminary work performed previously, which also demonstrated that the acidic fraction of plantain NSP exhibited significant inhibitory activity against the epithelial adhesion of S. Typhimurium (Parsons *et al.* 2014). Indeed, other studies have shown that pectin also exhibits anti-adhesive activity against other gut pathogens including *C. jejuni* and *H. pylori* (Ganan *et al.* 2010; Lee *et al.* 2006b). In addition, it has previously been demonstrated that pectin also has the ability to inhibit haemagglutination by bacteria, such as that caused by *S. areus* and *P. acnes*, but not that affected by *E. coli* and *L. acidophilus* (Lee *et al.* 2006b).

Interestingly, we also present data to suggest that maltodextrin, derived from the starch component of the plantain source material, which is added back to soluble plantain fibre during the bulk manufacturing process to aid spray drying and aid solubilisation, possesses some inhibitory activity against the epithelial adhesion of *C. difficile*, ETEC and *S*. Typhimurium *in vitro*. However, whilst maltodextrin is present in whole and neutral plantain NSP fractions, inhibitory activity is instead correlated with the presence of pectic material. Additionally, pre-incubation of intestinal epithelial cells with maltodextrin has no effect on the epithelial cell damage and inflammation induced by bacterial components, whereas these cellular responses are significantly suppressed by plantain NSP. We therefore suggest that maltodextrin only contributes slightly to the inhibitory activity of plantain NSP *in vitro*. Furthermore, it is likely that *in vivo*, maltodextrin would undergo almost complete

fermentation before reaching the colon (Quezada-Calvillo *et al.* 2007a; Quezada-Calvillo *et al.* 2007b), and as such, would have little or no protective effect.

11.1.4 Soluble plantain NSP inhibits bacterial adhesion via an interaction with the intestinal epithelium, however, the molecular mechanism underlying its inhibitory activity remains unclear

As a result of the work presented in Chapter 9, we provide further insight into the molecular mechanism underlying the inhibitory activity of plantain NSP. We demonstrate that soluble plantain NSP inhibits the adhesion of *C. difficile*, ETEC and S. Typhimurium via an interaction with intestinal epithelial cells, rather than through interaction with bacterial cell-surface adhesins.

Our group had shown previously that pre-treatment of human ileal FAE with plantain NSP was associated with a marked increase in transmucosal I_{sc} (Parsons *et al.* 2014). These preliminary results suggested that the inhibitory effect of plantain NSP might be mediated by increased epithelial chloride secretion, which is a recognised part of our innate immune system countering the invasion of luminal bacteria (Colgan 2013). As an example, secretagogue elicited epithelial chloride secretion and associated fluid transport was shown to be effective in reducing *Salmonella* internalisation to, and translocation across colonocytes (Keely *et al.* 2012). In Chapter 9, we confirm our previous findings and show that pre-incubation of intestinal epithelial cells with 2.5 – 20 mg/mL plantain NSP causes a significant increase in cellular chloride channel activation. However, pre-treatment of intestinal epithelial cells with chloride channel inhibitors, whilst blocking plantain NSP induced Cl^-

secretion, had little effect on plantain NSP blockade of bacterial adhesion. Similarly, the chloride channel activators used in this study did not act to block bacterial adhesion. Collectively, these findings indicate that whilst plantain NSP induces increased cellular chloride secretion, this has little or no effect on its inhibitory activity within an *in vitro* setting. We therefore suggest that the inhibitory activity of plantain NSP is mediated by an alternative mechanism.

Another potential mechanism underlying plantain NSP's inhibitory activity might include its ability to mimic the protective colonic mucus barrier, which is mainly composed of highly glycosylated MUC2, key to formation of the two distinct mucus layers that separate luminal bacteria from the epithelium (Johansson *et al.* 2011; Johansson *et al.* 2008). MUC2 *O*-glycans have recently been shown to have an anti-inflammatory and mucosally protective effects on the intestinal mucosa, which is mediated via interaction with cell-surface galectin-3, and formation of a complex with dectin-1 and the inhibitory IgG Fc (crystallisable fragment) Gamma Receptor-IIB (FcγRIIB). This interaction triggers the translocation of β-catenin into the dendritic cell nucleus, which results in consequent reduced activation of NFκB, reduced secretion of pro-inflammatory cytokines (IL-6, IL-8, IL-12p70 and TNF) and enhancement of anti-inflammatory IL-10 (Shan *et al.* 2013). Although studies of the anti-inflammatory and tolerogenic effects of MUC2 glycans described in this study focused predominantly on dendritic cells, preliminary data in relation to similar effects in intestinal epithelial cells was included, as demonstrated by increased epithelial expression of Thymic stromal lymphopoietin (Tslp), which also induces tolerogenic effects in dendritic cells (Shan *et al.* 2013). Previous studies have shown that pectic oligosaccharides, from citrus and ginseng, can interact with galectin-3 (Gao *et al.* 2013; Gunning *et al.* 2009; Sathisha *et al.* 2007), and recent

data from our own group has demonstrated that plantain NSP interacts with recombinant galectin-3 too (Shapanis A, Gastroenterology Research Unit, Department of Cellular and Molecular Physiology, University of Liverpool; unpublished data). Supporting a role for plantain NSP (and likely the pectic component) having a similar effect to that seen by MUC2 *O*-glycans, we illustrate here that pre-treatment of intestinal epithelial cells with plantain NSP induces the activation and nuclear translocation of β-catenin, as demonstrated by immunohistochemistry. Conversely, we demonstrate that in SW620^{Gal3-} cells (where galectin-3 expression has been knocked down by 82%) (Duckworth *et al.* 2015), plantain NSP retains its ability to significantly reduce *C. difficile* epithelial adhesion, as well as down-regulate the IL-8 response to bacterial flagellin in SW620^{Gal3-} cells. Taken alone, these results might suggest that soluble plantain fibre does not mimic MUC2 glycans, and thus, its inhibitory activity is not mediated by cell-surface galectin-3 expression. However, it is worth noting that these experiments are somewhat limited as they have only been performed once. Furthermore, as galectin-3 expression is not completely abolished in SW620^{Gal3-} cells, a residual interaction might still take place between plantain NSP and galectin-3, which might have an impact on bacterial adhesion. As a result of these inconclusive findings, the role of cell-surface galectin-3 expression on the inhibitory activity of plantain NSP is currently unclear. Further work is therefore necessary to characterise the molecular mechanism underlying the bioactivity of plantain NSP.

11.2 FINAL CONCLUSIONS

Soluble plantain fibre, particularly its pectin fraction, can significantly inhibit the epithelial adhesion of diarrhoeal pathogens *C. difficile* and ETEC, as well as *C. difficile* spores.

Furthermore, soluble plantain NSP also down-regulates the epithelial cell damage and inflammatory response induced by bacterial components, such as *C. difficile* TcdA and TcdB, as well as bacterial flagellin and LPS. These findings provide convincing evidence to support our hypothesis that soluble plantain fibre can inhibit harmful interactions between bacteria and the human intestinal epithelium, and thus, have a beneficial effect on intestinal health.

Importantly, inhibition of these cellular responses occurs at soluble fibre concentrations readily achievable within the human distal colon following dietary supplementation (Roberts *et al.* 2010). Modelling of soluble plantain NSP breakdown using mixed faecal microbiota from healthy volunteers has shown that 25-75% of ingested plantain NSP is likely to avoid fermentation in the human colon (Backman 2009; Roberts *et al.* 2010). Assuming passage of 1 litre of fluid daily into the caecum, we have estimated that readily achievable oral dosing of humans with 5 g soluble plantain NSP twice daily will achieve effective luminal concentrations of approximately 10 mg/mL and 7.5 mg/mL in the caecum and rectum, respectively (Roberts *et al.* 2010).

Overall, these studies provide convincing evidence to suggest that soluble plantain fibre, acting as a 'contrabiotic' (Simpson and Campbell 2015), could be developed as a potential prophylaxis or treatment against intestinal pathogens causing CDI and traveller's diarrhoea. In addition, dietary supplementation with soluble plantain NSP may also confer a therapeutic benefit in IBD.

Soluble plantain fibre could be formulated with dairy products (e.g. milk, a milkshake or yoghurt) or a fruit juice (e.g. orange juice or similar) to produce a palatable drink that would be highly suitable sufferers of diarrhoeal conditions (Simpson and Campbell 2015). Other potential formulations include powder mixes, which could be combined with further

ingredients added for nutritional or medical reasons, or for improved palatability. Soluble fibre could also be presented as food supplements or food additives, incorporated into slow release tablets, or alternatively, be administered as combination therapies alongside pre- or probiotic portions.

11.3 IMPLICATIONS FOR FUTURE WORK

We show here that the inhibitory activity of plantain NSP is mediated by its acidic, pectic fraction. Thus, it would be interesting to next screen a range of dietary pectins for their anti-inflammatory effects, as well as for their ability to inhibit the *in vitro* epithelial adhesion of gut pathogens (including *Salmonella* spp., AIEC and *C. difficile*). Total pectin could be extracted from a taxonomic spectrum of edible plants, with particular emphasis on those that have exhibited inhibitory activity in our own studies, such as plantain bananas, leek and broccoli. The pectin fractions that exhibit the highest bioactivity could then be subjected to further fractionation to identify specific inhibitory oligosaccharides. Indeed, if considerable bioactivity is shown *in vitro*, then pectin and pectic polysaccharide components could be assessed for their protective effect against inflammatory disease in both animal and human models. It is important to note that some pectin components are more rapidly degraded in the colon than others (Holloway *et al.* 1983). If biologically active pectin components are identified, it might therefore be necessary to develop an enteric-coated formulation to prevent their breakdown in the proximal colon.

Whilst the work performed in this thesis has provided a significant insight into the mechanism underlying the inhibitory activity of plantain NSP, further work is required to

establish if it is mediated by an interaction with galectin-3 via the MUC2-galectin3-Dectin 1 signalling pathway. As galectin-3 expression was only knocked down by 82% in the SW620^{Gal3-} cells used in our studies (Duckworth *et al.* 2015), it would be beneficial to repeat the experiments using a cell line that is completely devoid of cell-surface galectin-3 expression. In addition, it might be useful to treat intestinal epithelial cells with pre-designed siRNAs targeting the *GAL3* gene to assess if this has an effect on the inhibitory effect of plantain NSP. It would also be possible to use these methods to examine the effect of Dectin-1 suppression, as well as other key tolerogenic signalling partner moieties involved in the MUC2-galectin3-Dectin 1 pathway, such as FcRn (FcγRIIB in dendritic cells) and β-catenin.

We present promising findings to suggest that soluble plantain fibre can inhibit the *in vitro* epithelial interactions of *C. difficile* bacterium, toxins and spores. As such, it would next be beneficial to assess if dietary supplementation with soluble plantain fibre has a protective effect in an *in vivo* CDI model. Syrian hamsters represent a common CDI model as they exhibit many of the clinical symptoms that are observed in human CDI (Best *et al.* 2012). Similar to humans, infection in Syrian hamsters must be induced by disrupting the microbiota through the administration of antibiotics, most commonly clindamycin, followed by challenge with toxigenic *C. difficile* strains or spores. This results in haemorrhagic caecitis, which manifests as 'wet tail' and is followed by death (Best *et al.* 2012). To assess the effect of plantain NSP, hamsters would have either soluble plantain fibre or control maltodextrin supplemented in their diet for one week prior to antibiotic administration and subsequent infection with *C. difficile* spores, then throughout the study. At 12, 24 and 36 h post-challenge, hamsters would be culled for post-mortem analysis, where the caecum and colon removed aseptically and

their contents analysed for total bacterial load, as well as the presence of *C. difficile* toxins (Buckley *et al.* 2011).

To assess if the protective effect of plantain NSP translates into human clinical benefit, it would also be necessary to conduct a placebo-controlled clinical trial, where we could assess plantain NSP for its ability to prevent CDI in high-risk populations, such as those receiving broad-spectrum antibiotics (Ananthakrishnan 2011). This might include patients who are admitted to hospital with community-acquired pneumonia. Primary endpoints of this study would include the occurrence of diarrhoea, as well as laboratory analysis of stools to confirm the presence of *C. difficile* toxins (Iv *et al.* 2014).

Further work is also warranted to assess if dietary supplementation with plantain NSP has a protective effect in an *in vivo* colitis model, and similarly, whether this translates into a therapeutic benefit in human UC. Indeed, it has been shown previously that dietary intervention with green dwarf banana flour (*Musa* spp. AAA) reduces the severity of TNBS-initiated colitis in mice (Scarminio *et al.* 2012). It would therefore be interesting to determine if the oral administration of plantain soluble fibre (and pectic oligosaccharide fractions) has a similar protective effect in this particular colitis model. In addition, the impact of plantain NSP dietary supplementation on amelioration of spontaneous colonic inflammation observed in Muc2-deficient mice (Wenzel *et al.* 2014).

A future pilot phase IIA “placebo-blinded” study has also been proposed, in which patients with mild to moderately active UC will receive plantain NSP (15 or 30 g/day) or placebo (15 g/day maltodextrin). The primary endpoint of this clinical trial would be remission at 6 weeks based on a patient-reported outcome measure derived from the Mayo Clinic score

(Lewis *et al.* 2008), as well as reduction in faecal calprotectin concentration (Leiper *et al.* 2011). Considerable emphasis would also be placed on palatability and dosing of plantain NSP.

If the *in vivo* studies described above suggest that plantain NSP is a promising anti-colitic agent, further studies could also be performed to evaluate its ability to prevent colon carcinogenesis. This could be assessed using the mouse model of colon carcinogenesis induced by the alkylating agent azoxymethane (Rosenberg *et al.* 2009).

Further studies are also required to determine if soluble plantain NSP exerts a protective effect against ETEC infection *in vivo*. However, the development of therapies to treat ETEC infection has been limited by the lack of viable small-animal models, and as a result, further advances in this field will have to take place before we commence this work (Allen *et al.* 2006).

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APPENDICES

APPENDIX 1 – RECIPES OF COMMONLY USED BUFFERS AND SOLUTIONS

BACTERIAL CULTURE:

Luria Burtani (LB) agar

5 g	Yeast extract
10 g	Tryptone
10 g	NaCl
15 g	Agar
1 L	Distilled water

Brain heart infusion agar supplemented with 0.1% L-cysteine and 5 mg/mL yeast extract (BHIS)

37 g	Brain heart infusion broth
15 g	Agar
1 g	L-cysteine
5 g	Yeast extract
1 L	Distilled water

Tryptic glucose yeast (TGY) broth

30g	Tryptic soy broth
20g	Glucose
10g	Yeast extract
1g	L-cysteine
1L	Distilled water

Sorbitol MacConkey (SMC) broth

90 g	Peptone
5 g	Proteose peptone
1 g	$(NH_4)_2SO_4$
1.5 g	Tris base
1 g	L-cysteine
1 L	Distilled water

Sorbitol MacConkey (SMC) agar

90 g	Peptone
5 g	Proteose peptone
1 g	$(NH_4)_2SO_4$
1.5 g	Tris base
1 g	L-cysteine
15 g	Agar
1 L	Distilled water

CHLORIDE CHANNEL ACTIVITY ASSAYS:

Iodine loading buffer

0.88 g	NaI
8.9 mg	$CaCl_2$
10 mg	NaH_2PO_4
9.5 mg	1 mM MgCl2
89.6 mg	KI
100 mL	Distilled water

Detection buffer 1

19.8 g	Arsenic (III) acid
50 mL	25% ammonia
50 mL	Sulphuric acid
25g	Ammonium chloride
900 mL	Distilled water

Detection buffer 2

10 g	Ammonia Ce (IV)-sulfate
26 mL	Sulphuric acid
474 mL	Distilled water

IMMUNOBLOTTING:

5 x SDS-PAGE sample buffer

1 mL	0.5 M Tris-HCl
0.8 mL	Glycerol
1.6 mL	10% SDS
0.4 mL	Beta-mercaptoethanol
0.2 mL	0.05% (w/v) bromophenol blue
4 mL	Distilled water

SDS-PAGE transfer buffer

14.4 g	Glycine
3.03 g	Tris base
200 mL	Methanol
800 mL	Distilled water

SDS-PAGE running buffer

1 g	Sodium dodecyl sulphate (SDS)
14.4 g	Glycine
3.03 g	Tris base
1 L	Distilled water

Phosphate buffered saline (PBS)

8 g	NaCl
0.2 g	KCl
1.44 g	Na_2HPO_4
0.24 g	KH_2PO_4
1 L	Distilled water

Adjust pH to 7.4

APPENDIX 2 – MONOSACCHARIDE COMPOSITION OF NSP FIBRES SELECTED FOR STUDY IN THIS THESIS

NSP source	Uronic acid composition (g/100 g NSP)	Monosaccharide composition (μg/mg NSP)						
		Rhamnose	Fucose	Xylose	Arabinose	Mannose	Galactose	Glucose
Apple	19.9	3.2	0.0	0.0	6.1	2.3	3.2	3.7
Bean	31.7	2.8	0.0	0.0	17.5	1.8	2.8	2.1
Blueberry	19.2	6.3	0.0	0.0	14.1	4.7	5.5	8.2
Broccoli	17.5	1.2	0.0	0.0	2.6	2.3	7.8	5.3
Celery	45.8	2.4	0.0	0.0	9.7	1.6	6.6	0.8
Leek	23.0	11.4	0.0	0.0	4.1	8.1	1.4	32.8
Pear	32.7	1.4	0.0	0.0	1.4	1.9	1.4	3.1
Pepper (Red)	35.9	4.5	0.0	0.0	6.7	11.1	2.8	11.7
Plantain (Green)[1]	26.8	2.4	2.6	6.7	9.0	18.7	8.6	25.2
Strawberry	30.4	5.9	0.0	0.0	12.4	1.2	2.1	1.6
Tomato	29.9	7.0	0.0	0.0	29.7	4.4	8.5	9.6

[1] Sourced from Ecuador (ripeness stage 1). Average values obtained from plantains grown over three consecutive seasons

APPENDIX 3 – BACTERIAL CHARACTERISTICS OF ULCERATIVE COLITIS (UC) *E. COLI* STRAINS SELECTED FOR STUDY IN THIS THESIS

E. coli isolate	Hemagluttination status[1]	Afa/Dr cluster	Curli Fimbriae	Phylotype
HM250	**+**	**-**	**+**	B2
HM295	**-**	**-**	**-**	B2
HM378	**-**	**-**	**-**	B2
HM380	**-**	**-**	**+**	B2
HM387	**-**	**-**	**+**	B2

[1] Determined by the ability to haemagluttinate red blood cells (taken from [Subramanian et al. 2008])