Probiotics and their Interaction with Bacteria associated with Neonatal Necrotising Enterocolitis in an *In-vitro* Model

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Abstract

Neonatal necrotising enterocolitis (NEC) is one of the most devastating diseases affecting new born premature infants [5]. Globally it affects 1-5% of all neonatal intensive care admissions and is a leading cause of neonatal morbidity and mortality. The aetiology of NEC is not entirely understood, although it is clearly a multifactorial disease involving: bacterial colonisation of the neonatal gastrointestinal tract, immune system function and immaturity of the infant. Recent practice in neonatal units has moved focus from treatment to prevention of NEC before onset of disease. Supplementation of probiotic bacteria has been identified as a protective treatment and clinical trials have identified probiotics that reduce the incidence and severity of NEC.

The commercial probiotic formulation Infloran, containing *Lactobacillus acidophilus* and *Bifidobacterium longum* ss. *infantis* that is currently in use in the Dunedin public hospital neonatal intensive care unit, for infants of birth low weight (< 1,500 g). In this project, the Infloran probiotics inhibited the growth of four NEC-associated strains: *Cronobacter sakazakii* 50, *Cronobacter sakazakii* 2029, *Enterobacter cloacae* and *Klebsiella pneumoniae* ss. *oxytoca* *in vivo*. Inhibition was observed in solid media and probiotic conditioned media assays where the Infloran probiotics were able to completely inhibit the growth of the NEC-associated strains, putatively by lowering the pH (~4). *In vitro* attachment assays on an intestinal cell line (HT-29) identified localised and diffuse adherence patterns of the NEC-associated strains. When in co-culture with the Infloran probiotics, the attachment pattern changed to an aggregative adherence pattern. This aggregative pattern was observed, regardless of the NEC-associated strain or control strain used. An *In-vitro* neonatal model of the intestinal lumen was created using an anaerobic atmosphere and neonatal milk formula. This neonatal formula, pre-incubated with Infloran, was able inhibit the growth of the NEC-associated strains. A larger inhibitory effect was observed with longer pre-incubation of the Infloran probiotics.

In conclusion, this project has identified mechanisms: pH modulation, co-aggregation and substrate utilisation by which Infloran exerts its protective effect on pre-term neonates at risk of NEC.
Acknowledgements

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# Table of Contents

Abstract ................................................................................................................. ii  
Acknowledgements ............................................................................................... iii  
Table of Contents ....................................................................................................... iv  
Table of Figures ......................................................................................................... ix  
Table of tables .......................................................................................................... xii  
List of Abbreviations ............................................................................................... xiii  

1  Introduction ......................................................................................................... 1  

1.1  Neonatal Necrotising Enterocolitis ................................................................. 1  
1.1.1  Diagnosis and Treatment ........................................................................... 1  
1.1.2  Pathogenesis ............................................................................................... 3  
1.1.3  Hypothetical Model of NEC ....................................................................... 5  

1.2  Probiotics ......................................................................................................... 7  
1.2.1  *In vivo* Research ..................................................................................... 8  
1.2.2  *In vitro* Research ................................................................................... 11  

1.3  Introduction to Project .................................................................................... 12  

2  Materials and Methods ...................................................................................... 15  

2.1  Stock Materials and Standard Methods ......................................................... 15  
2.1.1  Stock Solutions ......................................................................................... 15  
2.1.2  Antibiotics ................................................................................................. 16  
2.1.3  Bacterial Culture Media ............................................................................. 16  
2.1.4  Bacterial Storage ....................................................................................... 16  
2.1.5  Drop Plate Counts .................................................................................... 17  
2.1.6  Bacterial Strains ....................................................................................... 18  
2.1.7  Bacterial Growth on Solid Media ............................................................... 20  
2.1.8  Bacterial Growth in Liquid Broth ............................................................... 20  
2.1.9  Dilution Curves ......................................................................................... 21
Results ................................................................................................................................................. 41

3.1 Solid Media Antagonism Assays ........................................................................................................ 41
3.1.1 Spot Antagonism ............................................................................................................................. 41
3.1.2 ‘P-Typing’ Assays .......................................................................................................................... 41
3.1.3 Soft Agar Overlays .......................................................................................................................... 41
3.1.4 Soaked Plate Antagonism ................................................................................................................ 41
3.1.5 Probiotic Supernatant Supplemented Plate Antagonism Assays ..................................................... 41

3.2 Co-Aggregation Assays ........................................................................................................................ 43
3.2.1 Auto-aggregation ............................................................................................................................... 43
3.2.2 Aggregation with *Lactobacillus acidophilus* ................................................................................... 43
3.2.3 Aggregation with *Bifidobacterium infantis* .................................................................................... 43
3.2.4 Aggregation with Mixed Probiotics ................................................................................................... 44
3.2.5 *L. casei* Control ............................................................................................................................... 44
3.2.6 Triphenyl Tetrazolium Chloride (TTC) ............................................................................................ 44

3.3 Broth Antagonism Assays ....................................................................................................................... 46
3.3.1 *Lactobacillus acidophilus* Supernatant ............................................................................................. 46
3.3.2 *Bifidobacterium infantis* Supernatant ............................................................................................... 50
3.3.3 Lactic acid supplemented MRS ........................................................................................................ 54

3.4 Attachment Assays with ^3^H-Thymidine ............................................................................................. 58
3.4.1 Optimal Attachment time .................................................................................................................. 58
3.4.2 Further ^3^H-Thymide Assays .......................................................................................................... 61

3.5 Attachment Assays with Giemsa Stain .................................................................................................. 61
3.5.1 Single Strain Attachment ................................................................................................................... 61
3.5.2 Co-culture Attachment ...................................................................................................................... 72

3.6 Invasion Assays ...................................................................................................................................... 96
3.6.1 *Salmonella* Typhimurium Optimisation .......................................................................................... 96
3.6.2 Single Strain Invasion, NEC, Controls and Probiotics ....................................................................... 99
3.6.3  Single Strain Invasion, Log Phase Cultures ........................................ 102

3.6.4  Probiotic Co-culture Invasion ................................................................. 105

3.7  Neonatal Formula Growth Experiments ....................................................... 108

3.7.1  Co-culture Growth Curves in Neonatal Formula ........................................ 108

3.7.2  Probiotic Pre-incubation in Neonatal Formula ......................................... 110

4  Discussion ........................................................................................................... 113

4.1  Probiotic Inhibition of NEC-associated Strains ............................................... 113

4.1.1  Probiotic Strains from Infloran & Inhibition of Growth of NEC-associated Strains on Solid Media ................................................................. 113

4.1.2  Auto-aggregation and Co-aggregation of the Probiotics from Infloran and the NEC-associated Strains ................................................................. 113

4.1.3  Probiotic Strains from Infloran & Inhibition of Growth of NEC-associated Strains in Liquid Media ................................................................. 114

4.2  3H-Thymidine Attachment Assays with the Infloran Probiotic Strains and NEC-associated Strains ................................................................. 116

4.3  Giemsa Attachment Assays with the Infloran Probiotic Strains and NEC-associated Strains ......................................................................................... 117

4.3.1  Single Strain Attachment Assays ................................................................. 117

4.3.2  Co-attachment Assays .................................................................................. 119

4.4  Invasion Assays with the Infloran Probiotic Strains and NEC-associated Strains ......................................................................................... 120

4.5  Neonatal Formula Growth Assays .................................................................... 122

4.5.1  Co-culture Growth Curve ........................................................................... 122

4.5.2  Probiotic Pre-incubation ............................................................................. 122

4.6  Conclusions ..................................................................................................... 124

4.7  Future Directions ............................................................................................. 124

5  References ......................................................................................................... 126

6  Appendix ............................................................................................................ 141

6.1  M’Farland 0.5 Opacity Standard .................................................................... 141
6.2  $^3$H-Thymidine Co-attachment experiments ........................................ 142
6.3  Giemsa Attachment Assays Pictures ..................................................... 145
6.4  Neonatal Formula Ingredients .................................................................. 145
6.4.1 Pfizer S-26 Gold .................................................................................. 145
Table of Figures

Figure 1. Comparison NEC progression at the intestinal barrier of pre-term neonates to the healthy intestinal barrier function of full term infants. Adapted from Brooks et al. 2013 (Benirschke, 1973; Boccia, Stolfi, Lana, & Moro, 2001; H. J. L. Brooks, McConnell, & Broadbent, 2013; Cheromcha & Hyman, 1988). .............................................................................................................. 6

Figure 2. Comparison NEC progression at the intestinal barrier of pre-term neonates to the healthy intestinal barrier function of full term infants. Adapted from Brooks et al. 2013 (H. J. L. Brooks et al., 2013) .............................................................................................................. 6

Figure 3. Broth antagonism assays for *K. pneumoniae* ss. *oxytoca* (A), *E. coli* O111 (B) and *C. freundii* (C) ................................................................................................................................. 49

Figure 4. Broth antagonism assays for *C. sakazakii* 50 (A), *C. sakazakii* 2029 (B) and *E. cloacae* (C) .................................................................................................................................................. 51

Figure 5. Broth antagonism assays for *K. pneumoniae* ss. *oxytoca* (A), *E. coli* O111 (B) and *C. freundii* (C) ............................................................................................................................................. 53

Figure 6. Broth antagonism assays for *C. sakazakii* 50 (A), *C. sakazakii* 2029 (B) and *E. cloacae* (C) .................................................................................................................................................. 55

Figure 7. Broth antagonism assays for *K. pneumoniae* ss. *oxytoca* (A), *E. coli* O111 (B) and *C. freundii* (C) ............................................................................................................................................. 57

Figure 8. ³H-Thymidine incubation time optimisation for all strains at an MOI of 1:100...... 59

Figure 9. *Cronobacter sakazakii* 50, Giemsa stained with HT-29 ........................................ 64

Figure 10. *Cronobacter sakazakii* 2029, Giemsa stained with HT-29 ................................... 65

Figure 11. *Enterobacter cloacae*, Giemsa stained with HT-29 ............................................. 66

Figure 12. *Enterobacter cloacae*, Giemsa stained with HT-29 ............................................. 67

Figure 13. *Escherichia. coli* O111, Giemsa stained HT-29. .................................................... 68

Figure 14. *Citrobacter freundii*, Giemsa stained HT-29 ........................................................ 69

Figure 15. *Bifidobacterium infantis*, Giemsa stained HT-29 ................................................ 70

Figure 16. *Lactobacillus acidophilus*, Giemsa stained HT-29 ................................................ 71

Figure 17. *Cronobacter sakazakii* 50 in co-culture with *Bifidobacterium infantis*, Giemsa stained HT-29 .................................................................................................................................................. 73

Figure 18. *Cronobacter sakazakii* 50 in co-culture with *Lactobacillus acidophilus*, Giemsa stained HT-29 .................................................................................................................................................. 74

Figure 19. *Cronobacter sakazakii* 50 in co-culture with both Infloran probiotics, Giemsa stained HT-29 .................................................................................................................................................. 75
Figure 20. *Cronobacter sakazakii* 2029 in co-culture with *Bifidobacterium infantis*, Giemsa stained HT-29. ................................................................................................................................. 77
Figure 21. *Cronobacter sakazakii* 2029 in co-culture with *Lactobacillus acidophilus*, Giemsa stained HT-29 cells ............................................................................................................................ 78
Figure 22. *Cronobacter sakazakii* 2029 in co-culture with both Infloran probiotics, Giemsa stained HT-29 ........................................................................................................................................... 79
Figure 23. *Enterobacter cloacae* in co-culture with *Bifidobacterium infantis*, Giemsa stained HT-29 ................................................................................................................................................ 81
Figure 24. *Enterobacter cloacae* in co-culture with *Lactobacillus acidophilus*, Giemsa stained HT-29 .............................................................................................................................................. 82
Figure 25. *Enterobacter cloacae* in co-culture with both Infloran Probiotics, Giemsa stained HT-29 ............................................................................................................................................. 83
Figure 26. *Klebsiella pneumoniae* ss. *oxytoca* in co-culture with *Bifidobacterium infantis*, Giemsa stained HT-29 ................................................................................................................................. 85
Figure 27. *Klebsiella pneumoniae* ss. *oxytoca* in co-culture with *Lactobacillus acidophilus*, Giemsa stained HT-29 ................................................................................................................................. 86
Figure 28. *Klebsiella pneumoniae* ss. *oxytoca* in co-culture with both Infloran probiotics, Giemsa stained HT-29 ............................................................................................................................................. 87
Figure 29. *Escherichia coli* O111 in co-culture with *Bifidobacterium infantis*, Giemsa stained HT-29 .............................................................................................................................................. 89
Figure 30. *Escherichia coli* O111 in co-culture with *Lactobacillus acidophilus*, Giemsa stained HT-29 .............................................................................................................................................. 90
Figure 31. *Escherichia coli* O111 in co-culture with both Infloran probiotics, Giemsa stained HT-29. ................................................................................................................................................ 91
Figure 32. *Citrobacter freundii* in co-culture with *Bifidobacterium infantis*, Giemsa stained HT-29 ................................................................................................................................................ 93
Figure 33. *Citrobacter freundii* in co-culture with *Lactobacillus acidophilus*, Giemsa stained HT-29 ................................................................................................................................................ 94
Figure 34. *Citrobacter freundii* in co-culture with both the Infloran Probiotics, Giemsa stained HT-29 ................................................................................................................................................ 95
Figure 35. *Salmonella* Typhimurium invasion time and MOI optimisation experiments ................... 98
Figure 36. Single strain invasion of HT-29 cells by all NEC strains, controls and probiotics ................. 101
Figure 37. Single strain invasion with Log phase cultures of control and probiotic strains. 104
Figure 38. Invasion of HT-29 cells by NEC strains and control strains co-cultured with *Lactobacillus acidophilus* and *B. infantis* in a 1:1 ratio. ................................................................. 107

Figure 39. Co-culture growth curves in Neonatal formula with the NEC strains and the Infloran probiotics .................................................................................................................. 109

Figure 40. Growth of the NEC strains in Pfizer S-26 formula pre-incubated with probiotic. 111

Figure 41. Growth of NEC strains in Nestle PreNAN Gold pre-incubated with Probiotic... 112

Figure 42. Total viable counts for all NEC-associated, controls and Infloran strains......... 141

Figure 43. ³H-Thymidine attachment, all strains in co-culture with probiotics 1:1 .......... 143

Figure 44. ³H-Thymidine attachment, *C. sakazakii* strains in co-culture with probiotics..... 144
Table of tables

Table 1. Bell’s Clinical Staging for the diagnosis of NEC in neonates ........................................ 2
Table 2. Culture Media Used in this study ....................................................................................... 17
Table 3. All bacterial strains used in this study ................................................................................ 19
Table 4. M’Farland Opacity Standards used in this study ................................................................. 23
Table 5. Tissue culture types used in this study ................................................................................ 23
Table 6. Co-Aggregation scoring table ............................................................................................. 27
Table 7. Summary of solid media antagonism experiments for the detection of inhibitory activity in probiotic culture supernatants against NEC associated bacteria and control strains. ......................................................................................... 42
Table 8. Auto- and co-aggregation scores from probiotic bacteria, NEC-associated and control strains ................................................................................................................................. 45
Table 9. Summary of $^{3}$H-Thymidine optimal attachment time, mean percentage attachment and SEM. ................................................................................................................................. 59
Table 10. Labelling efficiency of all strains with $^{3}$H-Thymidine ........................................................ 59
Table 11. Quantification of associated and stacked bacteria in Giemsa stained HT-29 cells. .............. 62
Table 12. Invasion of HT-29 cells by S. Typhimurium: effect of different incubation times and MOI expressed as the mean percentage of the initial inoculum with SEM ................................. 97
Table 13. Invasion of HT-29 cells by S. Typhimurium: effect of different incubation times and MOI expressed as the mean total recovered viable (cfu/mL) bacteria with SEM .......................... 97
Table 14. Single strain invasion of HT-29 cells by all NEC-associated and control strains, expressed as total recovered cfu/mL with mean and SEM ......................................................... 100
Table 15. Single strain invasion of HT-29 cells by all NEC-associated and control strains expressed as percentage of initial inoculum recovered, mean and SEM ................................................................. 100
Table 16. Log phase Single strain invasion, controls and probiotics only expressed as total recovered cfu/mL with mean and SEM ................................................................................................................................. 103
Table 17. Log phase Single strain invasion, controls and probiotics only expressed as percentage of initial inoculum with mean and SEM. ......................................................................................... 103
Table 18. Probiotic Co-culture Invasion of HT-29 expressed as total recovered cfu/mL, mean and SEM. ................................................................................................................................................. 106
Table 19. Probiotic Co-culture Invasion of HT-29 expressed as percentage of initial inoculum, mean and SEM. ................................................................................................................................. 106
Table 20. $^3$H-Thymidine attachment, mean and SEM of single co-attached probiotics and single strains
Table 21. $^3$H-Thymidine Co-attachment, C sakazakii strains and controls only. Mean and SEM

**List of Abbreviations**

- ^°^C: Degrees centigrade
- Caco-2: Human colon epithelial cell line
- cfu: Colony forming units
- DMEM: Delbco's modified eagle medium
- DMSO: Di-methyl sulfoxide
- $E_h$: Redox potential
- EPEC: Enteropathogenic E. coli
- FCS: Fetal calf serum
- GI: Gastrointestinal
- Hep-2: HeLa derivative, cervical epithelial cell line
- HT-29: Human colorectal carcinoma cell line
- HT-29 MTX: A derivative of the HT-29 Cell Line, mucus secreting goblet cells
- IEC-6: Rat small intestine epithelial cell line
- IFN: Interferon
- IgG: Immunoglobulin G
- IL: Interleukin
- iNOS: Inducible nitric oxide synthase
- LA: Lactic acid
- LAB: Lactic acid bacteria
- mL: Millilitres
- MyD88: Myeloid differentiation primary response factor
- NEC: Necrotising enterocolitis
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa-light chain-enhancer</td>
</tr>
<tr>
<td>NICU</td>
<td>Neonatal intensive care unit</td>
</tr>
<tr>
<td>NZRM</td>
<td>New Zealand type culture collection</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>pH</td>
<td>Power of hydrogen</td>
</tr>
<tr>
<td>PROM</td>
<td>Premature rupture of membranes</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>sIgA</td>
<td>Secretory immunoglobulin A</td>
</tr>
<tr>
<td>spp</td>
<td>Species</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>TTC</td>
<td>2,3,5-triphenyl-2H-tetrazolium chloride</td>
</tr>
<tr>
<td>VLBW</td>
<td>Very low birth weight</td>
</tr>
<tr>
<td>YAMC</td>
<td>Young adult mouse colon</td>
</tr>
<tr>
<td>μL</td>
<td>Microlitre</td>
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1 Introduction

1.1 Neonatal Necrotising Enterocolitis

Neonatal necrotising enterocolitis (NEC) is an inflammatory disease of the lower gastrointestinal (GI) tract, usually starting at the ileocaecal junction with an aetiology that remains unclear, although a number of hypotheses have been proposed [6]. NEC most commonly occurs in pre-term (<33 weeks gestation) and very low birth weight infants (<1,500 g) (VLBW) [7, 8]. The incidence varies from <1 to 5% of neonatal intensive care unit admissions [9]. Infant care practices have improved vastly over the last 50 years, although in spite of this, the incidence of NEC has stayed constant at between 70 and 80%. This is thought to be due to an increased incidence of pre-term births with a shorter gestation periods [4, 8, 10, 11]. Apart from prematurity and low birth weight, other risk factors include delivery by caesarean section, formula feeding as opposed to breast milk, length of stay in a neonatal intensive care unit (NICU), congenital birth defects and intestinal ischemia [12-15].

1.1.1 Diagnosis and Treatment

Diagnosis is based on a well-established set of clinical staging criteria originally devised by Bell et al in 1978. Although there have been revisions, they remain the gold standard for diagnosis of NEC [16-18] (Table 1). Although not limited to these early stages the initial signs and symptoms include feeding intolerance, blood in the stool, general lethargy and irritability. Later stages include ileus, pneumatosis intestinalis, and positive blood cultures with progressively more severe symptoms such as perforation of the intestines and septic shock, as the disease progresses.[19, 20]. Even so, NEC is still a loosely defined disease and treatment generally on a case by case basis. Once NEC is suspected, there are a number of strategies for treatment. These include cessation of enteral feeding, intravenous broad spectrum antibiotics, parenteral nutrition, and in advanced cases, surgery to remove diseased tissue. Surgery is required in 20 to 40% of cases and comes with significant morbidities, as well as mortality of up to 50% in some centres [7, 21]. Morbidities include short bowel syndrome, neurodevelopmental issues, poor growth and liver failure due to prolonged parenteral nutrition [10, 22, 23].
Table 1. Bell’s Clinical Staging for the diagnosis of NEC in neonates

Adapted from Bell et al. (1978) [16]

<table>
<thead>
<tr>
<th>Stage I, Suspected</th>
</tr>
</thead>
<tbody>
<tr>
<td>a) Historical factors leading to perinatal stress</td>
</tr>
<tr>
<td>b) Systemic factors- temperature instability, lethargy, apnea, bradycardia.</td>
</tr>
<tr>
<td>c) Gastrointestinal manifestations-poor feeding, increasing pregavage residuals, emesis (may be bilious or test positive for occult blood), mild abdominal distension, occult blood may be present in stool (no fissure).</td>
</tr>
<tr>
<td>d) Abdominal radiographs show distension with mild ileus.</td>
</tr>
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<table>
<thead>
<tr>
<th>Stage II, Definite</th>
</tr>
</thead>
<tbody>
<tr>
<td>a) Any one or more historical factors.</td>
</tr>
<tr>
<td>b) Above signs and symptoms plus persistent occult or gross gastrointestinal bleeding; marked abdominal distension.</td>
</tr>
<tr>
<td>c) Abdominal radiographs show significant intestinal distension with ileus; small bowel separation (oedema in bowel wall or peritoneal fluid), unchanging or persistent &quot;rigid&quot; bowel loops, pneumatosis intestinalis, portal vein gas.</td>
</tr>
</tbody>
</table>

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<thead>
<tr>
<th>Stage III, Advanced</th>
</tr>
</thead>
<tbody>
<tr>
<td>a) Any one or more historical factors.</td>
</tr>
<tr>
<td>b) Above signs and symptoms plus deterioration of vital signs, evidence of septic shock or marked gastrointestinal haemorrhage.</td>
</tr>
<tr>
<td>c) Abdominal radiographs may show pneumoperitoneum in addition to others listed in II c.</td>
</tr>
</tbody>
</table>
1.1.2 Pathogenesis

There are two major schools of thought as to the pathogenesis of NEC firstly the specific pathogen theory, and secondly, the abnormal colonisation theory. Many retrospective studies and controlled trials have looked for a single causative agent [24-28]. Although outbreaks in some individual NICU’s involved a predominance of a single species, there has never been a consistently identified organism related to every NEC case or outbreak. While not as attractive as the single agent theory, most now agree that NEC is a result of an abnormal colonisation of the GI tract [4, 29]. This occurs late in onset NEC usually 7-20 days postpartum, with the major risk factors being: VLBW, significant prematurity (<33 weeks gestation) and formula feeding, especially if by nasogastric tube [12, 30, 31].

There is also another type of NEC referred to as early onset NEC which occurs both in full term and preterm infants and is related to ischaemic damage of the GI lining [32, 33].

Until recently it was thought bacterial colonisation of the neonate started from the moment of delivery. Current research indicates that initial colonisation takes place when bacteria traverse the vagina and cervix and go on to colonise the amniotic membranes. This sometimes results in membrane infection, premature rupture of the amniotic membranes (PROM) and infection of the foetus. This has been implicated as one of the major causes for preterm birth and carries with it serious risk for the mother and infant [34-36]. In full term and pre-term infants who are vaginally delivered, further bacterial exposure occurs when passing through the birth canal. Microbes are also introduced from the mother’s commensal microbiota via breast feeding and from elsewhere by interaction with other people [37]. The first colonisers are facultative aerobes which begin the process of using oxygen and bringing the \( E_h \) down to more anaerobic levels [38].
Bacteria isolated during the first 10 days of infant life include *Bacteroides* spp., *Streptococcus* spp., *Staphylococcus* spp., *Klebsiella* spp., *Escherichia coli* and lactobacilli [39-41]. By 4-7 weeks these populations have moved to a predominance of *Bifidobacterium* spp. and smaller amounts of *Enterobacter* spp., *Enterococcus* spp. and *Clostridium* spp. [42] [43] In formula fed infants a similar progression occurs although often there are more *Enterobacteriaceae* and *Clostridium difficile* with lower total numbers of *Bifidobacterium* spp. [44].

1.1.2.1 Gut Motility

One of the first symptoms that will be noticed in a suspected NEC infant is ileus, the slowing or complete halt of peristalsis: the rhythmical smooth muscle function of the gut. In the foetus GI motility does not fully mature until late in the 3rd trimester [45]. At approximately 34 weeks gestation the proper muscle function is fully developed [46] and consequently prematurity is often accompanied by poor gut motility [47, 48]. Due to this poor motility formula or breast milk will be very slow to pass through the GI tract. Coupled with very low or absent stomach acid and poor absorption of nutrients in the small intestine, this will provide a nutrient rich environment for the bacteria introduced to the GI tract. This undigested bolus of substrate may be the origin of the bloom of bacteria associated with NEC [49-51].

1.1.2.2 Neonatal Immune Function

Neonates both preterm and full term have had little, or no stimulation of their adaptive immune system. Thus they must rely on their innate immune system to protect them in this new environment and instruct the adaptive immune response [23, 52]. The first line of defence is non-specific barriers such as gastric acidity as well as other digestive enzymes which can inhibit bacteria and break down toxins and other antigenic material. Mucus secretion and motility of this mucus layer along with sIgA from the mother’s breast milk help to opsonise and trap bacteria in the mucus layer and flush them out of the GI tract [12, 53]. Many of these first line defences are dysfunctional due to immaturity in the pre-term neonate. Toll like receptors (TLR) are another major arm of the innate immune system. TLR recognise highly conserved bacterial, viral and other pathogen derived antigens, a few examples include: TLR-5 which recognises subunits of bacterial flagella; TLR-3 which recognises viral double stranded RNA; and TLR-4 which recognises LPS present on all Gram negative bacteria [54]. With the exception of TLR-3, stimulation of TLR’s activates the MyD88 pathway which goes on to express NF-κB. NF-κB migrates to the nucleus and upregulates
genes associated with inflammation such as TNF-α and IL-8[29, 55, 56]. In term infants this reaction is tempered by the expression of IκB which binds NF-κB and prevents it from entering the nucleus. This along with the physical barrier of mucus promotes tolerance to the microbiota in the full term infant. In the preterm neonates this constitutive expression of IκB is often at very low levels and may lead to excessive inflammation at the gut lining [57, 58]. Other arms of the immune system like platelet activating factor and growth factors like TGF-β are also extensively involved.

1.1.3 Hypothetical Model of NEC

Many risk factors are involved in the development of NEC and the innate immune system appears to play a substantive role in the formation of the disease [56]. Poor mucus secretion is commonly observed in preterm infants and may allow for direct interaction between commensal bacteria (or potential pathogens) and the toll like receptors on epithelial cells. These TLR trigger the innate immune response which leads to inflammation, loosening of the tight junctions between cells, apoptosis and necrosis of epithelial cells, which allow bacteria to translocate through to the other side of the epithelium (Figure 1) [55, 57]. Other factors may assist the translocation of bacteria such as; ischaemic damage to the epithelium due to complications with labour, microbial cytotoxins or other toxic chemicals, infection with enteric viruses, and a bloom of a select number of bacterial species in the gut which can lead to increased phagocytosis by the epithelial cells and antigen presenting cells [59]. The epithelium of a healthy full term infant is very different. In contrast to the pre-term infant, there is much more mucus secreted, which is much more mobile and can trap commensals and potential pathogens, and move them out of the GI tract. Antimicrobial substances such as lactoferrin and lysozyme as well as slgA from the mother’s colostrum and milk are present which help to inhibit colonisation of the epithelia [53, 60, 61]. Commensals are tolerated by the enterocytes, which helps to
a. Progression of bacterial overgrowth that can lead to NEC in a pre-term neonate. Lack of gut motility combined with poor mucus secretion allows bacteria to adhere to and translocate through epithelial mucus secretionally, where inflammation will be set up due to upregulation of inflammatory cytokines from both the epithelial cells themselves, and professional antigen presenting cells such as macrophages. This leads to apoptosis and necrosis of epithelia and loosening of tight junctions from inflammation which will allow bacteria to translocate via the paracellular invasion route.

b. Normal intestinal homeostasis with good motility of the mucus layer along with diverse bacterial flora and the mothers secretory IgA as well as a physical separation between the commensal bacteria and the epithelium.

Figure 1. Comparison NEC progression at the intestinal barrier of pre-term neonates to the healthy intestinal barrier function of full term infants. Adapted from Brooks et al. 2013 [1-4].
reduce the TLR mediated innate immune response. Indeed the ability of the innate immune system to recognise and differentiate between normal, usually harmless, commensals and potential pathogens may be a very large factor in the pathogenesis of NEC [1, 62].

1.2 Probiotics

In recent years the approach to treatment of NEC has changed from reactive to a proactive by identifying those infants most at risk and preventing the disease before it starts. Healthy infants usually have a predominance of *Bifidobacterium* spp. in the GI tract which has been shown to have an immunoregulatory role along with other lactic acid producing bacteria (LAB) such as *Lactobacillus* spp. [63; Tannock, 2002 #121, 64-66]. This evidence prompted researchers and carers to look into using these species as probiotics to assist the neonatal gut.

The World Health Organisation (WHO) defines probiotics as "live micro-organisms which, when administered in adequate amounts, confer a health benefit on the host" [67]. The word probiotic comes from the Latin and Greek, ‘pro’ ‘biotic’ meaning ‘for’ ‘life’. In the case of probiotic administration to neonates, the probiotics used are usually isolates from healthy full term infants. Species currently in use include: *L. casei, L. rhamnosus, L. acidophilus, B. bifidus, B. longum, Saccharomyces boulardii*, some strains of *E. coli* as well as many others [68, 69]. These bacterial species have all been tested as formulations for neonates. A recent Cochrane collaboration review by AlFaleh et al. (2009) strongly recommended a change in practice for premature infants of VLBW to be given probiotic supplementation as part of normal practice. Their meta-analysis showed a 32% reduction in the relative risk of developing Bells Stage II or III NEC, and a 43% reduction in the relative risk of mortality [70].

The two most commonly used probiotic genera are *Bifidobacterium* and *Lactobacillus*. Recent research has investigated the effect these species have on the intestinal homeostasis and immune function [20, 71]. The effect of probiotics include, decreasing the pH of the gut to inhibit the growth of pathogenic bacteria, secretion of bacteriocin peptides, stimulation of the epithelial cells to secrete defensins and increase mucus production, as well as physically blocking bacterial attachment and invasion [72-74]. *L. acidophilus* and *B. infantis* are both members of a group of bacteria collectively referred to as lactic acid bacteria (LAB). pH is well documented as a critical factor in the growth of many types of bacteria, and as a major factor for the health of mucosal surfaces in the human body. Previous work has demonstrated
the ability of LAB to lower pH and prevent the attachment and invasion of GI pathogens such as *Salmonella enterica* subsp. *enterica* serovar Typhimurium (S. Typhimurium), [72]. Others have shown LAB to reduce the luminal pH in model organisms as well as in human neonates [75, 76]. *Lactobacillus* spp. have also been shown to produce hydrogen peroxide, a potent antibacterial agent [77-79]. Other effects of probiotics include maturation of the intestinal lining with the production of butyrate and other compounds that help improve tight junction integrity and modulation of the immune response [75, 80, 81]. Production of cytokines such as IL-10 and TGF-β, TNF-α and IFN-γ is influenced by probiotics and a role in activating T-regulatory cells has been proposed [52, 82, 83].

1.2.1 *In vivo* Research

There have been two main methods used to investigate the function of the probiotics administered to infants. The first being large trials of neonates who have received probiotic treatment or placebo [84-86]. The second being, specific models of infection, either animal models or human tissue culture [87-90]. Many trials have been undertaken to study the effectiveness of probiotic supplementation, but as a whole they are hampered by variable study sizes, inconsistent use of double blind and randomisation. More recently there have been reports of parents giving their neonate off the shelf probiotic formulations due to fears that their child may be in the control group, thus defeating the purpose of the trial. [6, 91-93].

As an example, the largest *in vivo* study so far was conducted over a two year period, by Hoyos (1999), and had a cohort of over 2,500. In the first year infants had no probiotic supplementation and in the second year all infants, regardless of birth weight, were supplemented with *Lactobacillus acidophilus* and *Bifidobacterium infantis* (Infloran, Swiss Serum and Vaccine Institute Berne, Berne, Switzerland) at $2.5 \times 10^8$ (colony forming units) cfu of each strain per day. This was not a double blind trial, but the sheer size of the study gives the results some weight. The total number of NEC cases dropped from 85 infants in the control group in year one to 37 infants in the supplemented group in year two with a P-value of $<0.0002$ and mortality of those with NEC dropped from 25 to 7 infants. Staff also noted better feeding tolerance and a reduction in diaper dermatitis [94].

Another study using the Infloran probiotics is by Lin et al 2005. They had a cohort of 367 neonates over four and a half years. The study was a full randomised, double blinded trial with both the parents and the primary carers unaware of treatment status of the infant. Of the
cohort, 180 infants received the probiotic formulation Infloran mixed with breast milk and 187 breast milk alone. There was a significant reduction in the incidence of both NEC and infant death in the Infloran treated group versus the control group; 9 of 180 versus 24 of 187 respectively with a P value of 0.009. There were only 6 cases of severe NEC (Bells Stage III) and they were all in the control group [95]. It is worth noting that Lin specified the use of breast milk in both study groups, whereas Hoyos made no mention of whether the infants received formula or breast milk.

Not all studies involving probiotics have had such positive results, Dani et al 2002 reports a double-blind study of 585 infants across 12 different NICU’s in Italy. In this study all infants born at <33 weeks gestation and or <1,500g were divided up into either treatment or control groups. Those in the treatment group were supplemented with 6 x 10^9 cfu of Lactobacillus rhamnosus GG daily, until the infants left hospital. At the completion of the study there was no significant difference noted between the treatment and control cohorts in terms of urinary tract infections, NEC or sepsis [96]. Once again as in Hoyos study there was no mention as to whether the infants were on formula or breast milk.

An example closer to home was undertaken in Perth Australia by Patole et al 2014. Their study was another randomised double-blinded study, with a small cohort of only 159 neonates. They supplemented with 3 x 10^9 cfu of Bifidobacterium breve M-16V per day, mixed with either expressed mothers milk or distilled water. At the completion of the study the authors were able to confirm they could isolate significantly larger numbers of B. breve from the stool of treated infants, although they did point out their study was not specifically powered to see if there was a reduction in NEC. As such, only one case of NEC (Bells class II) was observed in the control group, and it was not considered a significant outcome [97].

These four studies are a small sample of some trials that have been undertaken with probiotics and they demonstrate some of the problems with consistency in the application of and design of probiotic trials. Differing studies have returned both negative and positive results as to the effectiveness of supplementation with probiotics and the differences in study design make it very hard to directly compare and contrast their results. As previously mentioned a Cochrane meta-analysis has taken all this into account and came to the conclusion that probiotics should be mandatory for all infants of VLBW [98].
Trails using animal models are another useful tool in investigating the efficacy of supplementation with probiotic bacteria. These studies include the use of mice, rats and piglets as approximations of the infant system. The primary issue with these models is that a hypoxic event to induce intestinal ischemia is often required to imitate NEC-like conditions. This type of model, though easy to use, is more representative of early onset NEC, which is much less common than late onset NEC [99, 100].

Pigs are used as an animal model as they are considered the closest non-primate species to humans, with a similar omnivorous diet and are of a similar size. Pigs also naturally harbour a large population of *Lactobacilli* spp throughout the throat, stomach and GI tract.[88]. This animal model also has its issues, as pigs have a large caecum where the primary fermentative digestion occurs, whereas in humans, it primarily occurs in the large intestine [101]. A study by Siggers et al. (2008) showed enhanced intestinal maturation and a reduction of potential pathogens in the GI tract of pre-term piglets that were fed a combination of *Bifidobacterium* spp and *Lactobacillus* spp. Interestingly the addition of the probiotic formulation also enhanced the growth of naturally occurring *Lactobacillus* species in the GI tract [87].

Hunter (2009), undertook a study using both a hypoxic rat pup model of NEC and the IEC-6 rat intestinal cell line [102]. In both systems they treated with *Cronobacter sakazakii*, a bacterium commonly associated with NEC and sepsis outbreaks in NICU’s and also regularly isolated from powdered infant formula. They also treated with the probiotic species *Lactobacillus bulgaricus* with the aim of reducing the expression of inducible nitric oxide synthase (iNOS). One theory of NEC states that nitric oxide production by intestinal cells is responsible for much of the gut barrier failure and tissue necrosis observed in NEC and its production is upregulated by the presence of pathogenic bacteria [103]. In Hunters study the probiotic *L. bulgaricus* was added to the hypoxic rat pups and the IEC-6 cell line both pre-infection and simultaneously with infection of *C. sakazakii*. A significant reduction in the expression of iNOS and a reduction in tissue damage when pre-treating with *L. bulgaricus* was observed, compared to both simultaneous treatment and non-treatment (P= < 0.001). This was a very promising result, especially considering that a similar effect was observed in both the *in vivo* and *in vitro* model but, this research still has the drawback that it is a non-human model. Although animal models do provide useful insights into NEC pathogenesis and the effects of probiotic treatment, they are by no means a perfect system.
1.2.2 *In vitro* Research

It is not possible to conduct the type of *in vivo* trials performed on animals in human infants, for evident ethical reasons. Laboratory work to study the effect of probiotics can be performed *in vitro* with tissue culture cells of human origin such as HT-29, Caco-2 and Hep-2, which are all adult epithelial cells and are very useful as tools for studying the interaction between bacteria and the GI tract [104-106]. Additionally a large number of *in vitro* experiments use rat GI tissue culture lines such as IEC-6 [102]. Ideally an infant GI epithelial cell line would be used, but at this point no such cell lines exist. Primary tissue could also be used, but this presents other difficulties.

Studies such as those of Kim et al. (1998) demonstrate the ability of pathogenic bacteria to invade and cause apoptosis of multiple GI tract tissue culture cell lines. They showed that *Salmonella* Dublin, *S. Typhimurium* and an enteroinvasive *Escherichia coli* were able to invade, actively replicate, and cause apoptosis in HT-29 and Caco-2 tissue culture, as well as inducing the production of pro-inflammatory cytokines such as TNF-α [107]. A similar study by Ostad et al. (2009) found that lactobacilli isolated from one month old neonates, exclusively fed on their mother’s breast milk, were able to prevent the attachment of the pathogenic bacterial strains: *S. Typhimurium* and enteropathogenic *E. coli* to Caco-2 cells. In their study, they showed *L. acidophilus* and *L. casei*, isolated from neonatal faeces, paired with biochemically similar commercial strains of *L. acidophilus* and *L. casei*, along with a strain of *L. agilis*, were able to prevent adherence of *S. Typhimurium* and enteroinvasive *E. coli* to Caco-2 cell lines, regardless of whether the bacteria were alive or dead [89].

The individual GI tract is home to upwards of 400 different strains of bacteria and the ability of the body to tolerate this large population of organisms is integral to normal intestinal function[43, 108]. Probiotic bacteria have been shown to induce tolerance in the GI tract. Kimura et al. (1997) demonstrated that the predominant populations of *Lactobacillus* and *Bifidobacterium* isolated from human volunteers elicited a minimal humoral response in the serum of their study participants, with low serum IgG concentrations that were more genus specific than species specific [63]. Yan et al. (2002) demonstrated that *L. rhamnosus* GG was able to prevent apoptosis in both murine (YMAC) and human (HT-29) tissue culture cells that had been treated with the pro-inflammatory cytokines TNF and IL-1α [109]. Similar results were obtained with colon organ culture from 8 week old mice [110].
Furthermore, research has been conducted into directly detecting the attachment of probiotic bacteria when in co-culture with GI tract pathogens. Bernet et al. 1994 showed four different strains of \textit{L. acidophilus} attaching to HT-29-MTX, a mucus secreting subtype of HT-29 and Caco-2, in co-culture with enterotoxogenic and diffusely adhering \textit{E. coli}, as well as \textit{Salmonella} Typhimurium and \textit{Yersinia pseudotuberculosis}. Using radioactive $^{14}$C labelling and scanning electron microscopy they were able to show significant inhibition of cell association of the \textit{E. coli} and invasion of \textit{S. Typhimurium} and \textit{Y pseudotuberculosis}. [111].

When describing the attachment patterns of bacteria to tissue culture cells it is necessary to use the nomenclature for \textit{E. coli} attached to Hep-2 tissue culture cells, as to date there is no work specifically on NEC associated bacteria or probiotic attachment patterns. The three major \textit{E. coli} patterns are: diffuse, localised and entero-aggregative adhesion [90, 112, 113]. Diffuse adherence is seen when the bacteria cover the whole surface or most of the surface of an epithelial cell. Localised adherence has bacteria attached to one or a few sites on the cell surface [114]. Aggregative adherence has a characteristic ‘log-jam’ or ‘stacked-brick’ appearance that occurs on the surface of the cells and continues on the slide surface (if present), and like the other two is most prevalent on Hep-2 cells [115-117].

\textit{In vitro} research into NEC has focused either on the immunological effects of probiotic bacteria in interaction with GI pathogens or examined the gross morphological damage that pathogens can inflict on the GI tract and whether probiotics can mitigate or prevent that damage. However, relatively little research has focused directly on the interaction between putative NEC pathogens and probiotics strains of bacteria that are currently in use in NICU’s around the world. Thus, this study focused on these interactions between the probiotic bacteria \textit{L acidophilus} and \textit{B. infantis} with four different NEC associated bacteria: \textit{C. sakazakii} #50 isolated from powdered infant formula, \textit{C. sakazakii} #2029 isolated from a throat swab, \textit{Enterobacter cloacae} isolated from a confirmed NEC case and \textit{Klebsiella pneumoniae ss. oxytoca} isolated from a suspected case of NEC in the Dunedin public hospital NICU.

\subsection*{1.3 Introduction to Project}
The probiotic Infloran is used in several New Zealand Hospitals for the prevention of NEC. Although its efficacy in human trials has been reported, its exact mechanism of action has not been studied in detail [84, 95]. The NICU at Dunedin public hospital has been using Infloran
since 2011 for any infant born <33 weeks gestation and/or <1,500 g birth weight. From the first feeding the contents of an Infloran capsule are mixed into 3mL of either expressed breast milk or neonatal formula and given to the infant daily [118].

The project reported here, sought to investigate the interactions between this probiotic and Gram negative NEC associated bacteria isolated from infants at the hospital or obtained from the ESR collection. It is believed these bacteria multiply in standing milk formula and in the undigested formula in the bowel of NEC susceptible infants, attach to the epithelial surface exposed by lack of mucus, triggering an immune response. The bacteria then translocate the GI epithelium by intra-cellular or para-cellular routes. Unable to be cleared by the immature immune system, the bacteria begin to multiply in the sub-mucosal tissues producing the characteristic signs and symptoms of NEC [1, 62, 119].

There are a number of possible ways in which the Infloran probiotics could interact with Gram negative NEC associated bacteria. These include but are not limited to:

1) Competition for milk substrates and growth inhibition through the production of bacteriocins and/or lactic acid.

2) Prevention of attachment and therefore invasion through the intracellular or para-cellular routes. Known effects of probiotics which may prevent attachment and invasion including displacement through competition for binding sights and co-aggregation [81, 111, 120, 121]

3) Assisting the maturation of the immune system by inducing tolerance of bacteria by the mucosal tissues and associated immune cells [53, 83].
The hypothesis of this study is as follows: That the probiotic formulation Infloran will be able to inhibit the growth of the NEC associated bacteria and reduce or prevent the adherence and invasion of the same bacteria in the HT-29 adult colorectal tissue culture line.

Pursuant to this the study aims and objectives are to:

1. Identify if the probiotic strains from Infloran are able to inhibit the growth of the NEC strains on solid media.
2. Identify if the probiotic strains produce any anti-bacterial factors in liquid media and whether that media, conditioned or on its own will inhibit the growth of the NEC strains.
3. Observe the gross co-aggregation ability of the probiotic and NEC strains individually and in co-culture.
4. Enumerate NEC strains and the probiotics, individually and in co-culture adhering to HT-29 cells using \(^3\)H-thymidine labelling.
5. Characterise the adhesion patterns of the NEC strains and probiotics following incubation with HT-29 cells using Giemsa staining and microscopy, then characterise the interaction between the strains when in co-culture with the probiotics using Giemsa staining and microscopy in HT-29 cells.
6. Investigate the invasive characteristics of the NEC and probiotic strains for HT-29 cells individually and in co-culture.
7. Grow the probiotics in neonatal milk formula in co-culture with the NEC strains as a model of luminal growth in the infant GI tract and enumerate by the plate count method.
2 Materials and Methods
All experiments were conducted in triplicate with at least 3 replicates per experiment unless otherwise stated.

2.1 Stock Materials and Standard Methods

2.1.1 Stock Solutions
Unless otherwise stated all stock solutions were dissolved in deionised water from a Barnstead, NANOpure Diamond Dispenser (Thermo-Fisher Scientific, MA, USA). Sterilisation was performed by autoclaving at 121°C, 15 minutes. All chemicals were from Sigma-Aldrich (MO, USA) unless otherwise stated.

2.1.1.1 Phosphate Buffered Saline (PBS) 10 X

80 g NaCl
2 g KCl
14.4 g Na₂HPO₄
2.4 g KH₂PO₄
1 L H₂O

To make the 1 X working stock, 100 mL of the 10 X stock was mixed with 900 mL of autoclaved deionised water and passed through a 0.22 µm Millex-FG syringe filter (Merck-Millipore, Darmstadt, Germany).

2.1.1.2 Giemsa Buffer 100 X

29.62 g Na₂HPO₄
36.38 g NaH₂PO₄H₂O
500 mL H₂O

The 100 X working buffer was diluted down with deionised water and the pH adjusted to 6.8 with 1M HCl to get the working 1 X buffer.

2.1.1.3 Trypsin 2 X

8 mL 0.5% Tripsin-EDTA (10 X) (Gibco, Life Technologies, CA, USA)
32 mL PBS 1 X

Stored frozen in 2 mL aliquots at -20°C
2.1.1.4 **Inactivated Bovine Foetal Calf Serum (FCS)**

Gibco’s Foetal Calf Serum (FCS; Life Technologies, CA, USA) was inactivated in 20 mL aliquots by heating at 56°C for 40 minutes in a water bath. The aliquots were then frozen down to -20°C for storage.

2.1.1.5 **Neonatal Formulas**

Nestle Pre-NAN Gold (Vevey, Canton of Vaud, Switzerland) and Pfizer S-26 gold (New York City, NY, USA) were both used in this project. Direct contact was attempted with the manufactures to get the ingredient lists for these formulas, no response was given. A list of ingredients for Pfizer S-26 Gold was acquired from a parenting website and is available in appendix 6.4. It is worth noting this is not confirmed by the manufacturer.

2.1.2 **Antibiotics**

Penicillin 5,000 U/mL-Streptomycin 5,000 µg/mL (Gibco, Life Technologies, CA, USA)

Gentamicin 5,000 µg/mL (Gibco, Life Technologies, CA, USA)

Antibiotic stock solutions were stored at -20°C.

2.1.3 **Bacterial Culture Media**

All media powders were sourced from BD Biosciences (NJ, USA), dissolved in distilled water and autoclaved at 121°C for 15 minutes, as per the manufacturer’s instructions, unless otherwise specified. All agar plates were 85 mm in diameter. Media used in this study are listed in Table 2.

2.1.4 **Bacterial Storage**

All bacterial strains were stored at -80°C in the Microbank advanced bacterial storage system (Pro-Lab Diagnostics, ON, Canada) as per the manufacturer’s instructions. Once frozen, a single bead was removed with sterile forceps, while keeping the micro tube on dry ice. This was inoculated onto a solid medium and incubated to check the resulting culture for purity. Once confirmed as pure, the beads were removed and inoculated onto solid or liquid media as required.
2.1.5 **Drop Plate Counts**

Drop plate counts to assess bacterial numbers were performed using a modification of Miles and Misra method [122]. Ten-fold dilution series were prepared in Falcon 96-well microtitre plates (BD Biosciences, NJ, USA).

Once the dilution series was completed, an agar plate was divided into six even sections. In each section four 10 µL spots were placed for each desired dilution. Thus a range of six dilutions for each sample could be counted on a single plate. The plates were incubated according to the requirements of the inoculated strain.

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Name</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>TSA/B</td>
<td>Triptcase Soy agar/broth</td>
<td>General bacterial propagation</td>
</tr>
<tr>
<td>BHI</td>
<td>Brian Heart Infusion</td>
<td>General bacterial broth propagation</td>
</tr>
<tr>
<td>MRS</td>
<td>deMan, Rogosa and Sharpes</td>
<td>Propagation of anaerobic lactic acid bacteria</td>
</tr>
<tr>
<td>CBA**</td>
<td>Columbia Blood Agar, 5% Sheep Blood</td>
<td>Cultivation of fastidious and non-fastidious bacteria</td>
</tr>
<tr>
<td>MH-II*</td>
<td>Mueller-Hinton II</td>
<td>Growth curves and antagonism assays</td>
</tr>
<tr>
<td>MC</td>
<td>M¢Conkey agar</td>
<td>Selective and differential medium for gram-negative, lactose fermenting enteric species</td>
</tr>
</tbody>
</table>

*MH-II only required 10 minutes at 121˚C for sterilisation

** Plates were sourced from Fort Richard Laboratories (Auckland, New Zealand)
2.1.6 Bacterial Strains

The commercial probiotic Infloran (Swiss Serum and Vaccine Institute Berne, Berne, Switzerland), is currently in use at the NICU in Dunedin Public Hospital, New Zealand for the prevention of NEC in low birth weight infants (<1,500 g) and was kindly provided by Dr Roland Broadbent. The formulation contains *Lactobacillus acidophilus* and *Bifidobacterium longum ss. infantis* with each 250 mg capsule containing $4.5 \times 10^8$ cfu of viable *L. acidophilus* and $1 \times 10^8$ cfu of viable *B. infantis* [118].

Bacterial strains were selected from a collection of Enterobacteriaceae of faecal origin isolated from neonates at the Dunedin Hospital NICU who had confirmed stage II or III NEC or suspected stage I NEC. As *Enterobacter cloacae* and *Klebsiella oxytoca* were the most common isolates from the infants, it was decided to use single, randomly selected isolates of these species as representative NEC associated organisms. Patient details, isolation and identification methods have been previously described [62]. To complement the above, two strains of *Cronobacter sakazakii*, which has been identified as a NEC-associated pathogen in a number of studies was included. *C. sakazakii* associated NEC often originates from powdered infant formula that was prepared incorrectly [123, 124]. As Brooks et al. (2006) had not isolated any *C. sakazakii* strains from during the course of their study, two strains from the New Zealand Reference Culture Collection at Environmental Science & Research (Porirua) were selected; *C. sakazakii #50* from a tin of dried milk and *C. sakazakii #2029* from a throat swab. [62].

An enteropathogenic *Escherichia. coli* O111 was used as a positive attachment control, as it is a known cause of neonatal diarrhoea [125, 126] and has previously been confirmed as a positive attachment control on HT-29 tissue culture cells by our research group. *Citrobacter freundii*, also isolated by Brooks et al. (2006) was selected as a negative control, as it had been previously identified as a non-attaching strain. *S. Typhimurium* was selected as a positive epithelial invasion control as it is a well-known invasive GI pathogen for all age-groups [62, 127].

Bacterial strains used in this study are summarised in Table 3.
### Table 3. All bacterial strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Collection Number/Type</th>
<th>Source</th>
<th>In Text</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cronobacter sakazakii 50</td>
<td>NCTC 8155</td>
<td>New Zealand Reference Culture Collection (NZRM)</td>
<td>C. sakazakii 50</td>
</tr>
<tr>
<td>Cronobacter sakazakii 2029</td>
<td>NCTC 11467</td>
<td>NZRM</td>
<td>C. sakazakii 2029</td>
</tr>
<tr>
<td>Enterobacter cloacae</td>
<td>Clinical Specimen, I1</td>
<td>Dr Brooks and McConnel Culture Collection</td>
<td>E. cloacae</td>
</tr>
<tr>
<td>Klebsella pneumoniae ss. oxytoca</td>
<td>Clinical Specimen, IX2</td>
<td>Dr Brooks and McConnel Culture Collection</td>
<td>KPSS. oxytoca</td>
</tr>
<tr>
<td>Citrobacter freundii</td>
<td>Clinical Specimen, V8</td>
<td>Dr Brooks and McConnel Culture Collection</td>
<td>C. freundii</td>
</tr>
<tr>
<td>Escherichia coli O111</td>
<td>ARCC BAA-2440</td>
<td>University of Otago Microbiology Department Culture Collection</td>
<td>E. coli O111</td>
</tr>
<tr>
<td>Escherichia coli DH5α (K12)</td>
<td>ATCC 10798</td>
<td>Prof John Tagg Culture Collection, C/- BLIS Technologies Ltd</td>
<td>E. coli K12</td>
</tr>
<tr>
<td>Salmonella enterica ss. enterica serovar Typhimurium</td>
<td>CDC 6516-60</td>
<td>University of Otago Microbiology Department Culture Collection</td>
<td>S. Typhimurium</td>
</tr>
<tr>
<td>Lactobacillus casei</td>
<td>ATCC 7469</td>
<td>Prof John Tagg Culture Collection</td>
<td>L. casei</td>
</tr>
<tr>
<td>Lactobacillus acidophilus</td>
<td>Infloran® Formulation</td>
<td>Prof Gerald Tannock Culture Collection</td>
<td>L. acidophilus</td>
</tr>
<tr>
<td>Bifidobacterium longum ss. infantis</td>
<td>Infloran® Formulation</td>
<td>Prof Gerald Tannock Culture Collection</td>
<td>B. infantis</td>
</tr>
</tbody>
</table>
2.1.7  **Bacterial Growth on Solid Media**

Unless otherwise mentioned bacterial strains were grown on solid media as follows:

2.1.7.1  **Aerobic Growth**

The NEC bacteria and controls were inoculated on to BHI agar plates and incubated aerobically at 35°C ± 2° for 18 hours.

2.1.7.2  **Anaerobic Growth**

The probiotic strains and the control *L. casei* were inoculated on to pre-reduced MRS agar plates and incubated anaerobically at 35°C ± 2° for 48 hours.

2.1.8  **Bacterial Growth in Liquid Broth**

Unless otherwise mentioned bacterial strains were grown in liquid broth as follows:

2.1.8.1  **Aerobic Growth**

The NEC strains and controls beads were inoculated into 10 mL universals of BHI broth and incubated with shaking, 200 rpm 35°C ± 2° for 18 hours. (New Brunswick G24 Gyratory Incubator, Eppendof, Hamburg, Germany).

2.1.8.2  **Log Phase Aerobic Growth**

If log phase cultures of the NEC and control strains were needed; 200 µL from overnight cultures as described in 2.1.8.1 was added into 10 mL of fresh BHI and incubated for a further 3 hours at 200 rpm, 35°C ± 2°.

2.1.8.3  **Anaerobic Growth**

The probiotic strains and the control *L. casei* beads were inoculated into 10 mL of pre-reduced MRS broth and incubated anaerobically for 48 hours at 35°C ± 2°.

2.1.8.4  **Log Phase Anaerobic Growth**

If log phase cultures of the anaerobes were required; they were grown as above in 2.1.8.3 but only for 24 hours.
2.1.9 Dilution Curves
All NEC and control strains were grown to stationary phase as described in either 2.1.8.1 or 2.1.8.3. Using PBS, two fold dilution series were performed and optical densities were recorded at 600nm with a Biochrom Novaspec II (Biochrom, Cambridge, UK). Viable plate counts were performed as described in 2.1.5. Using the viable count data optical density was plotted against the total number of viable bacteria for each dilution, thus generating a dilution curve.

2.1.10 M°Farland Opacity Standards
Initially dilution curves were performed to give estimations of bacterial numbers in solution. It was noticed that the optical density for a given cfu in the dilution curves was very similar to the cfu indicated in the M°Farland opacity standards. Thus it was decided to use the M°Farland opacity standards in place of the dilution curve data. Plate counts were performed to confirm this assumption and the data is summarised in Appendix 6.1. It has been previously noted there is up to 0.888 of a log10 variation in drop counts with the Miles and Mistra method at a 99% confidence interval [128].

When a known number of bacteria were required in suspension, cultures were diluted down to the relevant M°Farland opacity standard, shown in Table 4 [129]. The optical density at 600nm was checked in a Biochrom Novaspec II (Biochrom, Cambridge, UK). This method proved to be much faster than doing individual optical densities for each strain and also produced consistent cfu for a given M°Farland opacity standard.

2.1.11 Tissue Culture Media
All tissue culture work was performed with Dulbecco’s Modified Eagle Medium (Gibco, Life Technologies, CA, USA). For normal growth it was supplemented with heat-inactivated FCS at 10% and penicillin-streptomycin at 50 U/mL - 50 µg/mL (Gibco, Life Technologies, CA, USA).

2.1.12 Tissue Culture Cell Storage
Stock tissue culture cells were stored in Nalgene Cryogenic Tubes (Themo-Fisher Scientific, MA, USA), with 1 mL of DMEM supplemented with 10% DMSO and at a concentration of 1 x 10^6 cells/mL. The tubes were kept in vapour phase nitrogen (between -135°C and -190°C).
For resuscitation the tubes were removed from the liquid nitrogen and placed into a 37°C water bath as soon as possible. Once defrosted the contents were rapidly placed into a Falcon 25 cm² tissue culture flask (BD Biosciences, NJ, USA) with 9 mL of pre-warmed DMEM. The cells were incubated for 24 hours at 35°C ± 2°C with 5% CO₂. After 24, hours the medium was replaced to remove the DMSO contamination. The flasks were then treated as specified below (2.1.12).

2.1.13 Tissue Culture Cell Maintenance and Propagation
Tissue culture cells were propagated in Falcon 75 cm² Flasks (BD Biosciences, NJ, USA) containing 10 mL of supplemented DMEM, incubated at 35°C ± 2°C with 5% CO₂ in a humidified atmosphere. The medium was replaced every third day.

At ~80% confluence, the flasks were passaged according to the standard laboratory protocol. Cells were detached with 2 X trypsin and suspended in supplemented DMEM before centrifugation at 244 x g, the cells were re-suspended in fresh supplemented DMEM, stained with Trypan blue and counted using a haemocytometer. Fresh flasks were seeded with 2 mL at 2 x 10⁵ cells/mL plus 8 mL of supplemented DMEM. All tissue culture work was performed in an MSC-Advantage Class II Biosafety Cabinet (Thermo-Fisher Scientific, MA, USA).

2.1.14 Tissue Culture Type
The human tissue culture cell line used in this project was HT-29, which is a colorectal adenocarcinoma from a 44 year old Caucasian female. This cell line was chosen because of its ease of growth, and previous use as a model of the GI tract epithelium in probiotic studies.

In Table 5 there is a summary of the tissue culture cell used, its source and cell type.

2.1.15 Statistical Analyses
All statistics, where applicable were performed in Graphpad Prism 6 for Windows, version 6.03 (Graphpad, CA, USA).
Table 4. M²Farland Opacity Standards used in this study

<table>
<thead>
<tr>
<th>M²Farland Standard</th>
<th>~ CFU/ml</th>
<th>OD 600\text{nm}</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>1-1.5 \times 10^8</td>
<td>0.2</td>
</tr>
<tr>
<td>2.0</td>
<td>6 \times 10^8</td>
<td>0.45</td>
</tr>
<tr>
<td>3.0</td>
<td>9 \times 10^8</td>
<td>0.58</td>
</tr>
</tbody>
</table>

Table 5. Tissue culture types used in this study

<table>
<thead>
<tr>
<th>Designation</th>
<th>ATCC number</th>
<th>Source</th>
<th>Cell Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>HT-29</td>
<td>HTB-38</td>
<td>American Type Culture Collection</td>
<td>Colorectal adenocarcinoma, from a 44 year old Caucasian female. Adherent epithelial colon cell. Ultrastructural features include microvilli and microfilaments.</td>
</tr>
</tbody>
</table>
2.2 Inhibition and Antagonism Assays

2.2.1 Solid Media Antagonism

The purpose of these assays was to elucidate whether the probiotic strains produced any factors that could prevent the growth of the NEC and control strains on solid media.

2.2.1.1 Probiotic Supernatant Conditioned Media Preparation

As *L. acidophilus* and *B. infantis* were unable to be grown on MH-II agar, TSA or BHI agar and the NEC strains would not grow well on MRS (3 plus days to get colonies), supernatant from the log phase cultures of these anaerobes was used for all the experiments according to the following protocol. Separate universal bottles containing 10 mL of pre-reduced MRS media were inoculated with *L. acidophilus* and *B. infantis* were grown to log phase, as described in 2.1.8.4. At the end of the incubation the universals were removed and placed in 50 mL Falcon tubes and centrifuged in an Eppendorf 5804 Falcon centrifuge (Eppendof, Hamburg, Germany) at 3,220 x g for 10 minutes. The supernatant was collected and passed through a 0.22 µm syringe filter to remove any residual bacteria. The conditioned media were either used right away or stored for up to a week in a 4°C cold room.

2.2.1.2 Spot Antagonism Assay

Overnight broth cultures of the NEC strains and controls as specified in 2.1.8.1, were diluted down to McFarland 0.5 opacity standard and 100 µL of each NEC strain was spread on to MH-II agar and CBA plates, and allowed to dry. Once dry, two 20 µL spots of *L. acidophilus* and two 20 µL spots of *B. infantis* conditioned media (2.3.1.1) were evenly placed on each plate and allowed to dry. As a negative control 20 µL spots of MRS media (untreated) were spotted on to separate spread plates. A positive control was not used. Plates were then incubated aerobically at 35°C ± 2° for 18 hours.

2.2.1.3 ‘P-Typing’ Assays

This was an adaption of an established P-typing method [130]. Using both MH-II agar and CBA plates, a strip of 100 µL of probiotic supernatant was carefully pipetted along the centre line of the plates and allowed to dry. Overnight broth cultures of the NEC strains and controls (2.1.8.1) were diluted down to a McFarland 0.5 opacity standard with PBS. Using sterile swabs, right angled stripes of each strain was inoculated across the supernatant (2.3.1.1) strip. The plates were incubated overnight aerobically at 35°C ± 2°.
2.2.1.4 Soft Agar Overlays
Log phase broth cultures of the probiotic strains as described in 2.1.8.4, were diluted down to McFarland 0.5 with PBS and 100 µL of each strain were spread on to a pre-reduced MRS plate and allowed to dry. Overnight broth cultures of the NEC strains and controls (2.1.8.1) were diluted down to a McFarland 0.5 opacity standard with PBS and 100 µL were added to bijoux bottles containing 2 mL of molten (46°C) soft MH-II (1% agar). The molten agars were overlaid on to the MRS plates and allowed to set. An overlay of each NEC strain and control was applied to separate L. acidophilus and B. infantis seeded plates in triplicate. The plates were then incubated anaerobically for 24 hours at 35°C ± 2° in the anaerobic chamber.

2.2.1.5 Soaked Plate Antagonism Assay
This was essentially the reverse of the spot antagonism assays. Well dried MH-II agar plates had 1 mL of supernatant (2.3.1.1) evenly spread on them and were allowed to dry. The plates were then divided up into four even sections, to each one of these sections a 20 µL spot of bacterial suspension at 1 x 10⁸ cfu/mL of the NEC strain or one of the controls was added and the plates allowed to dry. Plates with 1 mL of unconditioned MRS were also inoculated with the same strains as above as a control. The plates were then incubated aerobically overnight at 35°C ± 2°.

2.2.1.6 Probiotic Supernatant Supplemented Plate Antagonism Assay
Double strength MH-II agar was autoclaved and allowed to cool to 46°C in a water bath. An equal amount of each probiotic supernatant was added to two 50 mL aliquots of the MH-II agar and briefly stirred. The plates were then poured and dried as per usual. Once set, the plates were divided into four separate quarters. Overnight cultures of the NEC and control strains (2.1.8.1) were diluted to a McFarland 0.5 opacity standard with PBS. To each quadrant, 20 µL of diluted NEC strain or control was pipetted and allowed to dry. The plates were then incubated overnight aerobically at 35°C ± 2°.

At the conclusion of the experiment all plates were checked for pH with Macherey Nagel TRITEST pH strips (Düren, Germany).
2.2.2 Co-Aggregation Assay

The purpose of these assays was to observe if the bacterial strains could aggregate with each other in PBS and whether or not visible clumps would form.

Log phase broth cultures of the probiotics, *L. acidophilus*, *B. infantis* and co-aggregation control *L. casei* (2.1.8.4), were diluted down to a McFarland 3.0 opacity standard with PBS. Log phase broth cultures of the NEC and control strains (2.1.8.1) were similarly treated. Using a Falcon 24 well flat bottomed plate (BD Biosciences, NJ, USA) 500 µL of each probiotic was mixed with 500 µL of each NEC and control strain. To detect auto-aggregation, each strain was mixed with 500 µL of PBS. A mix of the *E. coli* O111 with *L. casei* was the positive control. Finally 10 µL of 1% Triphenyl tetrazolium chloride (TTC) was added to each well to indicate active cellular metabolism. The plate was then incubated aerobically on a 35°C ± 2° gyratory incubator set at 100 rpm for four hours. The plates were then removed and observed both macro and microscopically and scored according to Table 6. This scoring system was based on an internal scoring method used by the authors’ research group. This experiment was purely observational and as such no statistics could be applied.
<table>
<thead>
<tr>
<th>Score</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No aggregation</td>
</tr>
<tr>
<td>1</td>
<td>Small aggregates with small clusters of bacteria, only visible with a microscope</td>
</tr>
<tr>
<td>2</td>
<td>Aggregates with large numbers of bacteria, may settle to bottom of well, only visible with microscope</td>
</tr>
<tr>
<td>3</td>
<td>Macroscopically visible clumps of bacteria that may settle to bottom of well</td>
</tr>
<tr>
<td>4</td>
<td>Large macroscopically visible clumps of bacteria that may have settled to bottom of well</td>
</tr>
<tr>
<td>5</td>
<td>A single small clump of bacteria with a gelatinous consistency ≤ 5 mm</td>
</tr>
<tr>
<td>6</td>
<td>A very large clump of bacteria with a gelatinous consistency 6 mm +</td>
</tr>
</tbody>
</table>
2.3 Broth Antagonism Assay

The broth antagonism assays were performed to investigate whether or not supernatant from the two probiotic strains could prevent or slow down the growth of the NEC and control strains in liquid media.

2.3.1.1 Probiotic Supernatant

Overnight broth cultures of the NEC strains and controls (2.1.8.1) were diluted down to a McFarland 0.5 opacity standard in PBS and centrifuged at 3,220 x g for 10 minutes. They were then re-suspended in double strength MH-II broth. For each strain, 50 µL of bacterial suspension were pipetted into each well in each row of a 96-well plate, one row for each strain and leaving the last 2 wells empty. The supernatants from the probiotics prepared as described in 2.3.1.1, were diluted down with MRS in a doubling dilution series to 1/16. Then along each row 50 µL duplicates of each dilution were added down to 1/16. In the last two wells were the positive and negative controls: The positive growth control was the strain from the respective row mixed with 50 µL of MRS broth. This was also a duplicate control for the presence of MRS as it was known to be deleterious to the growth of non-lactic acid bacteria. The negative control, to test for sterility, was 50 µL MH-II broth mixed with 50 µL MRS broth.

The 96-well plate was then placed in a TECAN Infinite M1000 Plate reader (Tecan Group, Männedorf, Switzerland), which was set to incubate at 35°C ± 2° with orbital clockwise shaking for 12 hours and read absorbance at OD_{600nm} every half hour using the iContol software (Tecan Group, Männedorf, Switzerland).

2.3.1.2 Lactic Acid supplemented Broth Antagonism Experiment

During the course of the broth antagonism experiments it was theorised that the primary antagonistic agent was the lactic acid produced by the probiotics. Thus this set of experiments was performed to test this theory.

The probiotic supernatant of both *L. acidophilus* and *B. infantis* had their pH read via pH meter (MP220, Mettler Toledo, OH USA) each time a new batch was extracted and found to average to ~4.5. As the lactic acid (LA) stock we had was only pH 5.0 thus we could only
bring the MRS pH down to that level. Thus the LA was titrated into the MRS until it had reached that pH. This amount was recorded and the experiment was performed as above.

If the lactic acid concentration had proved not to be inhibitory at 5.0, a more concentrated solution at pH 4.5 or lower of Lactic acid would have been sourced, this was not required. A difference of half a pH point was not considered to be a significant by the investigator.

2.4 Attachment Assays with Tritiated Thymidine

This assay is based on work by Aathithan et al. (2001), utilising radioactive thymidine to estimate attachment of multiple antibiotic resistant strains of *Staphylococcus aureus* to Hep-2 cells. [131]

When bacteria are incubated in the presence of $^3$H-Thymidine they will incorporate it into their cell structure [132, 133]. Thus, when $^3$H-Thymidine labelled bacteria are attached to HT-29 cells the counts per minute can be quantified and related back to the initial inoculum, to give an estimation of the number of attached bacteria. By comparing the count obtained for the original inoculum, the proportion of bacteria attached can be calculated.

2.4.1 Optimal Attachment Time

To work out the optimal attachment time, separated 96-well plates of HT-29 were inoculated with $^3$H-Thymidine labelled NEC, control and probiotic strains and incubated for 2, 3 and 4 hours. Unattached bacteria were then removed by washing. Following trypsinistaion, HT-29 cells were harvested onto a filter mat and subjected to scintillation counting.

2.4.1.1 Preparation of bacterial inoculum

All NEC strains, controls and the probiotics were inoculated separately tubes containing 2.5 mL of BHI broth supplemented with 25 µL of $^3$H-Thymidine (PerkinElmer, MA, USA) and incubated aerobically for 18 hours at 35°C ± 2°C with shaking at 200 rpm. The bacterial strains were then diluted down to a McFarland 0.5 opacity standard and centrifuged at 3,220 x g for 10 minutes. The supernatant was removed and the pelleted bacteria were resuspended in un-supplemented DMEM and placed in a 35°C ± 2°C incubator to gently warm them.
2.4.1.2 Preparation of HT-29 cells

A flask of HT-29 cells was trypsinised as described in 2.1.13 and four 96-well plates were seeded with 100 µL of 3 x 10^5 cells/mL per well. The outer ring of wells was not seeded and instead had 100 µL per well of PBS added. The plates were then placed in a 35°C ± 2° with 5% CO₂ incubator for 24 hours.

The following morning the plates were checked to make sure the cells were ≥ 60% confluent.

2.4.1.3 Attachment experiment

The DMEM medium was then removed from the HT-29 cells and wells were washed the times with pre-warmed PBS. In each plate, five replicates of each strain were added in rows, with 100 µL in each well. The remaining wells had 100µL of unlabelled bacteria added to two of them and unsupplemented DMEM in the other three. The plates were then incubated for 2, 3 and 4 hours at 35°C ±2° and 5% CO₂. During this time the left over bacteria were kept on ice for the duration of the experiment to enable scintillation counting of the original amount added.

At the end of each incubation, the plate was removed and the wells washed with PBS as above. Pre-warmed 2 X Trypsin, (100 µL) was added to each treatment well and incubated for 90 minutes at 35°C ± 2° with 5% CO₂. Following this, 100 µL in triplicate of the undiluted labelled bacteria were added to unused wells to give a value for the starting inoculum. (from the remaining bacteria on ice). The plate was then harvested on to a printed filter mat with a Tomtec MACH III manual cell harvester (CT, USA) according to the manufacturer’s instructions. The filter mat was then prepared for scintillation counting in the Wallac 1450 MicroBeta PLUS (PerkinElmer, MA, USA), described in method (2.4.3).
2.4.2 Co-culture Attachment
HT-29 cells were prepared as described in 2.1.4.2.

2.4.2.1 Preparation of bacterial inoculum
*L. acidophilus* and *B. infantis* were inoculated into two 2.5 mL universals of pre-reduced MRS broth with 25 µL of $^3$H-Thymidine. In addition two 10 mL universals of pre-reduced MRS were inoculated with the probiotics and no $^3$H-Thymidine. These were incubated at 35°C ± 2˚ anearobically for 24 hours. The NEC strains and control strains were inoculated into 5 mL universals of BHI broth with 50 µL of $^3$H-Thymidine. These were incubated for 18 hours at 35°C ± 2˚, 200 rpm aerobically.

The bacteria were then diluted down to a McFarland 0.5 opacity standard in a total volume of 10 mL, with the exception of the $^3$H-Thymidine treated probiotics, where only 5 mL was required. All strains were then centrifuged at 3,220 x g for 10 minutes, re-suspended in 5 mL of un-supplemented DMEM and placed in a 35°C ± 2˚ incubator to gently warm.

2.4.2.2 Preparation of HT-29 cells
HT-29 cells were prepared as described in 2.4.1.2

2.4.2.3 Co-culture attachment experiment
Each NEC and control was added in replicates of five (50µL per well) to the HT-29 cells in the 96-well plates. To this 50 µL of the non $^3$H-Thymidine treated probiotic was added, *L. acidophilus* to one set of plates and *B. infantis* another. The remaining wells had 100µL of unlabelled bacteria added to two of them and unsupplemented DMEM in the other three. Plates were then incubated for 3 hours aerobically at 35°C ± 2˚ with 5% CO$_2$. During the incubation the remainder of $^3$H-Thymidine treated bacterial strains were placed on ice.

At the end of the incubation the plates were removed and washed with PBS and trypsinsed as described in section 2.4.1.3. At the end of the incubation 50 µL of the left over treated bacterial strains were added in triplicate to the unused wells on the 96-well plates, with the exception of the probiotic strains where 100 µL was added. The plates were then harvested and prepared for scintillation counting, as described in 2.4.1.3.
2.4.3 Scintillation Counting

The printed filter mat was placed in a sample bag and sealed at the long end. The short end was then carefully cut off and 4.5 mL of BetaPlate Scint Fluid (PerkinElmer, MA USA) was added to the sample bag. Using a roller the fluid was evenly distributed over the mat, taking care to remove any bubbles. The short end of the sample bag was then sealed and the placed into a counting cassette for the Wallac 1450 MicroBeta PLUS, trimming the plastic as required. The cassette(s) were then loaded into the reader and the inbuilt ‘Thymidine’ protocol was used to measure the plates, outputting the results as counts per minute (CPM). The results were expressed as percentage of initial amount of bacteria added to each well, using the equation below.

\[
% \text{ attached} = \frac{\text{CPM of treatment well}}{\text{CPM of initial bacteria added}} \times 100
\]
2.5 Attachment Assays with Giemsa Staining
As the $^3$H-Thymidine assay proved highly variable and therefore unreliable, Giemsa staining was used to visualise the attachment of the NEC strains and probiotics directly. Giemsa was ideal as it stained bacteria and eukaryotic cells different colours when buffered to the correct pH.

2.5.1 Single Strain Attachment

2.5.1.1 Preparation of bacterial inoculum
The probiotic bacteria *L. acidophilus* and *B. infantis* were grown to log phase as described in 2.1.8.4.

The NEC and attachment control strains were grown to log phase as described in 2.1.8.2.

Once at log phase all strains were diluted down to McFarland 0.5 opacity standard, centrifuged for 10 minutes at 3,220 x g, washed with PBS, resuspended in unsupplemented DMEM and placed in a 35°C ±2˚ incubator to gently warm.

2.5.1.2 Preparation of HT-29 cells
A flask of HT-29 cells was trypsinised as described in 2.1.13 and seeded into Nunc 4-well chamber slides (Nalge Nunc, NY, USA) with 1 mL per well at 3 x $10^5$ cell/ml. The slides were then placed in a 35°C ± 2˚ with 5% CO$_2$ incubator for 24 hours.

Following the 24 hour incubation the chamber slides were checked to see if they were ≥ 60% confluence.

2.5.1.3 Single strain attachment experiment
The chamber slide wells were washed with pre-warmed PBS. For each bacterial strain 1 mL was added to three wells on the chamber slide with 1 mL of unsupplemented DMEM only added to the final well. The slides were then incubated at 35°C ± 2˚, with 5% CO$_2$ for 3 hours.

At the end of the incubation, the slides were washed, the HT-29 cells and bacteria fixed with 100% methanol, the well constructs and gaskets were removed, and the slides left to air dry. Once dry the slides were stained with Giemsa overnight as described in 2.5.3.
At the end of the incubation, the slides were carefully removed, gently rinsed in PBS and then rinsed in deionised water. They were left on the bench to air dry. Once dry, microscopic images were taken of the slides with an Olympus BX-51 microscope (see 2.5.4)

2.5.2 Co-Culture Attachment
The chamber slides containing HT-29 cells, and bacterial strains were prepared as previously described in single strain attachment (2.5.1.2 and 2.5.1.3.)

2.5.2.1 Co-culture attachment experiment
The chamber slides were washed with pre-warmed PBS. The bacteria were then added to the slides as follows: 500 µL of the NEC or control strain in the top three wells and 1 mL of DMEM in the remaining well. To two wells 500 µL of one of the probiotic strains were added, gently pipetting up and down to make sure they were evenly mixed with the NEC associated strain or control, in the third well 500 µL of DMEM was added to give a NEC or control only field. The slides were then incubated at 35°C ± 2°, with 5% CO₂ for three hours.

At the end of the incubation, the chamber slides were treated as described in section 2.5.1.3.

2.5.2.2 Co-culture attachment with both Infloran strains
Experiments were performed using a combination of the probiotics, except that when adding the bacteria to the slides they were added in 333 µL amounts in the following ratio; 1 NEC/Control : 1 L. acidophilus : 1 B. infantis. All strains were added simultaneously and gently pipetted up and down to ensure an even mixture.

2.5.3 Giemsa stain preparation and staining
To make the Giemsa stain, Pre-made Giemsa Solution (Sigma-Aldrich MO, USA) and 1 X Giemsa buffer were mixed in a 1:200 ratio. This solution was used fresh in falcon tubes (50 mL) filled with 45 mL of the stain and two slides added to each tube back to back. These were then incubated at room temperature in the dark for 18 hours.
2.5.4 **Olympus BX-51 Microscope**

For every Giemsa stained slide, images were taken on an Olympus BX-51 Microscope (Olympus, Tokyo, Japan) with an Olympus DP70 camera attached. Using the 100 X oil immersion lens, 5 or more representative fields were taken for each section of the slide. The pictures were 4,080 x 3,072 resolution and in an uncompressed tagged image file (.tif) format.

2.5.5 **Counting of Bacteria Using ImageJ**

To assist with quantification of attached bacteria the freeware ImageJ (sourced from [http://rsbweb.nih.gov/ij/](http://rsbweb.nih.gov/ij/), National Institutes of Health, MD, USA) was used. From the drop down ‘Plugins’ menu: ‘Analyse’ and then ‘Cell Counter’ was selected. This was used to manually designate the HT-29 cells and the different types of bacteria with different coloured dots by the investigator and thus quantify the numbers attached bacteria to 50 HT-29 cells. While doing this, notes were taken about the observed attachment patterns of the different NEC bacteria and probiotic strains.
2.6 Invasion Assays

Invasion assays were performed to compare the ability of the NEC stains and probiotics to invade the HT-29 cells via the intracellular route. *S. Typhimurium*, a human intestinal pathogen known to invade intestinal cells, was used as a positive control. *E. coli* K12 was used as a negative control due to it being a well characterised lab strain unable to invade gut epithelial cells. The effect of co-culturing the probiotics and NEC strains was investigated to observe if the interaction would affect their invasive ability.

NB. For all invasion assays the limit of detection was $5 \times 10^2$ cfu/mL

2.6.1 Optimal Invasion Time and MOI

The purpose of these experiments was to optimise the attachment time and number of bacteria required for the positive invasion control *S. Typhimurium* and check the negative control *E. coli* K12.

2.6.1.1 Preparation of HT-29 cells

A flask of HT-29 cells was trypsinised as described in 2.1.13 and seeded into three 24-well plates with 1 mL per well at a concentration of $3 \times 10^5$ cell/ml. They were then incubated at 35°C±2˚ + 5 % CO₂ for 24 hours.

The following morning the plates were checked to confirm the HT-29 cells were at $\geq 60\%$.

2.6.1.2 Preparation of Bacterial Inoculum

The controls: *Salmonella* Typhimurium and *E. coli* K12 grown to log phase as described in 2.1.8.1.

At the end of the incubation period the bacteria were diluted down to McFarland 0.5 opacity standard and centrifuged at 3,220 x g for 10 minutes. The pallets were then re-suspended at room temperature in unsupplemented DMEM and 10 fold dilutions, from $10^0$ – $10^{-2}$ were performed. The bacterial suspensions were all placed in a 35°C±2˚ incubator to gently warm.
2.6.1.3 Invasion time and MOI experiment
The 24-well HT-29 plates were washed in pre warmed PBS. For each bacterial strain 200 µL of diluted or undiluted bacterial suspension was added in triplicate to each plate, the undiluted strains were labelled as an MOI of 1:100 (1 HT-29 cell to 100 bacteria) and the dilute strains MOI of 1:10 and 1:1 respectively. The plates were then incubated for 2, 3 and 4 hours aerobically at 35°C ± 2˚, with 5% CO₂.

At the end of each time point, the plate was washed with pre-warmed PBS. Then 200 µL of Gentamicin at 200 µg/mL were added to each well to kill external bacteria, and the plate incubated for a further two hours aerobically at 35°C ± 2˚, with 5% CO₂. A separate 24 well plate had 200 µL of each bacterial strain added in duplicate and 200 µL of the gentamicin added to control for bacterial sensitivity to the antibiotic.

After the antibiotic incubation the treatment plates were washed again as above and then 200 µL of 1% Triton X-100 was added to each well to lyse the HT-29 cells and they were incubated on the bench for ~30 minutes. The wells were checked for cell lysis, and if lysed, the contents of each well was pipetted up and down twice, to ensure an even mix and the 200 µL of the lysed cells was removed into a 96-well plate for dilution. Dilution plating and estimation of bacterial numbers was performed as previously described in 2.1.5. The drop plates were incubated as described in 2.1.7.

2.6.2 Single Strain Invasion
These experiments were conducted with the same method as 2.6.1, with the following modifications. The incubation time was set at 3 hours, MOI’s of 100:1 and 10:1 were used. The strains tested were the NEC strains, the Infloran probiotic strains as well as the positive invasion control S. Typhimurium and the negative invasion control E. coli K12.

2.6.3 Co-Culture Invasion
The methods for co-culture invasion were the same as the single strain invasion (2.6.2), except for: The MOI being set at 10:1 in all wells, and in the co-cultured wells probiotics and test strains were added in a 1 B. infantis: 1 L. acidophilus: 1 NEC or control strain ratio.
2.7 Neonatal Formula Growth Experiments

These assays were performed with the intent of creating a model similar to that which may occur in the infantile gut in the presence of undigested neonatal formula, the probiotic strains and NEC strains and how this would affect the NEC strains growth. At the point where these experiments were proposed and performed, it was very late in the project. As such these experiments should only be regarded as preliminary.

2.7.1 Co-culture Growth Curve in Neonatal Formula

2.7.1.1 Preparation of bacterial inoculum

The NEC strains and probiotic strains were inoculated and grown to stationary phase growth as described in 2.1.8.

Once their incubations were finished the NEC and probiotic strains were diluted down to McFarland 0.5 opacity standard to a total volume of 5 mL for the NEC strains and 10mL for the probiotic strains. They were centrifuged at 3,220 x g for 10 minutes, the supernatant carefully removed and the pallets re-suspended in 5 mL of PBS.

2.7.1.2 Growth curve in neonatal formula experiment

Universals with 5 mL of neonatal formula were prepared. In quadruplicate 50 µL of each NEC strain was added to get a final concentration of $1 \times 10^6$ cfu/mL (total of 16 universals). To eight of these universals 25 µL of each probiotic was added to give a final concentration of $1 \times 10^6$ cfu/ml. A 100 µL sample was taken from each universal, diluted to $10^{-9}$ and plated for estimation of starting bacterial numbers as described in 2.1.5

The universals were then incubated aerobically with shaking for 12 hours at 35°C ± 2°, 200 rpm. Further samples were taken as described above at 3, 6, 9 and 12 hours.

Two experiments were then undertaken, once as above with the ratio of NEC to probiotic strains 1:1 and a second with the probiotics concentrated to give a ratio of NEC to probiotic strains of 1:10.
2.7.2 Probiotic pre-incubation in neonatal formula

This protocol was designed to more accurately model the situation in the infant gut by performing the experiment in anaerobic conditions, and having a pre-incubation step with the probiotic strains to model the adding of Infloran from the very first feeding of the infant.

2.7.2.1 Preparation of bacterial inoculum

The probiotic strains were incubated to stationary phase growth as described in 2.1.8.3. The NEC strains were also incubated to stationary growth as described in 2.1.8.1.

Following the incubation the probiotic strains were diluted down to McFarland 2.0 opacity standard with PBS to a total volume of 30 mL. They were centrifuged at 3,220 x g for 10 minutes, and resuspended in 15 mL of pre-reduced neonatal formula to give a final concentration of ~1 x 10^9 cfu/ml.

The NEC strains were diluted down to McFarland 0.5 opacity standard with PBS. Then centrifuged at 3,220 x g for 10 minutes, resuspended and kept in the cold room (~4˚C) until required.

2.7.2.2 Pre-incubation in neonatal formula experiment

Three 24-well plates had 1 mL of each probiotic added to each of four wells, and topped up with pre reduced formula to give a total volume of 3 mL. Four more wells had 3 mL of pre reduced formula added to them. The plates were then incubated anaerobically for 3 hours at 35˚C ± 2˚.

After the 3 hour incubation 30 µL of each NEC strain was added in duplicate, 30 µL in the probiotic treated well and 30 µL in the formula only well. The plate was incubated for a further 18 hours in the same conditions. The above was repeated for the other two plates at 6 and 12 hour time points, followed by each being incubated for a further 18 hours.

As each 24-well plate finished its incubation, samples were taken from each well. The samples were then diluted in a 10 fold dilution series with 10^-6-10^-9 plated out on MRS and TSA for estimation of bacterial numbers, as described in 2.1.7.

This experiment was repeated three times with neonatal formulas. Pfizer S-26 Gold (New York, NY) for term infants and twice with Nestle PreNAN Gold (Vevey, Canton of Vaud,
Switzerland) for pre-term infants. In the final experiment the highest dilution was increased to $10^{11}$ cfu/mL.
3 Results

3.1 Solid Media Antagonism Assays

The results of all of these experiments are summarised in Table 7.

3.1.1 Spot Antagonism

Spot antagonism assays were negative for inhibition for all NEC and control strains with both B. infantis and L. acidophilus supernatants, regardless of whether the strains were grown on MH-II agar or CBA.

3.1.2 ‘P-Typing’ Assays

As with the spot antagonism assays there was no discernible reduction in growth with any of the tested strains, regardless of being grown on MH-II agar or CBA.

3.1.3 Soft Agar Overlays

There was no noticeable reduction in growth in any of the NEC or control strains when overlaid onto MRS inoculated with L. acidophilus or B. infantis. Notably the presence of the NEC and control strains did not impede the growth of the probiotic strains.

3.1.4 Soaked Plate Antagonism

No plate quadrants treated with probiotic supernatants showed any reduction in growth in either the NEC or control strains.

3.1.5 Probiotic Supernatant Supplemented Plate Antagonism Assays

Of all the solid media antagonism assays this was the only method that showed reduction in growth of the NEC and control strains. No strain showed any growth at all in either the L. acidophilus or B. infantis supernatants, but all strains grew normally on the MRS only control plates. The pH of the plate surface of both probiotic supernatant plates was 4-5, while the control MRS only plates had a pH of 6-7.
Table 7. Summary of solid media antagonism experiments for the detection of inhibitory activity in probiotic culture supernatants against NEC associated bacteria and control strains.

<table>
<thead>
<tr>
<th>Assay</th>
<th>C. sakazakii 50</th>
<th>C. sakazakii 2029</th>
<th>E. cloacae</th>
<th>K. pneumoniae ss. oxytoca</th>
<th>E. coli O111</th>
<th>C. freundii</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spot antagonism</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>‘P-Typing’</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Soft agar overlay</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Soaked plate antagonism</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Supernatant supplemented plate antagonism</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
</tbody>
</table>

Key:  
- Growth (No inhibition)  
+ Small amount of growth inhibition  
++ Complete inhibition of growth
3.2 Co-Aggregation Assays

The purpose of the co-aggregation assays was to assess the ability of all the NEC and control strains to self-aggregate and then compare this to their ability to aggregate with the one or both of the probiotic strains. These data are summarised in Table 8.

3.2.1 Auto-aggregation

Of the NEC strains only *K. pneumoniae* ss. *oxytoca* showed any observable auto-aggregation with microscopically observable clusters of bacteria settling to the bottom of the well. The positive attachment control *E. coli* O111 also had microscopically observable aggregation.

Positive aggregation control *L. casei* demonstrated macroscopically observable auto-aggregation and the probiotic strains showed a very high ability to auto-aggregate with both *B. infantis* and *L. acidophilus* showing single large clumps ≤5 mm.

3.2.2 Aggregation with *Lactobacillus acidophilus*

The positive NEC strains had a strong ability to aggregate with *L. acidophilus* with *C. sakazakii* 50, 2029 and *E. cloacae* observed to have single large clumps ≤5 mm. *K. pneumoniae* ss. *oxytoca* did not aggregate as well, with large macroscopically visible clumps settling to the bottom of the well.

The control strains were somewhat variable with; *E. coli* O111 forming large macroscopically visible clumps settling to the bottom of the well, a single large clump ≤5 mm. was observed for *C. freundii*. *S. Typhimurium* was observed with less aggregation, with clusters of aggregates settling to the bottom of the well when observed with a microscope.

3.2.3 Aggregation with *Bifdobacterium infantis*

As with the *L. acidophilus* three NEC strains *C. sakazakii* 50, 2029 and *E. cloacae* were observed as having single large clumps ≤5 mm. *K. pneumoniae* ss. *oxytoca* scored lower with macroscopically visible clumps settling to the bottom of the well.

The control strain *E. coli* O111 had a large clump of bacteria ≤5 mm that settled to the bottom of the well and *C. freundii* had microscopically visible clumps at the bottom of the well. *S.
Typhimurium was observed as having a small amount of aggregation with large numbers of microscopically visible clumps settling to the bottom of the well.

3.2.4 Aggregation with Mixed Probiotics
All strains showed significant aggregation with the mixed probiotics as all were scored with very large clumps of bacteria $\geq 6$ mm with gelatinous consistency.

Also the probiotics aggregated with each other with large clumps of bacteria $\geq 6$ mm with gelatinous consistency.

3.2.5 L. casei Control
The L. casei control behaved as expected when co-aggregating with the E. coli O111, a single large clump $\leq 5$ mm was consistently observed.

3.2.6 Triphenyl Tetrazolium Chloride (TTC)
As TTC is only cleaved by actively metabolising cells to give a red substrate [134], it was a good indicator as to whether the bacteria were still actively metabolising while aggregated with the probiotics. In all combinations, the NEC and control bacteria appeared to be actively metabolising, although the red colour did not look as strong when the strains were mixed with both probiotics. The probiotics strains showed no red colour when self-aggregated or mixed with each other. It was not possible to quantify the colour metric change from the TTC as the non-homogeneous nature of the particulate matter from the aggregates made optical density readings unreliable.
Table 8. Auto- and co-aggregation scores from probiotic bacteria, NEC-associated and control strains

<table>
<thead>
<tr>
<th></th>
<th>Self (PBS)</th>
<th>L. acidophilus</th>
<th>B. infantis</th>
<th>Mixed Probiotic</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. sakazakii 50</td>
<td>0</td>
<td>5</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>C. sakazakii 2029</td>
<td>0</td>
<td>5</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>E. cloacae</td>
<td>0</td>
<td>5</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>K. pneumoniae ss oxytoca</td>
<td>2</td>
<td>4</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>Escherichia coli O111</td>
<td>2</td>
<td>4</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>Citrobacter freundii</td>
<td>0</td>
<td>5</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>Salmonella Typhimurium</td>
<td>0</td>
<td>2</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>Lactobacillus acidophilus</td>
<td>5</td>
<td>n/a</td>
<td>6</td>
<td>n/a</td>
</tr>
<tr>
<td>Bifidobacterium infantis</td>
<td>5</td>
<td>6</td>
<td>n/a</td>
<td>n/a</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Self (PBS)</th>
<th>E.coli O111</th>
<th>(Positive Control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactobacillus casei</td>
<td>5</td>
<td>4</td>
<td></td>
</tr>
</tbody>
</table>

Key

0  No Aggregation
1  Small aggregates with small clusters of bacteria, only visible with a Microscope
2  Aggregates with large numbers of bacteria, may settle to bottom of well, only visible with microscope
3  Macroscopically Visible clumps of bacteria that may settle to bottom of well
4  Large macroscopically visible clumps of bacteria that may have settled to bottom of well
5  A single Clump of bacteria with a gelatinous consistency, ≤5 mm
6  A very large clump of bacteria with a gelatinous consistency, 6 mm +
3.3 Broth Antagonism Assays

To complement the solid media antagonism assays, broth antagonism was also performed, once again using supernatant from the probiotic bacteria as their growth was not supported by MH-II media.

Two-way ANOVA in the form of the Tukey's multiple comparisons test with a 95% confidence interval was used to assess the statistical significance of the results.

3.3.1 Lactobacillus acidophilus Supernatant

For all strains tested the positive growth control was observed to have a normal growth curve for 12 hour incubation with a starting inoculum of 1 x 10^8 cfu/mL.

3.3.1.1 Cronobacter sakazakii 50

After 5.5 hours incubation, the optical density readings for wells containing 1/8 and 1/16 dilutions of the probiotic supernatant were significantly higher compared to the undiluted supernatant/negative control wells giving p-values of 0.016 and 0.0170 respectively. At 6.5 hours the 1/4 dilution gave significantly higher readings compared with the undiluted supernatant/negative controls with a P-value of 0.041. By the end point of the experiments the dilutions 1/4 - 1/16 were all significantly different from the undiluted supernatant/negative control wells with P-values of <0.0001 (Figure 2.A).

3.3.1.2 Cronobacter sakazakii 2029

Significantly higher optical density readings compared to the undiluted supernatant/negative control were obtained for the 1/8 and 1/16 dilutions of the probiotic supernatant at 5.5 hours (P=0.01), the 1/4 dilution at 6.5 hours (P=0.01). At 12 hours, all dilutions from 1/4 to 1/16 had significantly higher optical density readings that the undiluted supernatant/negative control (P=< 0.0001) (Figure 2.B).

3.3.1.3 Enterobacter cloacae

At the 5 hour time point, the 1/8 and 1/16 dilutions gave significantly higher optical density readings than the undiluted supernatant/ negative control (P=< 0.05). The 1/4 dilution at 6.5 hours also had a higher reading (P=0.026). At 12 hours all dilutions from 1/4 to 1/16 were significantly higher than the undiluted supernatant/negative control (P= <0.0001). (Figure 2.C).
Strains were grown in MRS broth with the addition of two-fold serial dilutions of *L. acidophilus* supernatant. Optical density readings performed over a 12 hour incubation period indicated inhibition of growth of the test strains (equivalent to the negative control) by undiluted *L. acidophilus* supernatant. Dilution of the *L. acidophilus* supernatant diminished its inhibitory activity for the test bacterial strains as indicated by a trend towards increased optical density over time which reached statistical significance for certain time point/dilution combinations.

Figure 2. Broth antagonism assays for *C. sakazakii* 50 (A), *C. sakazakii* 2029 (B) and *E. cloacae* (C).
3.3.1.4 *Klebsiella pneumoniae ss. oxytoca*

Undiluted supernatant had showed no significant growth with *Klebsiella pneumoniae ss. oxytoca*, although there was some noticeable growth from the 7.5 hour point. By the 7.5 hour time point the OD was significantly higher than the undiluted supernatant/negative control for the 1/4 to 1/16 dilutions (P=< 0.038) (Figure 3.A).

3.3.1.5 *Escherichia coli* O111

At 4.5 hours, the 1/4 to 1/16 dilutions were all significantly higher than the undiluted supernatant with regard to optical density (P=< 0.035) The 1/2 dilution and undiluted supernatant had no significant growth (Figure 3.B).

3.3.1.6 *Citrobacter freundii*

By the 6.5 hour time point, the OD of the 1/4 to 1/16 dilutions were all significantly higher than the OD of the undiluted supernatant (P=< 0.049) The 1/2 dilution and undiluted supernatant had no significant growth (Figure 3.C).
Strains were grown in MRS broth with the addition of two-fold serial dilutions of *L. acidophilus* supernatant. Optical density readings performed over a 12 hour incubation period indicated inhibition of growth of the test strains (equivalent to the negative control) by undiluted *L. acidophilus* supernatant. Dilution of the *L. acidophilus* supernatant diminished its inhibitory activity for the test bacterial strains as indicated by a trend towards increased optical density over time which reached statistical significance for certain time point/dilution combinations.

**Figure 3. Broth antagonism assays for *K. pneumoniae ss. oxytoca* (A), *E. coli* O111 (B) and *C. freundii* (C)**
3.3.2  *Bifidobacterium infantis* Supernatant
As with the *L. acidophilus* assays, in all strains tested the positive growth control was observed to have a normal growth curve for 12 hour incubation with a starting inoculum of 1 x 10⁸ cfu/mL.

3.3.2.1  *Cronobacter sakazakii 50*
At 4 hours the 1/8 and 1/16 dilutions had significantly higher OD than the undiluted supernatant/negative control (P= < 0.034). The 1/2, 1/4 and undiluted supernatants had no significant growth (Figure 4.A).

3.3.2.2  *Cronobacter sakazakii 2029*
At 4 hours the OD of the 1/8 and 1/16 dilutions all significantly higher than the undiluted supernatant/negative control (P= < 0.019) Undiluted, 1/2 and 1/4 diluted supernatants had no significant growth (Figure 4.B).

3.3.2.3  *Enterobacter cloacae*
At 3.5 hours, the 1/8 and 1/16 dilutions all had significantly higher OD than the undiluted supernatant/negative control (P= < 0.015). The 1/2, 1/4 and undiluted supernatant had no significant growth (Figure 4.C).
Strains were grown in MRS broth with the addition of two-fold serial dilutions of *B. infantis* supernatant. Optical density readings performed over a 12 hour incubation period indicated inhibition of growth of the test strains (equivalent to the negative control) by undiluted *B. infantis* supernatant. Dilution of the *B. infantis* supernatant diminished its inhibitory activity for the test bacterial strains as indicated by a trend towards increased optical density over time which reached statistical significance for certain time point/dilution combinations.

**Figure 4. Broth antagonism assays for C. sakazakii 50 (A), C. sakazakii 2029 (B) and E. cloacae (C)**
3.3.2.4 *Klebsiella pneumoniae* ss. *oxytoca*
At 6 hours, both the 1/8 and 1/16 dilutions had significantly higher OD than the undiluted supernatant/negative control (P < 0.044). The undiluted supernatant, 1/2 and 1/4 dilutions all had no significant growth (Figure 5.A).

3.3.2.5 *Escherichia coli* O111
At 5 hours, both the 1/8 and 1/16 dilutions had significantly higher OD than the undiluted supernatant/negative control (P < 0.045). The undiluted supernatant, 1/2 and 1/4 dilutions had no significant growth (Figure 5.B).

3.3.2.6 *Citrobacter freundii*
At the 3 hour sample point both the 1/8 and 1/16 dilutions had significantly higher OD than the undiluted supernatant/negative control (P < 0.019). At the 10 hour sample the 1/4 dilution was significantly higher (P = 0.039). The undiluted supernatant and 1/2 dilutions had no significant growth (Figure 5.C).
Figure 5. Broth antagonism assays for *K. pneumoniae* ss. *oxytoca* (A), *E. coli* O111 (B) and *C. freundii* (C)

Strains were grown in MRS broth with the addition of two-fold serial dilutions of *B. infantis* supernatant. Optical density readings performed over a 12 hour incubation period indicated inhibition of growth of the test strains (equivalent to the negative control) by undiluted *B. infantis* supernatant. Dilution of the *B. infantis* supernatant diminished its inhibitory activity for the test bacterial strains as indicated by a trend towards increased optical density over time which reached statistical significance for certain time point/dilution combinations.
3.3.3  Lactic acid supplemented MRS

3.3.3.1  *Cronobacter sakazakii 50*
At the 1.5 hour time point the 1/4, 1/8 and 1/16 dilution OD were significantly higher than the undiluted LA supplemented media/negative control (P= < 0.012). The undiluted LA supplemented media and 1/2 dilution had no significant growth (Figure 6.A).

3.3.3.2  *Cronobacter sakazakii 2029*
At the 1.5 hour time point the 1/4, 1/8 and 1/16 dilution OD were significantly higher than the undiluted LA supplemented media/negative control (P= < 0.039). The undiluted LA supplemented media/negative control and 1/2 dilution had no significant growth (Figure 6.B).

3.3.3.3  *Enterobacter cloacae*
At the 2 hour time point the 1/4, 1/8 and 1/16 dilution OD were significantly higher than the undiluted LA supplemented media/negative control (P= < 0.025). The undiluted LA supplemented media/negative control and 1/2 dilution had no significant growth (Figure 6.C).
Strains were grown in MRS broth supplemented with lactic acid with the addition of two-fold serial dilutions with MRS broth. Optical density readings performed over a 12 hour incubation period indicated inhibition of growth of the test strains (equivalent to the negative control) by undiluted lactic acid supplemented MRS. Dilution of the supplemented MRS diminished its inhibitory activity for the test bacterial strains as indicated by a trend towards increased optical density over time which reached statistical significance for certain time point/dilution combinations.

Figure 6. Broth antagonism assays for *C. sakazakii* 50 (A), *C. sakazakii* 2029 (B) and *E. cloacae* (C)
3.3.3.4 *Klebsiella pneumoniae ss. oxytoca*
The 1/4, 1/8 and 1/16 dilution OD were significantly higher than the undiluted LA supplemented media/negative control at the 1.5 hour time point (P = < 0.0467). The undiluted LA supplemented media/negative control and the 1/2 dilution did not have any significant growth. (Figure 7.A)

3.3.3.5 *Escherichia coli O111*
At the 1.5 hour time point the 1/4, 1/8 and 1/16 dilutions were significantly higher than the undiluted LA supplemented media/negative control (P = < 0.016). The undiluted LA supplemented media/negative control and the 1/2 dilution had no significant growth (Figure 7.B).

3.3.3.6 *Citrobacter freundii*
At the 1.5 hour time point the 1/4, 1/8 and 1/16 dilutions were significantly higher than the undiluted LA supplemented media/negative control (P = < 0.043). The 1/2 dilution was significantly higher at 11.5 hours (P = < 0.034). The undiluted LA supplemented media/negative control did not show any significant growth (Figure 7.C).

3.3.3.7 Broth Antagonism Overview
The NEC-associated strains and controls all were sensitive to *L. acidophilus* and *B. infantis* supernatant conditioned media as well as LA supplemented MRS media. This effect on growth appeared to be removed with dilution of the conditioned or LA supplemented media.
Strains were grown in MRS broth supplemented with lactic acid with the addition of two-fold serial dilutions with MRS broth. Optical density readings performed over a 12 hour incubation period indicated inhibition of growth of the test strains (equivalent to the negative control) by undiluted lactic acid supplemented MRS. Dilution of the supplemented MRS diminished its inhibitory activity for the test bacterial strains as indicated by a trend towards increased optical density over time which reached statistical significance for certain time point/dilution combinations.
3.4 Attachment Assays with $^3$H-Thymidine

The purpose of these assays was to incorporate $^3$H-Thymidine into the bacterial cells while they were growing, and use the radioactive signal to indirectly assess the proportion of bacteria that attached to the tissue culture cells. Then, probiotic strains were added to examine the effect on the baseline attachment levels.

Statistical significance was analysed by two-way ANOVA, using Tukey’s multiple comparisons test.

3.4.1 Optimal Attachment time

This experiment determined the effect of different incubation times on the attachment of NEC, control and probiotic bacteria with HT-29 cells. Table 9 shows the aggregate mean attached bacteria per well, expressed as a percentage of the original inoculum. The MOI was 100 bacterial cells per HT-29 cell. There was no significant difference between the three incubation times for the tested strains at a 95% confidence interval.

These data are all summarised in Figure 8, Table 9 and 10.
Table 9. Summary of $^3$H-Thymidine optimal attachment time, mean percentage attachment and SEM.

<table>
<thead>
<tr>
<th></th>
<th>2 Hours</th>
<th></th>
<th></th>
<th>3 Hours</th>
<th></th>
<th></th>
<th>4 Hours</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SEM</td>
<td>N</td>
<td>Mean</td>
<td>SEM</td>
<td>N</td>
<td>Mean</td>
<td>SEM</td>
<td>N</td>
</tr>
<tr>
<td><strong>C. sakazakii 50</strong></td>
<td>2.197</td>
<td>0.642</td>
<td>9</td>
<td>2.081</td>
<td>0.619</td>
<td>9</td>
<td>1.837</td>
<td>0.496</td>
<td>9</td>
</tr>
<tr>
<td><strong>C. sakazakii 2029</strong></td>
<td>2.662</td>
<td>1.142</td>
<td>9</td>
<td>2.008</td>
<td>0.288</td>
<td>9</td>
<td>1.955</td>
<td>0.607</td>
<td>9</td>
</tr>
<tr>
<td><strong>E. cloacae</strong></td>
<td>7.061</td>
<td>1.831</td>
<td>9</td>
<td>7.410</td>
<td>1.700</td>
<td>9</td>
<td>8.374</td>
<td>0.158</td>
<td>9</td>
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<tr>
<td><strong>K. pneumoniae ss. oxytoca</strong></td>
<td>4.310</td>
<td>2.082</td>
<td>9</td>
<td>5.294</td>
<td>1.392</td>
<td>9</td>
<td>4.007</td>
<td>2.130</td>
<td>9</td>
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<td><strong>E. coli O111</strong></td>
<td>8.019</td>
<td>4.861</td>
<td>9</td>
<td>8.913</td>
<td>2.190</td>
<td>9</td>
<td>11.339</td>
<td>5.244</td>
<td>9</td>
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<tr>
<td><strong>C. freundii</strong></td>
<td>2.447</td>
<td>0.399</td>
<td>9</td>
<td>4.359</td>
<td>0.498</td>
<td>9</td>
<td>6.060</td>
<td>3.201</td>
<td>9</td>
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<tr>
<td><strong>B. infantis</strong></td>
<td>27.692</td>
<td>10.082</td>
<td>9</td>
<td>23.532</td>
<td>2.169</td>
<td>9</td>
<td>18.597</td>
<td>6.540</td>
<td>9</td>
</tr>
<tr>
<td><strong>L. acidophilus</strong></td>
<td>3.944</td>
<td>2.540</td>
<td>9</td>
<td>18.471</td>
<td>14.341</td>
<td>9</td>
<td>5.952</td>
<td>3.968</td>
<td>9</td>
</tr>
</tbody>
</table>

Table 10 shows the labelling efficiency of all strains with $^3$H-Thymidine. Note there were marked differences between the strains.

Table 10. Labelling efficiency of all strains with $^3$H-Thymidine

<table>
<thead>
<tr>
<th>Strain</th>
<th>Labelling Efficiency, mean CPM for $2 \times 10^7$ cfu</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>C. sakazakii 50</strong></td>
<td>7.19E+03</td>
</tr>
<tr>
<td><strong>C. sakazakii 2029</strong></td>
<td>6.56E+03</td>
</tr>
<tr>
<td><strong>E. cloacae</strong></td>
<td>2.86E+03</td>
</tr>
<tr>
<td><strong>K. pneumoniae ss. oxytoca</strong></td>
<td>4.98E+03</td>
</tr>
<tr>
<td><strong>C. freundii</strong></td>
<td>1.20E+04</td>
</tr>
<tr>
<td><strong>E. coli O111</strong></td>
<td>2.06E+03</td>
</tr>
<tr>
<td><strong>L. acidophilus</strong></td>
<td>1.52E+03</td>
</tr>
<tr>
<td><strong>B. infantis</strong></td>
<td>1.49E+02</td>
</tr>
</tbody>
</table>
There was no statistical difference between the three tested incubation times. The positive attachment control *E. coli O111* had attachment between 8% and 11.3%, while the negative attachment control *C. freundii* had attachments ranging from 4.4% to 6.1%.

Figure 8. 3H-Thymidine incubation time optimisation for all strains at an MOI of 1:100
3.4.2 Further $^3$H-Thymide Assays
When conducting further assays with $^3$H-Thymidine many problems were encountered with the negative and positive controls. Often the controls were outside of the expected range, to the extreme that the negative control returned a higher percentage count per minute than the positive control. Due to this variability the results had to be omitted from the main body of the text. Summary tables and figures are in the appendix (6.2.).

3.5 Attachment Assays with Giemsa Stain
These assays used direct imaging of the HT-29 cells and the attached bacteria with pH controlled Giemsa stain to differentiate the bacteria from the tissue culture cells. Attachment was only quantified in the single strain attachment, as quantification proved to be very difficult in the co-culture attachment.

3.5.1 Single Strain Attachment
The quantified attached bacteria have been divided into two broad categories:

1. Directly associated bacteria, which were seen associated with the surface of the HT-29 cells.
2. Indirectly associated and stacked bacteria, which were seen at the edges of the cells and often had more bacteria stacked off to the side of them.

The directly and indirectly attached bacteria on 50 HT-29 cells per strain is summarised in table 11. NB. Individual indirectly associated stacks on a bacterium were counted, thus multiple stacks could be counted per cell.

3.5.1.1 Cronobacter sakazakii 50
There were small amounts of bacteria attached directly to the HT-29 cells and the bulk of the bacteria observed were in small clusters around the edge of the HT-29 cells. The larger of the stacks observed were described as having localised adherence (Figure 9.).

3.5.1.2 Cronobacter sakazakii 2029
C. sakazakii 2029 had broadly the same results as C. sakazakii 50. Although the indirectly associated stacks were larger in C. sakazakii 2029, with many more bacteria in the 11+ categories. The larger of the stacks observed were described as having localised adherence (Figure 10.).
Table 11. Quantification of associated and stacked bacteria in Giemsa stained HT-29 cells

<table>
<thead>
<tr>
<th></th>
<th>0-5</th>
<th>6-10</th>
<th>11-15</th>
<th>16-20</th>
<th>21-30</th>
<th>31-40</th>
<th>41+</th>
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<tbody>
<tr>
<td>Directly Associated Bacteria</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>C. sakazakii</em> 50</td>
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<td>0</td>
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</tr>
<tr>
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<td>1</td>
<td>0</td>
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<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>E. cloacae</em></td>
<td>30</td>
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<td>3</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>K. pneumoniae ss. oxytoca</em></td>
<td>6</td>
<td>17</td>
<td>23</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>E. coli</em> O111</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>7</td>
<td>14</td>
<td>27</td>
</tr>
<tr>
<td><em>C. freundii</em></td>
<td>50</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>B. infanis</em></td>
<td>4</td>
<td>12</td>
<td>13</td>
<td>9</td>
<td>10</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td><em>L. acidophilus</em></td>
<td>35</td>
<td>11</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Indirectly Associated and Stacked Bacteria</th>
<th>0-5</th>
<th>6-10</th>
<th>11-15</th>
<th>16-20</th>
<th>21-30</th>
<th>31-40</th>
<th>41+</th>
</tr>
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<tbody>
<tr>
<td><em>C. sakazakii</em> 50</td>
<td>43</td>
<td>18</td>
<td>3</td>
<td>2</td>
<td>1</td>
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<td>0</td>
</tr>
<tr>
<td><em>C. sakazakii</em> 2029</td>
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<td>9</td>
<td>6</td>
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<tr>
<td><em>E. cloacae</em></td>
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<td>6</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>K. pneumoniae ss. oxytoca</em></td>
<td>32</td>
<td>40</td>
<td>16</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td><em>E. coli</em> O111</td>
<td>5</td>
<td>11</td>
<td>10</td>
<td>15</td>
<td>8</td>
<td>5</td>
<td>9</td>
</tr>
<tr>
<td><em>C. freundii</em></td>
<td>47</td>
<td>5</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>B. infanis</em></td>
<td>22</td>
<td>19</td>
<td>13</td>
<td>8</td>
<td>6</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td><em>L. acidophilus</em></td>
<td>42</td>
<td>7</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

For the directly associated bacteria each count refers to a single HT-29 tissue culture cell with the indicated number of bacteria directly attached on 50 HT-29 cells. The counts in the indirectly associated and stacked bacteria refer the number of bacteria in a stack. Thus if there were multiple stacks on a single HT-29 tissue culture cell, they were recorded as such and the total number of stacks can be more than 50, for the 50 HT-29 tissue culture cells observed.
3.5.1.3  

**Enterobacter cloacae**

*E. cloacae* had an even spread of numbers of bacteria directly and indirectly attached to the HT-29 cells. Directly attached bacteria did not follow any particular pattern, but the larger stacks of indirectly associated bacteria had a localised adherence pattern (Figure 11.).

3.5.1.4  

**Klebsiella pneumoniae ss. oxytoca**

*K. pneumoniae ss. oxytoca* showed the most attachment out of all the NEC associated strains, with large numbers of directly and indirectly attached bacteria. The primary pattern of attachment was diffuse adherence (Figure 12.).

3.5.1.5  

**Escherichia coli O111**

As *E. coli* O111 was the positive attachment control, it had the highest numbers of directly and indirectly associated bacteria. Much more than any of the NEC associated strains or the probiotic strains. Though *E. coli* O111 is described in the literature as having localised adherence, the investigator noted all major types of attachment were observed: local adherence, diffuse adherence and aggregative adherence (Figure 13.).

3.5.1.6  

**Citrobacter freundii**

*C. freundii* was the negative attachment control and pursuant to that it has very few directly associated bacteria with all HT-29 cells counted having 5 or less bacteria. There were some indirectly associated stacks, but this was a very small amount. It is also worth noting that there were a lot of bacteria attached directly to the surface of the slide and not associated with the HT-29 cells. As such no attachment pattern was assigned to *C. freundii* (Figure 14.).

3.5.1.7  

**Bifidobacterium infantis**

The probiotic *B. infantis* had a very even spread of bacteria directly and indirectly attached. The adherence pattern was the classic ‘stacked brick or log jam’ associated with aggregative adherence (Figure 15.).

3.5.1.8  

**Lactobacillus acidophilus**

The probiotic *L. acidophilus* had relatively low total numbers of indirectly and directly attached bacteria. There was no consistent pattern of adherence observed, as sometimes the *L. acidophilus* displayed a diffuse attachment pattern and in other areas a localised attachment pattern was observed (Figure 16.).
A typical field of HT-29 cells with attached C. sakazakii 50. Overall, low attachment levels were observed with occasional large stacks showing localised adherence patterns. A. HT-29 cell. B. C. sakazakii 50

Figure 9. Cronobacter sakazakii 50, Giemsa stained with HT-29
A field of *C. sakazakii* 2029 displaying limited direct attachment to HT-29 cells, but some indirectly associated stacks can be seen towards the top of the picture. The primary pattern observed was localised attachment A. HT-29 cell. B. *C. sakazakii* 2029

**Figure 10. Cronobacter sakazakii 2029, Giemsa stained with HT-29**
Figure 11. *Enterobacter cloacae*, Giemsa stained with HT-29.

An *E. cloacae* field showing indirectly associated stacks of bacteria localised adherence towards the centre and to the bottom right. The primary observed pattern was one of localised adherence. **A.** HT-29 cell. **B.** *E. cloacae*
A typical field of *K. pneumoniae* ss. *oxytoca* attached to HT-29 cells, displaying a diffuse attachment pattern. **A.** HT-29 cell. **B.** *K. pneumoniae* ss. *oxytoca*.

**Figure 12.** *Enterobacter cloacae*, Giemsa stained with HT-29.
A typical field of E. coli O111 adherence showing all three major types of attachment: diffuse, localised and aggregative attachment. **A.** HT-29 cell. **B.** E. coli O111.

**Figure 13.** *Escherichia. coli* O111, Giemsa stained HT-29.
Figure 14. *Citrobacter freundii*, Giemsa stained HT-29
A typical field of *C. freundii* and HT-29 cells. There are very few directly or indirectly associated bacteria, with no discernable attachment pattern. **A.** HT-29 cell. **B.** *C. freundii.*
Figure 15. *Bifidobacterium infantis*, Giemsa stained HT-29
A typical field of Giemsa stained *B. infantis* on HT-29 cells displaying an aggregative attachment pattern. **A.** HT-29 cell. **B.** *B. infantis.*
Figure 16. *Lactobacillus acidophilus*, Giemsa stained HT-29.
A typical field of Giemsa stained *L. acidophilus* with HT-29 cells, though this field showed elements of diffuse attachment; there was no consistent pattern of attachment observed. **A.** HT-29 cell. **B.** *L. acidophilus*
3.5.2 Co-culture Attachment

There were two sets of co-culture experiments performed. One set with the NEC and control bacteria mixed with one of the two probiotics. The second was a mix of the NEC and control strains with the full Infloran formulation: *Lactobacillus acidophilus* and *Bifidobacterium infantis* in equal amounts.

These fields were not counted as there was too much activity observed to be accurately counted, thus only the overall attachment pattern was observed and recorded.

3.5.2.1 *Enterobacter sakazakii* 50

3.5.2.1.1 *Bifidobacterium infantis*

When mixed with *B. infantis*, *C. sakazakii* 50 no longer displays sparse attachment or the occasional stack of localised adherence seen in the single strain attachment assay (Figure 10), but co-aggregation with the *B. infantis* in an aggregative adherence pattern is observed. Often the *B. infantis* are along the edges of these aggregations with some located in the centre (Figure 17.).

3.5.2.1.2 *Lactobacillus acidophilus*

With *L. acidophilus* similar patterns are observed, with heavy co-aggregation between the two strains. Overall the aggregations appear to be larger than those with *B. infantis*. Once again an aggregative adherence pattern is present (Figure 18.).

3.5.2.1.3 Combination with both Infloran Probiotics

When combined with both probiotics, large aggregates are observed again with the *L. acidophilus* and *B. infantis* are both involved. An aggregative adherence pattern was displayed, and this is present both in direct association with the HT-29 cells and on the slide surface (Figure 19.).
Cronobacter sakazakii 50 formed large associations with Bifidobacterium infantis in an aggregative adhesion pattern with HT-29 cells, often with the C. sakazakii towards the middle and B. infantis clustering around the edges. A. HT-29 cell. B. C. sakazakii 50, light purple slightly translucent rod. C. B. infantis darker purple, slightly bulbous rod.
Figure 18. Cronobacter sakazakii 50 in co-culture with Lactobacillus acidophilus, Giemsa stained HT-29.

C. sakazakii 50 formed large aggregations with L. acidophilus in an aggregative adherence pattern with HT-29 cells. A. HT-29 cell. B. C. sakazakii 2029 light purple rod. C. L. acidophilus, large pink to purple rod.
Both probiotic strains and *C. sakazakii* 50 are observed to have an aggregative adherence pattern with HT-29 cells. 

**A.** HT-29 cell. 
**B.** *C. sakazakii* 50, small pink semi translucent rod. 
**C.** *B. infantis* light purple, slightly bulbous rod. 
**D.** *L. acidophilus*, large pink rod.

**Figure 19. Cronobacter sakazakii 50 in co-culture with both Infloran probiotics, Giemsa stained HT-29**
3.5.2.2 *Cronobacter sakazakii* 2029

3.5.2.2.1 *Bifidobacterium infantis*

Like *C. sakazakii* 50 a change was observed from areas of occasional localised adherence, increased co-aggregation with *B. infantis* in an aggregative adherence pattern with *C. sakazakii* 2029. With *B. infantis* primarily concentrated around the edges of the aggregations and *C. sakazakii* 2029 towards the centre (Figure 20.).

3.5.2.2.2 *Lactobacillus acidophilus*

Mixed in co-culture with *L. acidophilus* large aggregates were seen with *C. sakazakii* 2029, in an aggregative adherence pattern. It is worth noting that the *C. sakazakii* 2029 does not aggregate as closely with the *L. acidophilus* as with *B. infantis* (Figure 21.).

3.5.2.2.3 Combinations with both Infloran Probiotics

In co-culture with both probiotics very large aggregations were observed consisting of all 3 strains. Once again showing an aggregative adherence pattern, with the *C. sakazakii* 2029 closely associated with both probiotic strains (Figure 22.).
Figure 20. *Cronobacter sakazakii* 2029 in co-culture with *Bifidobacterium infantis*, Giemsa stained HT-29.

A. *C. sakazakii* 2029, lighter purple rod. B. *B. infantis*, darker purple bulbous rod. This picture is a typical field showing aggregative adherence with HT-29 cells.
Figure 21. *Cronobacter sakazakii* 2029 in co-culture with *Lactobacillus acidophilus*, Giemsa Stained HT-29 cells
A. *L. acidophilus*, large pink rods. B. *C. sakazakii* 2029, small light purple rods.
A typical Giemsa stained field showing aggregative adherence of the two strains with HT-29 cells.
Figure 22. *Cronobacter sakazakii* 2029 in co-culture with both Infloran probiotics, Giemsa Stained HT-29

A. *L. acidophilus*, large bright purple rods, B. *B. infantis*, small bright purple rods, C. *C. sakazakii* 2029, small light purple rods. A typical field of all three strains in co-culture showing the large aggregative adherence patterns with HT-29 cells.
3.5.2.3 *Enterobacter cloacae*

3.5.2.3.1 *Bifidobacterium infantis*

In the presence of *B. infantis*, *E. cloacae* changed from having small clusters of localised adherence to having large stacks of the two strains forming an aggregative adherence pattern. The majority of *B. infantis* cells were observed to be concentrating around the edges of the aggregations (Figure 23.).

3.5.2.3.2 *Lactobacillus acidophilus*

In co-culture with *L. acidophilus* the *E. cloacae* formed large clusters in an aggregative adherence pattern. When compared to the *B. infantis* co-culture (Figure 24) this was a less closely associated aggregation (Figure 24).

3.5.2.3.3 Combination with both Infloran Probiotics

When combined with both probiotic strains large clusters of all three strains were formed in an aggregative adherence pattern. *E. cloacae* were seldom observed without being associated with at least one of the probiotic strains (Figure 25.).
Figure 23. *Enterobacter cloacae* in co-culture with *Bifidobacterium infantis*, Giemsa stained HT-29

A. *E. cloacae*, light purple rods, B. *B. infantis* darker purple slightly bulbous rods. A typical Giemsa stained field of these two stains showing an aggregative adherence pattern with HT-29 cells.
A. *E. cloacae*, small light to mid purple rods, B. *L. acidophilus* large pink to dark purple rods. A typical Giemsa stained field of these two strains displaying an aggregative adherence pattern with HT-29 cells.

Figure 24. *Enterobacter cloacae* in co-culture with *Lactobacillus acidophilus*, Giemsa stained HT-29
Figure 25. *Enterobacter cloacae* in co-culture with both Infloran Probiotics, Giemsa stained HT-29

A. *L. acidophilus*, large pink rods, B. *B. infantis*, small bulbous rods, purple, C. *E. cloacae* small light purple rods. This is a typical Giemsa stained field of HT-29 with the Infloran probiotics and *E. cloacae* showing an aggregative adherence pattern.
3.5.2.4 Klebsiella pneumoniae ss. oxytoca

3.5.2.4.1 Bifidobacterium infantis

*K. pneumoniae* ss. *oxytoca* co-cultured with *B. infantis*, changed from having diffuse attachment pattern to having an aggregative adherence pattern. It was also observed that the *B. infantis* were not as concentrated to the edges of the aggregations as had been seen in the other three NEC strains, or as closely associated with *K. pneumoniae* ss. *oxytoca* (Figure 26.).

3.5.2.4.2 Lactobacillus acidophilus

When incubated in the presence of *L. acidophilus*, a change from diffuse to aggregative adherence was observed. *K. pneumoniae* ss. *oxytoca* did look to have a slightly closer association with *L. acidophilus* than the other NEC strains (Figure 27).

3.5.2.4.3 Combined with both Infloran Probiotics

When mixed with both Infloran probiotic strains *K. pneumoniae* ss. *oxytoca* had a very similar pattern of adherence to the other three NEC strains. It was observed that *K. pneumoniae* ss. *oxytoca* formed very close associations with both probiotic strains and formed an aggregative adherence pattern (Figure 28.).
Figure 26. *Klebsiella pneumoniae ss. oxytoca* in co-culture with *Bifidobacterium infantis*, Giemsa stained HT-29

A. *K. pneumoniae ss. oxytoca*, short purple rods. B. *B. infantis*, bulbous rods with a dark purple colour. This is an example of a typical field of co-attachment with *B. infantis* and *K. pneumoniae ss. oxytoca* in an aggregative adherence pattern with HT-29.
A. *L. acidophilus*, pinkish purple large rods, B. *K. pneumoniae ss. oxytoca* small short purple rods. This is an example of a typical Giemsa stained field of HT-29 cells with *L. acidophilus* and *K. pneumoniae ss. oxytoca* showing an aggregative adherence pattern.
Figure 28. *Klebsiella pneumoniae* ss. *oxytoca* in co-culture with both Infloran probiotics, Giemsa stained HT-29

A. *L. acidophilus*, large bright purple rods. B. *B. infantis*, dark purple bulbous rods. C. *K. pneumoniae* ss. *oxytoca*, short light purple rods. This is a typical Giemsa stained field of HT-29 cells showing aggregative adherence of *K. pneumoniae* ss. *oxytoca* and the two Infloran probiotics.
3.5.2.5 *Escherichia coli O111*

3.5.2.5.1 *Bifidobacterium infantis*
When co-cultured with *B. infantis* there was no a difference observed in the attachment profile compared to *E. coli O111* on its own, with the exception that this time the probiotic bacteria also interacting with the *E. coli O111*. Again the *B. infantis* were concentrating towards the edges of the stacks of bacteria, with the *E. coli* in the centre (Figure 29.).

3.5.2.5.2 *Lactobacillus acidophilus*
As with the *B. infantis*, similar attachment patterns were observed when compared to the single strain attachment experiment. The exception being, *L. acidophilus* co-aggregating with the *E. coli O111* (Figure 30.).

3.5.2.5.3 Combination of both Infloran Probiotics
In combination with both probiotics there were heavy aggregations of the probiotics clustered around the edges of the HT-29 cells with an aggregative adherence pattern and the *E. coli O111* were concentrated directly on top of the HT-29 cells (Figure 31.).
Figure 29. *Escherichia coli* O111 in co-culture with *Bifidobacterium infantis*, Giemsa stained HT-29

A. *B. infantis*, dark purple bulbous rods, B. *E. coli* O111, light purple short rods. This is example of a typical Giemsa stained field of HT-29 cells showing aggregative attachment of *B. infantis* and *E. coli* O111.
A. *L. acidophilus*, large purple dark rods, B. *E. coli* O111, small short light purple rods. This is a typical Giemsa stained field of HT-29 cells showing attachment with *E. coli* O111 and *L. acidophilus* in an aggregative adherence pattern.
A. *L. acidophilus*, large purple rods, B. *B. infantis*, smaller purple rods with bulbous ends, C. *E. coli* O111, short rods with a darker purple colour. This is a typical Giemsa stained field of HT-29 cells showing aggregative adherence between *E. coli* O111 and *L. acidophilus*.

Figure 31. *Escherichia coli* O111 in co-culture with both Infloran probiotics, Giemsa stained HT-29.
3.5.2.6 *Citrobacter freundii*

3.5.2.6.1 *Bifidobacterium infantis*

In co-culture with *B. infantis* there was a change from sparse attachment, to heavy co-aggregation with, an aggregative adhesion pattern. Unlike the single strain there are *C. freundii* attached to the surface of the HT-29 cells (Figure 32.).

3.5.2.6.2 *Lactobacillus acidophilus*

When incubated with *L. acidophilus* there was still little attachment of the *C. freundii* observed directly to the HT-29 cells. There are large aggregates of *C. freundii* and *L. acidophilus* on the surface of the slide and these are in an aggregative adherence pattern (Figure 33.).

3.5.2.6.3 Combination with both Infloran Probiotics

When combined with both probiotics a similar pattern to what is seen in the NEC strains is observed with heavy clustering of both probiotics and the *C. freundii*. These were observed to have an aggregative adhesion pattern (Figure 34.).
Figure 32. *Citrobacter freundii* in co-culture with *Bifidobacterium infantis*, Giemsa stained HT-29

A. *C. freundii*, short rods with a light purple colour. B. *B. infantis*, bulbous rods with a dark purple colour. This field is a typical Giemsa stained field of HT-29 showing aggregative attachment between *C. freundii* and *B. infantis* with some HT-29 association.
Figure 33. *Citrobacter freundii* in co-culture with *Lactobacillus acidophilus*, Giemsa stained HT-29

A. *L. acidophilus*, large pink to purple rods, B. *C. freundii*, small dark purple rods. Typical Giemsa stained field of HT-29 cells showing aggregative attachment between *L. acidophilus* and *C. freundii* occurring on the chamber slide surface.
A. *L. acidophilus*, large ping to purple rods, B. *B. infantis*, small pink bulbous rods. C. *C. freundii* small dark purple rods, not as densely stained. This is a typical Giemsa stained field of HT-29 cells showing *C. freundii* forming aggregative adhesive patterns with the Infloran probiotics and considerable HT-29 association.
3.6 Invasion Assays

The purpose of the invasion assays was to establish whether the NEC strains and probiotics were capable of invading HT-29 cells, and to incubate the NEC strains in co-culture with the probiotics. This was to observe whether the presence of the probiotic strains would significantly change the invasion ability of the NEC strains.

Different control strains were used for this set of experiments: *Salmonella enterica* ss. *enterica* serovar Typhimurium as the positive invasion control and *Escherichia coli* K12 DH5α as the negative invasion control.

It was decided to show both the total recovered counts and the percentage invasion as it was felt the total recovered bacteria gave a clearer picture of what was going on, though the percentage of initial inoculum was also shown, as it is the convention for invasion assays.

3.6.1 *Salmonella* Typhimurium Optimisation

These experiments were performed to find the optimal invasion time and MOI for *S. Typhimurium* in HT-29. Both total recovered counts and percentage of the initial inoculum invading the HT-29 cells were calculated and results are summarised in Figure 35.

Statistical analysis of these data was performed with two-way ANOVA, using Tukey’s multiple comparison tests with a 95% confidence interval

3.6.1.1 Total Recovered Counts

There were no statistically significant differences between the different MOI and times of infection when comparing the total recovered cfu/mL. This data is summarised in Table 12 and Figure 35A.

3.6.1.2 Percentage of Initial Inoculum

As with the total recovered counts there were no statistically significant differences between the different MOI and incubation time. It is worth noting that the three hour incubation with a MOI of 10 : 1 had the largest mean percentage invasion. This data is summarised in Table 13 and Figure 35B.
Table 12. Invasion of HT-29 cells by S. Typhimurium: effect of different incubation times and MOI expressed as the mean percentage of the initial inoculum with SEM

<table>
<thead>
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<th>Incubation (h)</th>
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<th>MOI 10:1</th>
<th>MOI 1:1</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SEM</td>
<td>N</td>
</tr>
<tr>
<td>2</td>
<td>0.133</td>
<td>0.051</td>
<td>9</td>
</tr>
<tr>
<td>3</td>
<td>0.153</td>
<td>0.096</td>
<td>9</td>
</tr>
<tr>
<td>4</td>
<td>0.024</td>
<td>0.016</td>
<td>9</td>
</tr>
</tbody>
</table>

Table 13. Invasion of HT-29 cells by S. Typhimurium: effect of different incubation times and MOI expressed as the mean total recovered viable (cfu/mL) bacteria with SEM

<table>
<thead>
<tr>
<th>Incubation(h)</th>
<th>MOI 100:1</th>
<th>MOI 10:1</th>
<th>MOI 1:1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SEM</td>
<td>N</td>
</tr>
<tr>
<td>2</td>
<td>4.47E+5</td>
<td>1.73E+5</td>
<td>9</td>
</tr>
<tr>
<td>3</td>
<td>2.14E+5</td>
<td>2.27E+4</td>
<td>9</td>
</tr>
<tr>
<td>4</td>
<td>3.26E+4</td>
<td>5.24E+3</td>
<td>9</td>
</tr>
</tbody>
</table>
A. *E. coli* infection of HT-29 cells expressed as total counts (cfu/mL).

No statistical significance was found between the different time points and MOI’s.

B. *E. coli* infection of HT-29 cells expressed as percentage of the initial inoculum. As for the total counts there was no statistical significance found between the different conditions. It is worth noting that the three hour incubation and MOI of 10:1 was trending towards 5% invasion.

**Figure 35.** *Salmonella Typhimurium* invasion time and MOI optimisation experiments

A. *S. Typhimurium* invasion of HT-29 cells expressed as total counts (cfu/mL). No statistical significance was found between the different time points and MOI’s. B. *S. Typhimurium* invasion of HT-29 cells expressed as percentage of the initial inoculum. As for the total counts there was no statistical significance found between the different conditions. It is worth noting that the three hour incubation and MOI of 10:1 was trending towards 5% invasion.
3.6.2 Single Strain Invasion, NEC, Controls and Probiotics

From previous experiments (3.7.1) it was decided three hour incubation was optimal. The experiment was then carried out on all the NEC, control and probiotic strains with MOI of 100:1 (100 cfu per HT-29 cell) and 10:1 (10 cfu per HT-29 cell) (Figure 37.).

Statistical analysis of these experiments was performed with two-way ANOVA using Sidak’s multiple comparisons test.

3.6.2.1 Total Recovered Counts

At the MOI of 100:1, all NEC strains and the E. coli K12 showed significantly less invasion than the positive control S. Typhimurium (P= <0.0001). At the MOI of 10:1, the only NEC-associated strain not to have significantly lower invasion than S. Typhimurium was K. pneumoniae ss. oxytoca (P= < 0.001). No NEC strain was significantly different from another. When comparing the two different MOI’s to each other only S. Typhimurium had a significant difference (P= < 0.0001) (Figure 36 A).

This data is summarised in Table 14 and Figure 36A.

3.6.2.2 Percentage of Initial Inoculum

There was no statistically significant difference between the ability of the NEC strains to invade HT-29 at either MOI. The positive invasion control S. Typhimurium showed significantly higher invasion than all other strains at both MOI (P= < 0.001) When comparing the MOI only K. pneumoniae ss. oxytoca and S. Typhimurium were significantly different. In both cases the MOI of 10:1 was higher than the MOI of 100:1 (P= < 0.04) and <0.0001. (Figure 36 B)

This data is summarised in Table 15 and Figure 36B.
Table 14. Single strain invasion of HT-29 cells by all NEC-associated and control strains, expressed as total recovered cfu/mL with mean and SEM

<table>
<thead>
<tr>
<th></th>
<th>MOI 100:1</th>
<th></th>
<th></th>
<th>MOI 10:1</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SEM</td>
<td>N</td>
<td>Mean</td>
<td>SEM</td>
<td>N</td>
</tr>
<tr>
<td><em>C. sakazakii 50</em></td>
<td>3.76E+04</td>
<td>9.05E+03</td>
<td>9</td>
<td>3.55E+04</td>
<td>1.98E+04</td>
<td>9</td>
</tr>
<tr>
<td><em>C. sakazakii 2029</em></td>
<td>1.43E+05</td>
<td>8.17E+04</td>
<td>9</td>
<td>1.30E+04</td>
<td>2.21E+03</td>
<td>9</td>
</tr>
<tr>
<td><em>E. cloacae</em></td>
<td>1.20E+05</td>
<td>4.31E+04</td>
<td>9</td>
<td>3.40E+04</td>
<td>8.68E+03</td>
<td>9</td>
</tr>
<tr>
<td><em>K. pneumoniae ss. oxytoca</em></td>
<td>9.15E+04</td>
<td>2.74E+04</td>
<td>9</td>
<td>1.15E+05</td>
<td>6.49E+04</td>
<td>9</td>
</tr>
<tr>
<td><em>S. Typhimurium</em></td>
<td>3.32E+03</td>
<td>7.02E+02</td>
<td>9</td>
<td>5.00E+02</td>
<td>0.00E+00</td>
<td>9</td>
</tr>
<tr>
<td><em>E. coli K12</em></td>
<td>1.17E+06</td>
<td>2.80E+05</td>
<td>9</td>
<td>4.42E+05</td>
<td>6.95E+04</td>
<td>9</td>
</tr>
<tr>
<td><em>L. acidophilus</em></td>
<td>5.00E+02</td>
<td>0.00E+00</td>
<td>9</td>
<td>5.00E+02</td>
<td>0.00E+00</td>
<td>9</td>
</tr>
<tr>
<td><em>B. infantis</em></td>
<td>5.00E+02</td>
<td>0.00E+00</td>
<td>9</td>
<td>5.00E+02</td>
<td>0.00E+00</td>
<td>9</td>
</tr>
</tbody>
</table>

Table 15. Single strain invasion of HT-29 cells by all NEC-associated and control strains expressed as percentage of initial inoculum recovered, mean and SEM

<table>
<thead>
<tr>
<th></th>
<th>MOI 100:1</th>
<th></th>
<th></th>
<th>MOI 10:1</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SEM</td>
<td>N</td>
<td>Mean</td>
<td>SEM</td>
<td>N</td>
</tr>
<tr>
<td><em>C. sakazakii 50</em></td>
<td>0.019</td>
<td>0.008</td>
<td>9</td>
<td>0.191</td>
<td>0.100</td>
<td>9</td>
</tr>
<tr>
<td><em>C. sakazakii 2029</em></td>
<td>0.079</td>
<td>0.058</td>
<td>9</td>
<td>0.076</td>
<td>0.028</td>
<td>9</td>
</tr>
<tr>
<td><em>E. cloacae</em></td>
<td>0.053</td>
<td>0.026</td>
<td>9</td>
<td>0.151</td>
<td>0.052</td>
<td>9</td>
</tr>
<tr>
<td><em>K. pneumoniae ss. oxytoca</em></td>
<td>0.054</td>
<td>0.021</td>
<td>9</td>
<td>0.507</td>
<td>0.319</td>
<td>9</td>
</tr>
<tr>
<td><em>S. Typhimurium</em></td>
<td>0.002</td>
<td>0.001</td>
<td>9</td>
<td>0.000</td>
<td>0.000</td>
<td>9</td>
</tr>
<tr>
<td><em>E. coli K12</em></td>
<td>0.474</td>
<td>0.127</td>
<td>9</td>
<td>1.711</td>
<td>0.191</td>
<td>9</td>
</tr>
<tr>
<td><em>L. acidophilus</em></td>
<td>0.000</td>
<td>0.000</td>
<td>9</td>
<td>0.000</td>
<td>0.000</td>
<td>9</td>
</tr>
<tr>
<td><em>B. infantis</em></td>
<td>0.000</td>
<td>0.000</td>
<td>9</td>
<td>0.000</td>
<td>0.000</td>
<td>9</td>
</tr>
</tbody>
</table>
A. Single strain invasion expressed as total recovered viable bacteria (cfu/mL). At the MOI of 100:1 all NEC strains and the E. coli K12 values were all statistically significantly lower than the positive control S. Typhimurium with P-values of <0.0001. At the MOI of 10:1, the NEC strains C. sakazakii 50, 2029 and E. cloacae showed significantly less invasion than the positive control with P-values of <0.0093. B. Single strain invasion expressed as a percentage of initial inoculum. At the MOI of 10:1 the NEC strains were all significantly lower than the positive control with P-values of <0.0001. Comparing MOI’s in K. pneumoniae ss. oxytoca and S. Typhimurium had a statistically higher invasion at the 10:1 than the 100:1 MOI with P-values of <0.0001.
3.6.3 Single Strain Invasion, Log Phase Cultures

It was noted in the single strain experiments that the percentage invasion of the positive control was somewhat lower than what the literature suggests it should be (~5%). Thus it was decided to repeat the single strain invasion using log phase cultures instead of stationary phase cultures. It was noted during the experiments that there was no significant difference between the invasion ability of the NEC strains at stationary or log phase, thus those data have been omitted. (Figure 37.)

Statistical analysis was performed with two-way ANOVA using either Tukey’s or Sidak’s multiple comparisons tests.

3.6.3.1 Total Recovered Counts

At both MOI’s the positive control *S.* Typhimurium was statistically higher than the negative control *E. coli* K12 \( (P < 0.0001) \). *S.* Typhimurium was statistically higher at the 100:1 MOI than the 10:1 with a \( (P < 0.0001) \). (Figure 37 A)

This data is summarised in Table 16 and Figure 37A.

3.6.3.2 Percentage of Initial Inoculum

At both MOI the positive control *S.* Typhimurium was statistically higher than the negative control *E. coli* K12 \( (P < 0.005) \). At the MOI of 10:1 *S.* Typhimurium was significantly higher than the 100:1 \( (P < 0.0001) \). (Figure 37 B)

This data is summarised in table 17 and Figure 37B.
Table 16. Log phase Single strain invasion, controls and probiotics only expressed as total recovered cfu/mL with mean and SEM.

<table>
<thead>
<tr>
<th></th>
<th>MOI 100:1</th>
<th></th>
<th>MOI 10:1</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SEM</td>
<td>N</td>
<td>Mean</td>
</tr>
<tr>
<td><strong>E. coli O111</strong></td>
<td>1.78E+03</td>
<td>1.05E+03</td>
<td>9</td>
<td>2.33E+03</td>
</tr>
<tr>
<td><strong>S. Typhimurium</strong></td>
<td>9.85E+05</td>
<td>5.57E+04</td>
<td>9</td>
<td>6.08E+05</td>
</tr>
<tr>
<td><strong>L. acidophilus</strong></td>
<td>5.00E+02</td>
<td>0.00E+00</td>
<td>9</td>
<td>5.00E+02</td>
</tr>
<tr>
<td><strong>B. infantis</strong></td>
<td>5.00E+02</td>
<td>0.00E+00</td>
<td>9</td>
<td>5.00E+02</td>
</tr>
</tbody>
</table>

Table 17. Log phase Single strain invasion, controls and probiotics only expressed as percentage of initial inoculum with mean and SEM.

<table>
<thead>
<tr>
<th></th>
<th>1:100</th>
<th></th>
<th>1:10</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SEM</td>
<td>N</td>
<td>Mean</td>
</tr>
<tr>
<td><strong>E. coli O111</strong></td>
<td>0.003</td>
<td>0.001</td>
<td>9</td>
<td>0.024</td>
</tr>
<tr>
<td><strong>S. Typhimurium</strong></td>
<td>0.638</td>
<td>0.053</td>
<td>9</td>
<td>4.144</td>
</tr>
<tr>
<td><strong>L. acidophilus</strong></td>
<td>0.000</td>
<td>0.000</td>
<td>9</td>
<td>0.000</td>
</tr>
<tr>
<td><strong>B. infantis</strong></td>
<td>0.000</td>
<td>0.000</td>
<td>9</td>
<td>0.000</td>
</tr>
</tbody>
</table>
A. Log phase single strain invasion expressed as total recovered counts. At both MOI’s, the positive control gave statistically significantly higher total recovered counts than the negative control with P-values of <0.0001. *S.* Typhimurium was higher at the 100:1 MOI with a P-value of <0.0001.

B. Log phase single strain invasion expressed as percentage of initial inoculum. Again at both MOI’s *S.* Typhimurium gave significantly higher percentages than the negative control with P-values of <0.0045. *S.* Typhimurium was significantly higher at the 10:1 MOI than the 100:1 MOI, with a P-value of <0.0001. No invasion with the probiotic strains was detected, and was assumed to be below the level of detection.

Figure 37. Single strain invasion with Log phase cultures of control and probiotic strains.
3.6.4 Probiotic Co-culture Invasion
In this set of experiments the NEC and control strains were mixed 1:1 with the Infloran to observe the effect on invasion. An MOI of 10:1 was used for these experiments.

Statistical analysis was performed with two-way ANOVA using either Tukey’s or Sidak’s multiple comparisons tests. (Figure 38)

3.6.4.1 Total recovered Counts
There was no statistically significant difference between any of the strains with the probiotic added. In the single strains experiments, all four NEC strains and the negative control showed significantly less invasion than the positive control *S. Typhimurium* (P=< 0.0001). Only *S. Typhimurium* had a significant difference when comparing probiotics added with no probiotics added, (P= <0.0001). (Figure 38 A)

This data is summarised in Table 18.

3.6.4.2 Percentage of Initial Inoculum
When comparing the probiotic added to the single strains there was no significant difference in invasion. With no probiotic added, the NEC strains and *E. coli* K12 were all significantly less invasive than the positive control *S. Typhimurium*, with (P= <0.0001). Only *S. Typhimurium* had a significant difference when comparing probiotics added with no probiotics added, (P= <0.0001). (Figure 38 B)

This data is summarised in Table 19.
Table 18. Probiotic Co-culture Invasion of HT-29 expressed as total recovered cfu/mL, mean and SEM.

<table>
<thead>
<tr>
<th></th>
<th>Probiotic Added 1:1</th>
<th>No Probiotic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SEM</td>
</tr>
<tr>
<td><em>C. sakazakii</em> 50</td>
<td>1.88E+04</td>
<td>1.80E+04</td>
</tr>
<tr>
<td><em>C. sakazakii</em> 2029</td>
<td>1.04E+04</td>
<td>6.06E+03</td>
</tr>
<tr>
<td><em>E. cloacae</em></td>
<td>8.06E+03</td>
<td>5.64E+03</td>
</tr>
<tr>
<td><em>K. pneumoniae ss. oxytoca</em></td>
<td>6.89E+03</td>
<td>5.73E+03</td>
</tr>
<tr>
<td><em>E. coli</em> K12</td>
<td>5.83E+01</td>
<td>5.83E+01</td>
</tr>
<tr>
<td><em>S. Typhimurium</em></td>
<td>4.81E+04</td>
<td>1.55E+04</td>
</tr>
</tbody>
</table>

Table 19. Probiotic Co-culture Invasion of HT-29 expressed as percentage of initial inoculum, mean and SEM.

<table>
<thead>
<tr>
<th></th>
<th>Probiotic Added 1:1</th>
<th>No Probiotic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SEM</td>
</tr>
<tr>
<td><em>C. sakazakii</em> 50</td>
<td>0.873</td>
<td>0.817</td>
</tr>
<tr>
<td><em>C. sakazakii</em> 2029</td>
<td>0.808</td>
<td>0.549</td>
</tr>
<tr>
<td><em>E. cloacae</em></td>
<td>0.226</td>
<td>0.135</td>
</tr>
<tr>
<td><em>K. pneumoniae ss. oxytoca</em></td>
<td>0.324</td>
<td>0.273</td>
</tr>
<tr>
<td><em>E. coli</em> K12</td>
<td>0.003</td>
<td>0.003</td>
</tr>
<tr>
<td><em>S. Typhimurium</em></td>
<td>1.671</td>
<td>0.473</td>
</tr>
</tbody>
</table>
Figure 38. Invasion of HT-29 cells by NEC strains and control strains co-cultured with *Lactobacillus acidophilus* and *B. infantis* in a 1:1 ratio.

A. Probiotic co-culture invasion expressed as total recovered counts. For the NEC strains, no statistically significant difference was observed between experiments with and without the probiotic added. Invasion by the positive control *S. Typhimurium* was significantly greater without compared to with the probiotic with a P-value of <0.0001. B. Probiotic co-culture invasion expressed as percentage of initial inoculum. No statistical significance was observed between experiments with and without the probiotic added for the NEC strains. Statistical significance was observed in the positive control *S. Typhimurium* between experiments with and without the probiotic with a P-value of <0.0001.
3.7 Neonatal Formula Growth Experiments

The aim of these final experiments was to observe the effects of co-culture with the probiotics and NEC strains in the presence of neonatal formula. This was performed with two different methods; an aerobic growth curve and an anaerobic probiotic pre-incubation.

3.7.1 Co-culture Growth Curves in Neonatal Formula

This experiment was performed twice: once with Probiotic: NEC in a 1:1 ratio with Pfizer’s S-26 Gold formula and repeated with Probiotic: NEC in a 10:1 ratio with Nestle PreNAN Gold formula. Over the 12 hour time course of both experiments no significant difference in growth rate of the NEC strains was observed between the probiotic treatment and non-treatment experiments (Figure 39.).
A. Growth curve in Pfizer S-26 Gold formula with a 1:1 ratio of NEC strains to probiotics. No statistically significant difference in the growth rates was observed between the experiments with and without probiotics. B. Growth curve in Nestle PreNAN Gold formula with a 1:10 ratio of NEC to probiotics. No statistically significant difference was observed between probiotic treated and non-treated NEC growth rates.

Figure 39. Co-culture growth curves in Neonatal formula with the NEC strains and the Infloran probiotics
3.7.2 Probiotic Pre-incubation in Neonatal Formula
This experiment was performed three times, once with Pfizer S-26 Gold and twice with Nestle PreNAN Gold formulas. Due to a limited time was not possible to perform these experiments enough times to get meaningful statistics. However the results are trending towards statistically significance. Also note that in the second Nestle PreNAN Gold experiment lower dilutions were plated so numbers as low as $5 \times 10^2$ cfu/mL could be detected. In all three experiments the probiotics were recovered from the formulas at $\geq 1 \times 10^9$ cfu/mL on MRS, regardless of whether they were incubated with the NEC strains or on their own.

3.7.2.1 Pfizer S-26 Gold
All four NEC-associated strains grew to $>1 \times 10^9$ cfu/mL in the neonatal formula. When pre-treated with the Infloran probiotic strains for three, six and 12 hours there was no growth detected (Figure 40).

3.7.2.2 Nestle PreNAN Gold, Experiment One
As with the S-26 gold, the NEC associated strains grew to $>1 \times 10^9$ cfu/mL in the formula unsupplemented with probiotics. With zero hours pre-incubation with the probiotic strains, C. sakazakii 50, 2029 and E. cloacae did not grow beyond their initial inoculum. With three hours pre-incubation only K. pneumoniae ss. oxytoca was detectable. At six hours pre-incubation with the probiotics there was no growth above our detection limit (Figure 41 A).

3.7.2.3 Nestle PreNAN Gold, Experiment Two
The NEC-associated strains grew to $> 1 \times 10^9$ cfu/mL in the unsupplemented formula. As the detection limits were lower for this experiment, the NEC-associated strains were detected at every pre-incubation time. As before, the K. pneumoniae ss. oxytoca was the only strain to grow over the initial inoculated amount, and this growth decreased with further pre-incubation with the probiotic strains (Figure 41 B).
Following pre-incubation of the probiotic and formula milk for 3, 6 and 12 hours, the growth of NEC strains and Infloran probiotics was determined by viable count. The Infloran probiotics were consistently recovered at $10^9$ cfu/mL, regardless of whether they were incubated with the NEC strains or on their own. As there was only one experiment performed there was no ability to do statistics, although the results suggest the pre-incubation with probiotics was inhibitory.

Figure 40. Growth of the NEC strains in Pfizer S-26 formula pre-incubated with probiotic.
A. Nestle PreNAN Gold experiment 1, C. sakazakii 50, 2029 and E. cloacae did not grow beyond much beyond the inoculated amount (1 x 10^6 cfu/mL), even with the probiotics added simultaneously, indicating an inhibitory effect. K. pneumoniae ss. oxytoca, was able to grow, although this was reduced with longer periods of probiotic pre-incubation.

B. Nestle PreNAN Gold experiment 2. C. sakazakii 50, 2029 and E. cloacae were not able to grow beyond the initially inoculated amount (1 x 10^6 cfu/mL). K. pneumoniae ss. oxytoca growth was reducing with increasing length of probiotic pre-incubation. In both experiments the Infloran probiotics were consistently recovered at 10^9 cfu/mL.

Figure 41. Growth of NEC strains in Nestle PreNAN Gold pre-incubated with Probiotic
4 Discussion

Probiotic supplementation of neonates has been demonstrated by many studies to have beneficial effects for the host, as discussed in the introduction [70, 135, 136]. Few studies have looked at the direct interaction between probiotic and NEC associated bacteria, and there are no published studies for Infloran. The results described in this thesis have shed some light on this complex interaction, and suggest some possible mechanisms Infloran probiotic bacteria may use to exert their effect in the GI tract of the preterm neonate.

4.1 Probiotic Inhibition of NEC-associated Strains

Various probiotic bacteria have been identified as able inhibit potential pathogens via lactic acid and bacteriocin production, and competition for binding sites and resources [135, 137, 138]. However, there seems to nothing in the published literature specifically describes the inhibitory properties of the Infloran strains.

4.1.1 Probiotic Strains from Infloran & Inhibition of Growth of NEC-associated Strains on Solid Media

On solid media it was much more difficult to see an antagonistic effect, with four out of the five attempted assays showing no inhibition of the NEC strains. In light of the results from the broth antagonism assays, it is safe to assume the conditioned media was too dilute to have a significant effect on the pH at the surface of the plate. In the final antagonism experiment, the 1 : 1 mixing of the conditioned media to the double strength MH-II agar was enough to bring the media down to a low pH to where the NEC bacteria were inhibited. The difficulty of getting results in this set of experiments suggested that the inhibitory factors produced by L. acidophilus and B. infantis were not active at the low concentrations present in these assays. A limitation of these assays was the narrow range of culture conditions used. Tagg et al have shown that bacterial production of inhibitory substances is dependent on both the culture medium used and incubation conditions [130].

4.1.2 Auto-aggregation and Co-aggregation of the Probiotics from Infloran and the NEC-associated Strains

In the co-aggregation experiments the Infloran probiotics were observed aggregating with the NEC strains. The only NEC strain to be able to auto-aggregate was the K. pneumoniae ss. oxytoca as observed in comparison with the positive control E. coli O111, but the aggregates formed were small (visible only with a microscope), compared to those with the probiotics
(macroscopically visible). *K. pneumoniae* spp. with their polysaccharide capsules have been previously identified as able to aggregate, especially in response to stressful environments such as treatment with chlorine [139-141]. The *L. acidophilus* and *B. infantis* mixed separately with the NEC strains were able to show aggregates up to 5 mm. When the probiotic strains were mixed together as the Infloran formulation and added to the NEC strains even larger (6 mm +) aggregates were formed, and further evidence of this observation will be discussed in the Giemsa attachment assays. This in combination with the observation of a less intense red colour from the TTC is suggestive of the probiotic strains being able to form large aggregations with the NEC strains and also reduce their metabolism and therefore inhibit their growth. This inhibition has been observed in previous studies [121, 142]. The positive aggregation control was constantly observed with macroscopic aggregates of ≤ 5mm.

Once again there is no specific literature on the co-aggregation of the Infloran probiotics with potential GI pathogens. Tareb et al. (2013) characterised the co-aggregation of *L. rhamnosus* GG and *L. farciminis* with pathogens such as *E. coli*, *Salmonella* spp., *Campylobacter* spp. and *Listeria monocytogenes*. Tareb et al. discovered the probiotics adhesion was mediated by carbohydrate-lectin interaction.[143] Kos et al (2003) characterised the auto aggregation of the probiotic strain *L. acidophilus* 92 [144]. Their study found a strong correlation between the probiotics auto aggregation properties and the ability to adhere to epithelial cells, concluding that strains with this property would be better candidates for probiotic preparations as they are more likely to persist in the gastrointestinal tract. Both of the Infloran probiotic strains were able to auto aggregate, and this theoretically means they should be able to persist longer in the neonatal gut. Of course a potentially pathogenic strain such as *K. pneumoniae* ss. *oxytoca* is also able to auto aggregate and this would also increase their persistence in the GI tract as well.

### 4.1.3 Probiotic Strains from Infloran & Inhibition of Growth of NEC-associated Strains in Liquid Media

The broth antagonism assays in this study demonstrated the effect of Infloran probiotic conditioned media on the four NEC associated strains. Both *L. acidophilus* and *B. infantis* had similar stationary phase culture pH ~ 4.5. With all the NEC associated bacteria, the conditioned media were capable of significantly inhibiting their growth, and this effect was rapidly reversed with dilution of the media. Of the four NEC strains, the only one which was able to grow in the presence of such a low pH was *K. pneumoniae* ss. *oxytoca* and this was
towards the end of the incubation (Figure 4 A). *Klebsiella* spp. have been regularly implicated in cases of NEC [21, 62, 145], and have been previously identified as being able to resist low pH[146]. This ability to resist low pH was also observed in the neonatal formula growth assays, as will be discussed later on.

Following demonstration that conditioned media from the probiotics were able to prevent the growth of the NEC bacteria, thought to primarily be the result of lactic acid lowering the pH, it was decided to supplement the same media with lactic acid to a comparable pH. These experiments also showed a similar pattern of inhibition to the conditioned media, but the drop off in inhibition was much more marked with all the NEC strains displaying similar growth to the media-only control with the 1/4 dilution. Shiou et al. (2013) discuss a rodent model of NEC where a combination of *Lactobacillus acidophilus*, *Bifidobacterium infantis* and *Lactobacillus plantarum* conditioned media were able to protect the GI tract of the rats from damage due to hypoxia[147]. However, they did not investigate whether the conditioned media induced changes in the intestinal microflora.

Another significant observation is that the error bars were much tighter for the lactic acid supplemented media than the probiotic conditioned media. As it was not possible to do detailed analysis of the components within the conditioned media; this is only speculation, but it could be theorised that the variation seen with the dilutions was due to production of other antimicrobial compounds by the probiotics. This theory is supported by work of Corr et al. (2007) and Lee et al. (2008) who demonstrated the ability of multiple strains of probiotic bacteria to produce bacteriocins able to inhibit GI tract pathogens. Corr et al. demonstrated an *L. salivarius* producing a class II bacteriocin, Abp118, which is active against *Listeria monocytogenes* [148]. Lee (2008) demonstrated a cluster of sub class I broad spectrum lantibiotics in the genome of *Bifidobacterium longum* [149]. They demonstrated the presence of bacteriocins through a combination of sequencing and genetic knock out experiments. However the present study no bacteriocin production was detected in either the solid or liquid medium antagonism assays. There are also other metabolites produced by LAB such as acetic acid which may also contribute to antibacterial activity [150, 151].
4.2 \( ^3\)H-Thymidine Attachment Assays with the Infloran Probiotic Strains and NEC-associated Strains

As mentioned in the results, there was inconsistency between the \( ^3\)H-thymidine assays. After the attachment time optimisation experiments, which had fairly consistent results, the controls became what could be described as ‘bi-polar’. The positive and negative controls often gave the opposite result from what was expected. This was initially thought to be due to issues with purity of the cultures, or inconsistent labelling efficiency with the \( ^3\)H-thymidine. These possibilities were ruled out by gram stain, colony morphology and reviewing the labelling efficiency of the control stains. Further optimisation assays were performed by running just the controls on their own, to minimise investigator error, giving more consistent results.

It was decided to run the next phase of experiments with the NEC strains and probiotics in co-culture. Again problems were encountered with the control strains and although the results were suggestive of the probiotics having an effect on attachment, they had to be disregarded. This assay was based on papers where they had successfully investigated attachment of Gram positive cocci, not Gram negative rods, which could have contributed to the inconsistent results observed in this study [131, 160, 133, 152, 153].

In light of inconsistencies with this assay a change to attachment assays using Giemsa stained cells was made. During the Giemsa assays it was discovered the negative control had a strong affinity for attaching to the plastic of the chamber slides. This was proposed as the reason for the erroneous results, as the \( ^3\)H-labelled bacteria attached to the plastic chamber slide were giving false positives. The \( ^3\)H-Thimidine assay could not distinguish between the bacteria associated with the HT-29 and those simply attached to the surface of the 96-well plate. One possible way around this issue could have been growing the HT-29 tissue culture to 100% confluence, thus making sure there was no plastic slide available for the \( C. \) freundii bacteria to attach to. Unfortunately this was not attempted.

This phenomenon of getting erroneous results from \( ^3\)H-Thimidine has been reported before by Le Blay et al. (2004), where their study compared the efficacy of \( ^3\)H-thymidine, ELISA and total count methods. Le Blay et al. demonstrated that \( ^3\)H-thymidine was a good tool for estimating the attachment of \( E. \) coli O157, well-known for adhering to GI tract epithelia [117, 154], whereas, previous work with a \( Bifidobacterium longum \) strain, had shown it to have
poor adhesion [155]. In this study non-specific binding to plastic gave inaccurate results with $^3$H-thymidine and ELISA when compared to plate count and direct microscopy methods. They also mentioned that radio labelling of bacteria is a lot more efficient when used with 6-well plates, compared to the 96-well plates were used in this study [156]. The use of 96-well plates along with adherence to plastic may explain the inconsistencies observed in this project. Cunliffe et al. (1999) did work on bacterial attachment to synthetic surfaces. The *E. coli* they used was not particularly good at adhering to their synthetic surfaces. However, it was shown that other known GI tract pathogens such as *Listeria monocytogenes* and *Salmonella Typhimurium* were quite capable of adhering to synthetic surfaces [157].

The use of hotter isotopes such as $^{14}$C and $^{32}$P were considered as other options for labelling of the NEC bacteria and enumeration of attachment. In the end it was decided to attempt Giemsa staining and direct microscopic observation instead, as a significant amount of time had been spent already on the $^3$H-Thimidine attachment assays, and a different isotope was unlikely to alleviate the problems that had been experienced.

### 4.3 Giemsa Attachment Assays with the Infloran Probiotic Strains and NEC-associated Strains

The Giemsa staining and direct microscopic observation of the NEC strains and probiotics was much more successful than the $^3$H-thymidine assays. Giemsa gave good contrast between the HT-29 cells and the bacteria, and also differentially stained the NEC and probiotic strains. It also had a significant advantage over radio labelling as it was possible to both enumerate the number of bacteria attached to the HT-29 cells as well discern the patterns of attachment.

#### 4.3.1 Single Strain Attachment Assays

The attachment patterns observed with the NEC strains, controls and probiotics were all different and distinctive. As a recap, the three major types of attachment are: localised, diffuse and enteroaggregative adherence. Diffuse adherence is seen when the bacteria cover the whole surface or most of the surface of an epithelial cell. Localised adherence has bacteria attached to one or a few sites on the cell surface [114]. Enteroaggregative adherence has a characteristic ‘log-jam’ or ‘stacked-brick’ appearance that occurs on the surface of the cells and continues on the slide surface (if present).
The contrast between the NEC associated strains isolated by Brooks et al. (2006) and the \textit{C. sakazakii} strains from ESR, was quite stark [62]. The \textit{C. sakazakii} strains both had limited localised adherence and a low total number of attached bacteria, whereas the \textit{E. cloacae} and \textit{K. pneumoniae ss. oxytoca} had a much higher total attachment and showed more diverse patterns with a combination of diffuse and localised adherence. Considering that \textit{C. sakazakii} has been implicated in disease it is interesting to find they had less association \textit{in vitro} with GI epithelial derived cell line. \textit{C. sakazakii} 50 was isolated from a tin of dried milk, and many of the \textit{C. sakazakii} strains that have been implicated in NEC were thought to be from milk powder prepared for the infants [123, 124]. The \textit{C. sakazakii} 2029 was from a throat swab, and it is reasonable to assume it has the ability to survive attached to human epithelia, albeit from the upper GI tract. The \textit{E. cloacae} and \textit{K. pneumoniae ss. oxytoca} on the other hand were isolated from confirmed Bells criteria stage III and II NEC infants respectively. \textit{E. cloacae} was observed to have a more diffuse type adherence pattern with some localised indirect associations and the \textit{K. pneumoniae ss. oxytoca} a diffuse adherence pattern. Different strains of enteropathogenic \textit{E. coli} demonstrate both localised and diffuse attachment patterns, and both are represented in diarrheal disease in adults and infants [112, 158]. Thus the observation of these attachment patterns in the four NEC-associated strains investigated in this study is indicative of their ability to cause disease. As with most pathogenic processes association and adherence to the host cells are the first steps in the infection of a host.

The controls behaved as expected, in contrast to the results gained in the $^{3}$H-thymidine experiments, thereby confirming these strains as suitable negative and positive controls. It is interesting to note in the literature that our positive control, \textit{E. coli} O111, is an EPEC strain usually observed with a localised adherence pattern, though strains of that serotype have also been observed with diffuse adherence [114]. In this investigation, aggregative adherence was observed in addition to localised and diffuse adherence patterns. The negative control, \textit{C. freundii} had previously been identified by our research group to be a non-attaching strain. This was confirmed in the observed Giemsa fields. As previously mentioned, it was observed to have an affinity for attaching directly to the plastic of the microscope slides and this was theorised to be the primary reason for the erroneous results observed in the $^{3}$H-Thimidine assays.
The Infloran probiotics were both able to adhere to the HT-29 cells. It was noted *B. infantis* formed a very distinct pattern of aggregative attachment, whereas the *L. acidophilus* in contrast, showed variable attachment which sometimes looked like localised, and sometimes like diffuse attachment. If the probiotics can attach to the epithelia in vivo this theoretically may help them to persist in the GI tract on the patches of the mucosa not covered by the scantily produced mucus. Other researchers have identified adhesion to human cells in similar patterns to those observed in the present study. Bernet et al (1993), investigated the adhesion on *Bifidobacteria* spp. to Caco-2 cells, and although they did not describe the adherence pattern, their electron micrographs had a very similar adherence pattern to the *B. infantis* used in this study [159]. The attachment properties of *Lactobacilli* spp. are fairly strain specific and there are studies showing *L. acidophilus* with both localised and diffuse attachment, as well as direct attachment to mucous in the gut of pigs [160] [161]. The HT-29 cell line used in this study did not produce any mucus, this it was not possible to assess whether the *L. acidophilus* contained in the Infloran formulation could attach directly to mucus. A mucus secreting cell line like HT-29 MTX would be required for this experiment.

4.3.2 Co-attachment Assays
Consistent aggregative adherence patterns emerged when both Infloran probiotic strains and the NEC strains were in co-culture with HT-29 cells. This pattern was only seen when the two Infloran strains were incubated together. Observing the Giemsa stained fields with a single probiotic and a NEC or control strain, the patterning was still aggregative attachment, but often the association did not appear to be as close, or involve as many bacteria. This gives a putative reason for the previous observation in the co-aggregation assays, where much larger aggregates were observed when mixing the two Infloran probiotics with a given NEC or control strain.

Current literature does not note this particular pattern of adherence when *Lactobacilli* and *Bifidobacteria* are cultured together, although many papers have looked at the indirect effect when two or more probiotic strains are used together. Collado et al. (2008) demonstrated that various combinations of *Streptococcus thermophiles*, *Lactobacillus rhamnosus*, *Lactobacillus paracasei* and *Bifidobacterium lactis* were able to significantly displace *C. sakazakii* 2029 from preparations of human intestinal mucus[162]. *Lactobacillus GG* and *Lactobacillus delbrueckii* subsp. *bulgaricus* were shown by Ouwehand et al. (2000) to enhance the adherence of *B. lactis* in a human GI mucosal model [163]. Salminen et al. 2010 and
Timmerman et al. (2004) have produced very comprehensive reviews of probiotic interaction with various pathogens. They identified a number of preparations that display an additive effect when used together, although none of the mentioned papers have microscopic examples of the interaction on human GI tissue cells [69, 135]. Gopal et al. (2000) do have scanning electron microscope (SEM) pictures of *L. rhamnosus* and *B. lactis* adhering to mucus secreting HT-29-MTX cells, and demonstrate their ability to reduce the adherence and invasion of *E. coli* O157. However Gopal et al. do not show an SEM picture of all three strains together [164]. Bernet et al (2003) tested 13 different *Bifidobacterium* spp. and identified a *B. infantis* and *B. breve* that were able to inhibit the cell association of ETEC, EPEC and DA *E. coli* as well as an *S. Typhimurium* on Caco-2 cells in a dose dependant manner [159]. Unfortunately the authors did not have any electron or light micrographs of this interaction so it is not possible to directly compare their observations to those in this study.

### 4.4 Invasion Assays with the Infloran Probiotic Strains and NEC-associated Strains

As described in the introduction, NEC associated bacteria are believed to translocate from the intestinal lumen into the intestinal wall of susceptible neonates by the paracellular and transcellular routes [1]. The invasion assay used in the present study models the transcellular route, which requires internalisation of bacteria in the enterocyte. Brooks et al. isolated the *E. cloacae* and *K. pneumoniae* ss. *oxytoca* strains used in this study from the faeces of neonates with stage II and III NEC [62], but it is not known whether these particular isolates translocated in the affected infants. Nevertheless, they are representative of the Gram negative NEC-associated bacteria known to translocate in vivo, as evidenced by their occurrence in the bloodstream of infants with NEC [165].

*C. sakazakii* has been demonstrated in previous studies to have the ability to invade epithelia. Hunter et al. (2009) observed *C. sakazakii* invasion in both the GI epithelia of a hypoxic rat model and a rat intestinal cell line (IEC-6) [102]. As well as this Singamsetty et al. (2008) demonstrated invasion of human brain microvascular endothelial Cells (HBMEC) by *C. sakazakii* [166]. In the present study the NEC-associated stains were all observed to be able to invade HT-29 cells at a low level, ≤ 1% of initial inoculum. However when the total numbers of invading bacteria were taken into account, it was found the total CFU was similar to what Singamsetty observed in HBMEC, and they believed this to be a level capable of causing disease. Admittedly brain microvascular endothelial cells are not GI tract epithelia, but the
numbers were very close and worth mentioning. When the NEC strains were in co-culture with the probiotic strains it was impossible to see a significant effect as the numbers were simply too close to our detection limit, and too variable. In a personal communication with Dr Dowd the variability of invasion assays was discussed, and it was pointed out that invasion assays can have a significant amount of day to day variability. However, the relative level of invasion compared to the control should even out over the course of experiments (Dowd, GC, Personal communication, November 2013). However, it was observed that the probiotic formulation had a significant inhibitory effect on the invasiveness of S. Typhimurium, which was in agreement with previous studies which demonstrated an up to 2 x log\textsubscript{10} reduction in invasion and/or attachment [72, 89, 167]. The probiotics themselves showed no ability to invade HT-29 cells.

One possibility for the discrepancy between the observed invasiveness and the apparent \textit{in vivo} invasiveness of the NEC strains is the mechanism by which the NEC bacteria translocate through the GI epithelia. An invasive pathogen like S. Typhimurium invades the GI epithelium via the transcellular route, inducing uptake directly into the epithelial cells, hijacking the cellular surface receptors and their downstream regulation [127, 168]. Looking at the invasion assay results it can be inferred the NEC strains are not as capable invaders as the S. Typhimurium control. The small amount of invasion observed may have been due to the epithelia acting as antigen presenting cells as described by Hershberg et al. (2000) [169]. Hypothetically, NEC associated strains can translocate by causing localised inflammation, which will loosen the tight junctions between the GI epithelial cells and allow translocation through the para-cellular route [1]. As well as this, it is documented that preterm infants >33 weeks gestation have poor GI tract tight junctions [100, 170]. Therefore, this may allow the NEC associated strains to translocate the GI epithelial layer independently of direct invasion of the epithelial cells. Once translocated, the NEC strains can go on to cause pneumatosis intestinalis and other NEC related symptoms. Therefore, it could be postulated that one mechanism by which the Infloran probiotics assert their protection of the neonate is by the pathogen-probiotic aggregation acting as a physical block to para-cellular invasion. If the putative NEC causing strains are bound up to large amounts of \textit{L. acidophilus} and \textit{B. infantis} it will be much harder to pass through the poorly formed tight junctions, or loose tight junctions caused by localised inflammation.
4.5 Neonatal Formula Growth Assays

Growth of NEC-associated bacteria in undigested infant formula in the bowel of infants is believed to be a prerequisite for NEC [15, 171, 172]. Therefore it was of interest to determine whether the probiotic bacteria could inhibit the growth of the NEC strains in formula milk, and bring together several observations from the earlier assays.

4.5.1 Co-culture Growth Curve

This first experiment with neonatal formula was inadvertently undertaken in aerobic conditions. This allowed the NEC strains to overgrow the probiotic strains, and also did not account for the gut being an anaerobic or near anaerobic environment. Thus the results have to be disregarded.

4.5.2 Probiotic Pre-incubation

The probiotic pre-incubation experiment was a lot more successful. Unfortunately due to time constraints it was not possible to perform these experiments enough times to get meaningful statistics. Nevertheless, the trend observed was indicative of a significant reduction in growth of the all the NEC strains. Once again, K. pneumoniae ss. oxytoca displayed pH tolerance, as it was the only strain to grow beyond its initial inoculum as was indicated in the conditioned media experiments earlier, but this was still far below the level of growth the NEC-associated strains attained in the non-probiotic treated formula (1 x 10^9 cfu/ml or more). These experiments demonstrated the Infloran probiotic’s ability to create an acidified environment; pH ~4, despite the buffering capacity of milk. It seems likely that the acidity may have inhibited the growth of the NEC-associated bacteria. The inhibition of the NEC strains was more apparent with longer three and six hour pre-incubation times of the probiotics. This effect was independent of the pH, as the pH was consistent (pH ~4) regardless of the pre-incubation time. It was theorised this may have been due to the probiotic strains being able to produce more bacteriocins and bacteriocin like inhibitory substances (BLIS) with longer incubations, as has been previously described [82]. However, as noted earlier, bacteriocins were not detected in this present study. Another explanation is that the probiotics consumed growth substrates rendering them unavailable to the NEC strains, thus longer pre-incubation times result in less substrate for the NEC strains. The primary sugar contained in the neonatal formula is lactose and will be used preferentially by the probiotic bacteria, with the by-product of lactic acid. It is highly likely that the mechanism by which the growth of the NEC strains is inhibited is a combination of all the factors discussed above.
These results correlate with the observations in the conditioned media experiments, as growth of every NEC-associated strain was inhibited by the *L. acidophilus* and *B. infantis* conditioned media, and was also inhibited by the lactic acid treated media (pH 5). In these experiments pH ~4 was consistently observed in the formula growing the probiotic strains and this was accompanied by the NEC strains either staying at the level of their initial inoculum or being inhibited to point where fewer NEC-associated bacteria were detected than initially added. Also, as seen in the conditioned media assays *K. pneumoniae ss. oxytoca* was able to resist this lower pH.

As the neonatal formula was opaque, it was not possible to observe aggregations like those described in the co-aggregation assays and Giemsa attachment assays. In the light of our other observations it seems more than likely that they were present. This aggregative pattern could also explain the inhibition of the NEC strains in the milk formula. If the NEC strains were in a large aggregate surrounded by both each other and the Infloran probiotics it may limit access to substrate, and therefore inhibit growth, even though they may be in the presence of a large amount of undigested formula.

Of the two types of infant formula used the Pfizer S-26 Gold, designed for full term infants, and was observed to have a more inhibitory effect when the probiotics were in co-culture with the NEC-associated strains, than the second formula, Nestle PreNAN Gold designed for preterm infants. An attempt was made to find the ingredients list for the S-26 Gold on the Pfizer website and also by directly e-mailing the company. It was hoped the ingredients would reveal a pre-biotic or otherwise synergistic compound that could explain this observation. However neither approach was successful [86, 137, 173].

Of all the experiments performed for this study these experiments were the most interesting set of results. However time constraints prevented their completion. Although the experiments were interesting they are not without their limitations. The experiment was performed in a completely anaerobic environment, as discussed in the introduction the neonatal gut is an aerobic environment at birth, and the *Eh* gradually decreases as the ecology of the gut moves towards obligate anaerobes. NEC generally occurs 7-20 days post-partum, and at this stage the gut is not completely anaerobic, thus it may be more accurate to perform these experiments in a microaerophilic environment. This is unlikely to effect the growth of the
NEC strains which are all facultative anaerobes, but may change the growth rate of the obligate anaerobic Infloran probiotics.

4.6 Conclusions
This study has identified several possible mechanisms by which the Infloran probiotics could exert their protective effect on the preterm and VLBW infants guts. Inhibition of growth of the NEC-associated strains by the probiotics was observed in the solid media, liquid media and infant formula growth experiments. In all three types of experiment, this inhibition was considered to be in part due to acid production by the Infloran strains, although other factors such as substrate utilisation and secondary metabolites such as bacteriocins, were identified as possible causes. Evidence of an aggregative adherence pattern was observed in both the co-aggregation assays and Giemsa attachment assays, when the Infloran probiotics were mixed with the NEC-associated strains. In vivo these large aggregates may also inhibit the growth of the NEC-associated strains by physically blocking their access to substrate provided by the undigested milk formula. The aggregates may also prevent blocking adherence of NEC-associated bacteria to the intestinal mucosa, making them less likely to cause disease. The Infloran probiotics were able to significantly inhibit the invasion of S. Typhimurium in HT-29 cells, but no statistical difference was observed in trascellular invasion of NEC-associated with and without probiotic. The aggregations formed when Infloran probiotics interacted with NEC-associated bacteria may inhibit translocation via the paracellular route.

In conclusion, probiotic formulation Infloran was able to inhibit the growth of NEC-associated strains in an in vitro model of NEC, has demonstrated distinctive adherence patterns with the human cell line HT-29 and formed aggregates with the NEC-associated bacteria tested. Thus this thesis has identified possible mechanisms for the protective effect against NEC observed in vivo with Infloran.

4.7 Future Directions
While this study has identified some novel interactions between the Infloran probiotic formulation and the NEC-associated strains, further work is required to better define this interaction and confirm the same type of interaction occurs with other NEC-associated pathogens. The attachment experiments need to be repeated on a Caco-2 tissue cell line, as
Caco-2 is a large bowel undifferentiated cell line commonly used as a model for the small bowel of neonates [19, 174]. Scanning electron microscopy would be very informative and would give a 3D image of the cell to cell interaction between the different bacterial strains as well as the Caco-2/HT-29 cells. Caco-2 cells could be used to perform tight junction assays to elucidate if the NEC-associated strains are indeed translocating via the para-cellular route and whether this is inhibited by the probiotics. Further work to detect and identify the specific bacteriocins and BLIS *L. acidophilus* and *B. infantis* may produce. More repeats of the formula growth experiments are required to see if the observed inhibition was indeed significant and should also be repeated with other neonatal formula brands in use in NICU’s.
5 References


175. www.21food.com, S26 Gold Alpha Pro Step 1 Formula 900g, Milk Powder 900g. 2014.
6 Appendix

6.1 M\textsuperscript{c}Farland 0.5 Opacity Standard

![Total Plate Counts at McFarland 0.5](image)

**Figure 42. Total viable counts for all NEC-associated, controls and Infloran strains**

Viable drop plate counts from all strains at M\textsuperscript{c}Farland 0.5 opacity standard. All viable counts were statistically similar. Therefore the M\textsuperscript{c}Farland 0.5 opacity standard was considered to be equal to $1 \times 10^8$ cfu/mL.
6.2 $^3$H-Thymidine Co-attachment experiments

Table 20. $^3$H-Thymidine attachment, mean and SEM of single co-attached probiotics and single strains

<table>
<thead>
<tr>
<th></th>
<th><strong>B. infantis 1:1</strong></th>
<th><strong>L. acidophilus 1:1</strong></th>
<th><strong>Single Strains</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SEM</td>
<td>N</td>
</tr>
<tr>
<td><strong>C. sakazakii 50</strong></td>
<td>1.468</td>
<td>0.221</td>
<td>10</td>
</tr>
<tr>
<td><strong>C. sakazakii 2029</strong></td>
<td>0.674</td>
<td>0.119</td>
<td>10</td>
</tr>
<tr>
<td><strong>E. cloacae</strong></td>
<td>5.768</td>
<td>0.904</td>
<td>10</td>
</tr>
<tr>
<td><strong>K. pneumoniae ss. oxytoca</strong></td>
<td>1.226</td>
<td>0.337</td>
<td>10</td>
</tr>
<tr>
<td><strong>E. coli O111</strong></td>
<td>2.293</td>
<td>0.457</td>
<td>10</td>
</tr>
<tr>
<td><strong>C. freundii</strong></td>
<td>1.083</td>
<td>0.179</td>
<td>10</td>
</tr>
<tr>
<td><strong>B. infantis</strong></td>
<td>6.761</td>
<td>2.414</td>
<td>10</td>
</tr>
<tr>
<td><strong>L. acidophilus</strong></td>
<td>12.126</td>
<td>1.085</td>
<td>10</td>
</tr>
</tbody>
</table>
Significant differences were shown between probiotic treated and untreated *E. cloacae, K. pneumoniae* ss. *oxytoca, E. coli* O111 and *C. freundii*. All had P-values of <0.0003. There was no statistical difference shown between the single strain control treatments of *E. coli* O111 and *C. freundii*, thus this data had to be disregarded.
Table 21. $^3$H-Thymidine Co-attachment, *C sakazakii* strains and controls only. Mean and SEM

<table>
<thead>
<tr>
<th></th>
<th>L. acidophilus 1:1</th>
<th></th>
<th>B. infantis 1:1</th>
<th></th>
<th>Single Strains</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SEM</td>
<td>N</td>
<td>Mean</td>
<td>SEM</td>
</tr>
<tr>
<td><em>C. sakazakii 50</em></td>
<td>0.781</td>
<td>0.054</td>
<td>10</td>
<td>0.986</td>
<td>0.140</td>
</tr>
<tr>
<td><em>C. sakazakii 2029</em></td>
<td>0.845</td>
<td>0.061</td>
<td>10</td>
<td>1.599</td>
<td>0.189</td>
</tr>
<tr>
<td><em>E. coli O111</em></td>
<td>3.677</td>
<td>0.675</td>
<td>10</td>
<td>1.830</td>
<td>0.159</td>
</tr>
<tr>
<td><em>C. freundii</em></td>
<td>5.194</td>
<td>0.735</td>
<td>10</td>
<td>5.701</td>
<td>0.384</td>
</tr>
<tr>
<td><em>B. infantis</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>L. acidophilus</em></td>
<td></td>
<td></td>
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</tbody>
</table>

There were no significant differences between the probiotic treated and non-treated bacteria. The single strain positive and negative control was significantly different with a P-value of <0.0001, the negative control was higher than the positive control and as above the data had to be disregarded.
6.3 Giemsa Attachment Assays Pictures

The rest of the raw images taken for the Giemsa attachment assays are provided on the 32 Gb flash drive that came with this thesis.

6.4 Neonatal Formula Ingredients

6.4.1 Pfizer S-26 Gold

Lactose, vegetable oils [palm, coconut, oleic (sunflower or safflower), soybean], non-fat milk powder, emulsifiers (soy lecithin, monoglycerides), long chain polyunsaturated fatty acids from single-cell sources [arachidonic acid (AA), docosahexaenoic acid (DHA)], L-methionine, taurine, nucleotides (cytidine-5'-monophosphate, disodium uridine-5'-monophosphate, adenosine-5'-monophosphate, disodium inosine-5'-monophosphate, disodium guanosine-5'-monophosphate), antioxidants (mixed tocopherols concentrate, ascorbyl palmitate).

Minerals: Calcium carbonate, sodium citrate, magnesium chloride, potassium bicarbonate, calcium hydroxide, calcium chloride, potassium chloride, ferrous sulphate, zinc sulphate, copper sulphate, manganese sulphate; potassium iodide, sodium selenite.


S-26 Gold Alpha Pro protein source is alpha-lactalbumin enriched whey and non-fat milk. Contains milk and soy lecithin. [175]

NB. This is not from an official source, this it should only be viewed as an approximate guide to the ingredients.