INTERACTION BETWEEN *Shigella flexneri* AND INTESTINAL EPITHELIAL CELLS CO-INFECTED WITH SELECTED ENTERIC BACTERIA

BY

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DECLARATION

I, John Benjamin Ochieng, declare that the work presented herein is my original work and has not been presented for the award of any degree or publication anywhere

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I dedicate this study to my family for their perseverance and patience during the study and for creating enabling environment to pursue this course.

ABSTRACT

Bacteria, intestinal epithelium, and host innate immune responses are among the most critical interacting factors that determine the fate of bacterial infections and disease outcomes. Recent studies have described multiple infections with evidence of more severe diarrhoea and molecular detection methods, suggesting the association of certain pathogens and commensal bacteria with more aggressive *Shigella* infection. However, the interaction between *Shigella flexneri* and human intestinal epithelial cells co-infected with other intestinal commensal bacteria remains unknown. Therefore, the current study investigated the interaction between *S. flexneri* and human intestinal epithelial cells co-infected with selected intestinal commensal bacteria. This was an experimental study and the bacteria strains tested were selected based on co-infections data obtained from previous studies. The strains were obtained from American Type Culture Collection (ATCC) and routinely grown in Luria Battani agar medium. Human colonic T84 intestinal epithelial cells, which maintain phenotypic characteristics of colonic script cells, were maintained in Dulbecco's Modified Eagle Medium-F-12 (DMEM-F12) and polarized in transwell permeable support cell culture inserts. Polarized cells were infected apically with *S. flexneri* 2457T and selected enteric bacteria and assessed for trans-epithelial electric resistance (TEER), invasion, cytotoxicity and cytokine induction. Furthermore, the effects on cellular morphology on non-polarized cells were assessed by scanning electron microscopy. The strains were tested in triplicates and repeated thrice. The current study showed that interaction between *S. flexneri* and *Serratia marcescens* or *Citrobacter freundii* influences invasion, cytotoxicity and Interleukin-8 (IL-8) production by intestinal epithelial cells *in-vitro*. A synergistic invasion effect appeared where the intestinal epithelial cells were co-infected with *S. flexneri* and *S. marcescens*, and *S. flexneri* and *C. freundii* but the difference was not statistically significant between the bacteria strains tested ($p>0.05$). However, TEER dropped significantly in monolayers infected with *S. marcescens* ($p<0.0001$) and in those co-infected with *S. flexneri* and *S. marcescens* ($p<0.001$) accompanied with higher IL-8 response (444pg/mL). A similar IL-8 response appeared in cells co-infected with *S. flexneri* and *C. freundii* (508pg/mL). Lactate Dehydrogenase (LDH) concentration in supernatants from cells co-infected with *S. flexneri* and *S. marcescens* appeared much greater than the concentrations measured from cells co-infected with other enteric bacteria tested ($p<0.001$) suggesting increased cytotoxicity. A similar effect was demonstrated by scanning electron microscope (SEM) which revealed loss of microvilli and vacuolization in the monolayers following exposure to *S. marcescens.* Thus, this study demonstrated that (i) some bacteria previously considered non-pathogenic have the potential to interact with intestinal epithelial cells in culture to induce dramatic alterations similar to those produced by known enteric pathogens (ii) *S. flexneri* and *S. marcescens* or *C. freundii* co-infections can potentially cause severe enteric infection through elevated inflammatory responses and epithelial cell destruction, and (ii) *S. marcescens* manifested significant pathologic effect on epithelial cell morphology thus confirming cytotoxic effect. The finding from this study provides evidence that *S. flexneri* and *S. marcescens* or *C. freundii* can cause deleterious enteric infection in a synergistic manner. Therefore, epidemiologic studies should consider possible association between these microorganisms and diarrhoea in pathogenic states such as necrotizing enterocolitis and severe diarrhoea. The evidence of severe disease in co-infections calls for clinical and laboratory investigations to focus on a wider panel to include *S. marcescens* and *C. freundii* for diagnosis of infectious diarrhoeal diseases.

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DEFINITION OF TERMS

Apoptosis – is the programmed disintegration of cells into membrane-bound particles that are then eliminated by phagocytosis or by shedding.

Bacillary dysentery – is a form of severe bacterial diarrhoea caused by bacilli bacteria and associated with blood in the faeces and often accompanied by painful straining to pass stool, fever and malaise.

Enterobacteriaceae – are a large family of enteric bacteria, including many of the more familiar pathogens, such as *Shigella spp.* and *Escherichia coli*. They are facultative anaerobic gram negative bacilli, non-spore forming, oxidase and catalase negative.

Gastroenteritis – is a general term referring to inflammation or infection of the gastrointestinal tract.

Morbidity – is the state of being diseased or sick.

Mortality – refers to death.

Pathogen – refers to a biological agent that causes disease or illness to its host.

Virulence – refers to the degree of pathogenicity of a microbe, or in other words the relative ability of a microbe to cause disease.

ABBREVIATIONS

ATCC – American Type Culture Collection

DMEM – Dalbecco's Modified Eagle's Medium

FBS – Foetal Bovine Serum

HIV – Human Immunodeficiency Virus

IL – Interleukin

LDH – Lactate Dehydrogenase

PMN – Polymorphonuclear

SEM – Scanning electron microscope

TEER – Transepithelial Electric Resistance

TNF – Tumour Necrosis Factor

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CHAPTER ONE

INTRODUCTION

The interactions among bacteria, the intestinal epithelium, and host innate immune responses are among the most critical factors that determine the fate of bacterial infections and disease outcomes. In the gut epithelium, the elements that provide defence against infection include: commensal microbiota, epithelium integrity, and innate immune system. The commensal bacteria can compete with foreign bacteria and interfere with their colonization of the epithelial surface (Leser & Molbak, 2009). Both the epithelial monolayer and the mucosal surface act as physical and biological barriers against microbial invaders. The integrity of the epithelial monolayer is sustained by tight cell-cell junctions, and the mucosal surface is covered by a mucin layer containing various digestive enzymes, Muc2, secretory Immunoglobulin A (IgA), and other microbial agents including β-defensins, cathelicidins, bactericidal/permeability-increasing protein and chemokines (Mason & Huffnagle, 2009). Thus, the local innate host defence systems acts as the first line of defence in response to a pathogen when it comes in contact with the cell. The resolution of this encounter determines whether the interaction leads to infection and overt disease (Phalipon & Sansonetti, 1999).

Bacterial co-infections of the gastrointestinal tract have recently been recognized in surveillance and case control studies (Kotloff *et al.,* 2013; Nair *et al.*, 2010). The most prevalent bacterial aetiologies of diarrhoea in developing countries include *Shigella* spp.,

Salmonella spp., diarrhoeagenic *E. coli, Campylobacter* spp., *Vibrio cholerae* and *Aeromonas* spp. whereby *Shigella* spp. is the most prevalent (Kotloff *et al.,* 1999) with *S. flexneri* being the most dominant species (Brooks *et al.,* 2006).

Shigella is a genus in the family *Enterobacteriaceae* and are Gram-negative, non-motile, non-spore forming, rod-shaped bacteria. The genus *Shigella* is divided into four species (sub-groups) namely: *Shigella dysenteriae, Shigella flexneri, Shigella boydii* and *Shigella sonnei* (Murray, 1995). The sub-groups are further divided into 38 serotypes based on O antigen variations: 13 serotypes in *S. dysenteriae,* 18 in *S. boydii*, 6 in *S. flexneri* and 1 in *S. sonnei* (Ewing, 1986; Strockbine, 2005). Shigellosis is an acute infectious disease caused by all the four species of *Shigella*.

Shigella is believed to exploit M cells to cross the epithelial barrier and gain entry into the colonic epithelium and is unable to invade the epithelial cells through the apical route (Mounier, Vasselon, Hellio, Lesourd, & Sansonetti, 1992). M cells allow intact *Shigella* to traverse into the underlying sub-epithelial pocket at the basolateral pole and are engulfed by macrophages and instead of successfully destroying the bacteria in the phagosome, the macrophage succumbs to apoptotic death (Zychlinsky, Prevost, & Sansonetti, 1992). Prior to cell death, infected macrophages release Interleukin 1-beta (IL-1β) through the direct activation of caspase-1 by *Shigella* (Zychlinsky, Fitting, Cavaillon, & Sansonetti, 1994). The pro-inflammatory nature of this cytokine results in the recruitment of polymorphonuclear cells (PMNs) that infiltrate the infected site and destabilize the epithelium (Perdomo, Gounon, & Sansonetti, 1994). Loss of integrity of

the epithelial barrier allows more bacteria to traverse into the subepithelial space and gives these organisms access to the basolateral pole of the epithelial cells (Mounier, Vasselon, Hellio, Lesourd, & Sansonetti, 1992). *Shigella* can then invade the epithelial cells lining the colon, spread from cell to cell and disseminate throughout the tissue. However, it remains unknown whether other intestinal bacteria can contribute to the disruption of the tight junctions and allow *Shigella* spp. to transverse the intestinal monolayer and invade the cell. Thus, the current study investigated the individual effect of selected commensal intestinal bacteria on infected T84 cells.

The innate immune response provides an early defence against bacterial infection. Infection of monolayers of human colonic epithelial cell lines with *Shigella* results in coordinated expression and production of a specific array of pro-inflammatory cytokines, such as Interleukin-8 (IL-8), monocyte chemoattractant protein 1, granulocyte-macrophage colony-stimulating factor and tumour necrotic factor α (TNF-α). This response is aimed at protecting the host by evoking inflammation but may also be used by the pathogen to destroy the epithelium barrier and gain access to the intestinal milieu (Sansonetti, Arondel, Huerre, Harada, & Matsushima, 1999). The alteration of the tight junction during bacterial infection results in decrease in transepithelial electric resistance (TEER) (Strauman, Harper, Harrington, Boll, & Nataro, 2010).

Recent studies reported distinct cytokine pathways involved in the host responses to different enteropathogens (Jiang *et al.,* 2003). These findings suggest that a specific cytokine response adequate to resolve one pathogen infection may be inappropriate

against a different pathogen and can lead to prolonged infection and pathogenesis. Despite the extensive reports on the pathogenicity of *Shigella* and interaction with the intestinal epithelium, information on the effect of co-infection on invasiveness of *S. flexneri* on human intestinal epithelial cells remains unknown. Therefore, the current study assessed the effect of co-infection on invasiveness of *S. flexneri* on human intestinal epithelial (HIE) cells.

Bacterial pathogens have specialized mechanisms that enable them to invade the host cell and evade natural host defence and cause disease (Lu & Walker, 2001). Apoptosis and necrosis are forms of cell death observed in normal and disease pathogenesis. Human colonic epithelial cells undergo apoptosis when infected with invasive enteric bacteria and require bacterial entry and replication (Kim *et al.,* 1998). Apoptosis results in elimination of the epithelial cells from the mucosa with little inflammatory response. Necrosis occurs in response to cell injury by the invading bacteria and results in rapture of the cell membrane and release of cellular contents often accompanied by inflammatory response (Kono & Rock, 2008) as observed during acute *Shigella* colitis (Islam, Veress, Bardhan, Lindberg, & Christensson, 1997).

Induction of apoptosis and necrosis is considered to be a recognized virulence mechanism that causes tissue damage and facilitate colonization (Krzyminska, Ochocka, & Kaznowski, 2012). The rupture causes leakage of intracellular contents including cytoplasmic enzyme, lactate dehydrogenase (LDH) into extracellular milieu. The morphological changes of the infected cell during the development of apoptosis involve

shrinkage, round-up and detachment from culture plate (Krzyminska, Ochocka, & Kaznowski, 2012). Thus, when combined with morphological observation, measuring LDH release is useful in detecting cell death due to bacterial invasion. However, intestinal epithelial cells destruction in co-infection with *Shigella* and commensal intestinal bacteria is not fully documented.

Previous studies have indicated antagonistic and synergistic forms of interaction among the enteric bacteria. Protective bacteria can enhance the barrier function of naïve epithelial cells not exposed to any pathogen. Similarly, in an interaction study between representative strains of four predominant resident bacteria of the human colon, potent unilateral antagonism against common enteropathogens was evident (Ushijima & Ozaki, 1986). The probiotic nature of these bacteria is important in control of diarrhoea. More investigations are necessary to identify similar probiotics. On the other hand, *in-vitro* studies have demonstrated molecular basis for microbial interaction which could lead to increased severity of disease *in-vivo* in individuals who are co-infected with enteropathogens (Bukholm & Kapperud, 1987; Crane, Choudhari, Naeher, & Duffey, 2006). Investigation on the possible interaction with *Shigella* is necessary to help predict the severity and consequences of co-infection.

In vitro model systems using human intestinal cell lines grown on permeable filter supports with distinct upper (luminal) and lower (basal) chambers have been employed recently for invasion studies. The T84 cell line retains many morphological characteristics of the major population of cells found in intestinal scripts and functionally

similar to normal colon (Dharmsathaphorn & Madara, 1990). Growing intestinal T84 cell lines allows the cells to grow as columnar epithelial cells with a more or less organized brush border (depending on the cell line) and to polarize with distinct apical and basolateral membranes separated by intercellular tight junctions (McCormick, Fernandez, Siber, & Maurelli, 1999). Bacterial infection of the apical surface of cultured intestinal cells under such growth more closely mimics infection of the human intestinal epithelium. Thus, this study focused on selected bacterial species found in association with diarrhoea cases from previous epidemiologic and molecular studies (Kotloff *et al.*, 2013; Pop *et al.,* 2014). The commensal bacteria taxa found to be more associated with diarrhoea and severe shigellosis cases included *Serratia marcescens, Citrobacter freundii, Enterobacter cloacae* and *Klebsiella oxytoca*. T84 cell line growth system was used to study the co-infection of *Shigella* and the selected enteric bacteria *in vitro*.

1.1 Problem statement

Diarrhoea is one of the principle causes of morbidity and mortality among children in the developing world. It is estimated that diarrhoea accounts for 1.6 – 2.5 million deaths annually and a child in the developing world experiences an average of 3 episodes of diarrhoea per year (Kotloff *et al.,* 1999). Recent studies have begun to describe multiple infections with evidence of more severe diarrhoea in multiple infections (Albert, Faruque, Faruque, Sack, & Mahalanabis, 1999). In addition, an *in vitro* study on co-infection demonstrated that enterotoxigenic *E. coli* (ETEC) toxins and enteropathogenic *E. coli* (EPEC)-induced damage to the host cell enhanced virulence of each other which lead to

more severe cell damage (Crane, Choudhari, Naeher, & Duffey, 2006). On the other hand, some bacteria interact with epithelial cells to protect them from deleterious effect of invasive bacteria as was previously observed in a study in which *S. thermophilus* increased transepithelial resistance (TEER), contrasting markedly with the fall in resistance evoked by enteroinvasive *E. coli* (EIEC) infection (Resta-Lenert & Barrett, 2003). Likewise, infection of intestinal epithelial cell lines with invasive bacteria stimulated chloride secretion and decreases barrier function (Resta-Lenert & Barrett, 2002), induce membrane ruffling (MacComick 1993) or vacuolization and cytolysis due to secretion of toxins by the invading bacteria (Cherla, Lee, & Tesh, 2003; Nataro, Hicks, Phillips, Vial, & Sears, 1996). The pathology exhibited by other commensal bacteria could enhance invasion of the intestinal epithelial cell by *Shigella*.

Recently, a multi-site case control study conducted in Asia and Africa revealed multiple infections in both cases and control (Kotloff *et al.,* 2013). Moreover, molecular detection methods used in these studies suggested the association of certain pathogens and commensal bacteria with diarrhoea and more aggressive *Shigellosis* with *S. flexneri* being the most prevalent (Pop *et al.,* 2014). However, the individual effect of the selected commensal intestinal bacteria on infected T84 cells, the effect of co-infection on invasiveness of *S. flexneri* on human intestinal epithelial (HIE) cells and the effect of *S. flexneri* and the selected commensal intestinal bacteria on epithelial cell morphology remain unknown. Therefore, the current study investigated the interaction between *Shigella flexneri* and human intestinal epithelial cells co-infected with other enteric bacteria as a co-factor micro-organism in diarrheal disease.

1.2 General objective

To investigate the interaction between *Shigella flexneri* and human intestinal epithelial cells co-infected with other enteric bacteria as a co-factor micro-organism in diarrheal disease.

1.2.1 Specific objectives

1. To evaluate the individual effect of selected commensal intestinal bacteria on infected T84 cells.
2. To assess the effect of co-infection on invasiveness of *S. flexneri* on human intestinal epithelial (HIE) cells.
3. To determine the effect of *S. flexneri* and selected commensal intestinal bacteria on epithelial cell morphology.

1.2.2 Research questions

1. What are the individual effects of selected commensal intestinal bacteria on T84 cells?
2. What is the significance of co-infection on invasiveness of *S. flexneri* on human intestinal epithelial (HIE) cells?

3. What are the effects of *S. flexneri* and selected commensal intestinal bacteria on epithelial cell morphology?

1.3 Significance of the study

Polymicrobial infections include microbial interference and synergistic effects. Previous studies indicate associations between some pathogens affecting the human gastrointestinal tract (Kotloff *et al.,* 2013; Nair *et al.,* 2010). These interactions can occur between potential pathogens or between commensals and pathogens. Live interaction of commensal bacteria with intestinal epithelial cells can protect them from invasion by other pathogens (Ushijima & Ozaki, 1986). Data on the potential beneficial effects of such bacteria could help predict chances of an individual developing infective diarrhoea episode depending on the proportion of the protective bacteria or potential probiotics. In contrast, the presence of one microorganism can predispose the host to infection by *Shigella* consecutively. Previous epidemiological and molecular studies associated multiple infections with aggressive shigellosis whereby the enteric bacterial taxa detected in diarrhoea cases with *Shigella* infection included *Serratia marcescens*, *Citrobacter freundii*, *Enterobacter cloacae* and *Klebsiella oxytoca* (Pop et al., 2014). The observation of selective associations among some gastrointestinal pathogens raises the question of how *Shigella* interacts with these intestinal bacteria *in vivo*. Understanding the physical and chemical interaction between these microorganisms with intestinal epithelial cells in co-infection can help to define potential new therapeutic and prevention strategies.

1.4 Limitations

1. In this study, the T84 cells were infected apically while *Shigella flexneri* is known to infect epithelial cell more efficiently through the baselateral. This could have affected the invasion capability of the bacteria.
2. In the co-infection experiment, the proportion of each bacterial CFUs obtained from the invasion assay could not be established.

CHAPTER TWO

LITERATURE REVIEW

Diarrhoea is one of the major causes of infant morbidity and mortality worldwide. According to World Health Organization (WHO), diarrhoea accounts for 1.6 – 2.5 million deaths annually and each child in the developing world experiences an average of three episodes of diarrhoea per year (Kosek, Bern, & Guerrant, 2003; Kotloff et al., 1999). Globally, it contributes to 0.801 million (10.5%) deaths due to infectious disorders and most of which occur in Africa (Liu *et al.,* 2012)The intestinal epithelium in composed of physical intercellular structures called tight junctions and functions as a barrier that segregates luminal material from entering the tissues (Guttman & Finlay, 2009). Since epithelial cells are among the first cells entered by many enteric pathogens, the ability of epithelial cells to respond to selected enteric bacterial strains was investigated.

2.1 Effect of commensal intestinal bacteria on infected intestinal epithelial cells

Enteric infections, with or without diarrhoea, have profound effects on intestinal absorption, nutrition, and childhood development (Petri *et al.,* 2008). Diarrheal infections may be caused by an array of bacterial, viral and parasitic pathogens. Some infections are caused by a single defined pathogen, others do not have any defined cause and a substantial number are caused by multiple pathogens (Nair *et al.,* 2010). From previous

global enteric multisite studies (GEMS), multiple infection was common and the commensal bacteria taxa found to be more associated with diarrhoea and severe shigellosis cases included *Serratia marcescens*, *Citrobacter freundii, Enterobacter cloacae* and *Klebsiella oxytoca* (Kotloff *et al.,* 2013; Pop *et al.,* 2014). However, investigations linking these intestinal bacteria with potential to infect intestinal epithelial cells *in vitro* are lacking.

Many bacteria species of the normal intestinal flora are members of the family Enterobacteriaceae. They include the coliforms which are opportunistic pathogens responsible for a wide range of infections (Guentzel, 1996). The mechanisms used by most enteric bacteria to invade host epithelial cells have been studied extensively. In a previous review, it is evident that bacterial pathogens have evolved two major strategies to colonize the intestinal epithelium. Adherent microorganisms bind to the apical pole of the intestinal epithelium, whereas invasive microorganisms disrupt and invade the epithelium (Sansonetti, 2002). Breaching the epithelial barrier has profound effect on human health and disease as it results in decrease in transepithelial electric resistance (TEER) (Strauman, Harper, Harrington, Boll, & Nataro, 2010) and results into diarrhoea through a "leak –flux" mechanism whereby water enters the lumen of the intestine through the passive movement of both water and ions (Schmitz *et al.,* 2000) inflammation and pathogenic effects (Guttman & Finlay, 2009). Invading bacteria extensively disrupts the tight junction structure (Sakaguchi, Kohler, Gu, McCormick, & Reinecker, 2002), gain access to the basolateral pole of the epithelium, invade macrophages, initiate inflammation facilitating further invasion of intestinal epithelial

cells (Mounier, Vasselon, Hellio, Lesourd, & Sansonetti, 1992), and enhance transmigration of polymorphonulear (PMN) leucocytes across the intestinal crypt epithelium which is a hallmark of active intestinal disease (McCormick, Colgan, Delp-Archer, Miller, & Madara, 1993; Sansonetti, 2002).

Several bacteria induce their own uptake into epithelial cells, the uptake processes involve different host receptors and cell signalling pathways (Galan & Bliska, 1996). Moreover, these organisms have different intracellular localizations. Nonetheless, entry of these bacteria requires rearrangement of the epithelial cell actin cytoskeleton, and is impaired by inhibitors of actin polymerization (e.g., cytochalasin D). Infection with each of these organisms results in a qualitatively similar host response in regard to the upregulated expression of immunoregulatory genes in epithelial cells. This includes increased production of pro-inflammatory cytokines and chemokine which include TNF-α, IL-1α, and IL-1β (Eckmann *et al.*, 1993; Jung *et al.*, 1995; McCormick, Colgan, Delp-Archer, Miller, & Madara, 1993). Two subfamilies of chemokines, CXC and CC, are recognized based on the arrangement of the first two of the four conserved cysteine residues in their amino acid sequences. CXC chemokines typified by IL-8 have chemoattractant properties for inflammatory calls and act specifically on neutrophils while the CC group acts on monocytes but not neutrophil (Baggiolini, Loetscher, & Moser, 1995). Recent studies have demonstrated that human intestinal epithelial cells express and secrete IL-8 in response to bacterial invasion and there was a strict relationship between the number of intracellular bacteria and the amount of secreted IL-8 (Eckmann *et al.*, 1993; Jung *et al.*, 1995).

This programming of epithelial cells to produce pro-inflammatory molecules leads to attraction of and transepithelial migration of polymorphonuclear (PMN) leucocytes, thereby disrupting the permeability of the epithelium (McCormick, Miller, Carnes, & Madara, 1995). In the case of *Shigella*, this process facilitates bacterial invasion of epithelial cells via their basolateral pole which is more permissive to bacterial entry into the cell (Perdomo, Gounon, & Sansonetti, 1994). In contrast to the differential and regulated expression of pro-inflammatory cytokines, intestinal epithelial cells do not appear to express a number of cytokines such as IL-2, IL-4, IL-5, IL-12 , or IFN-γ that are more commonly associated with antigen-specific acquired immune responses (Jung *et al.*, 1995). This suggests that products secreted by intestinal epithelial cells are likely to play a more important role in initiating and regulating the innate mucosal inflammatory response rather than antigen-specific mucosal immune responses. However, the individual effect among most commensal intestinal bacteria on infected intestinal epithelial cells remains unknown.

T84 cells are human colon-derived epithelial cells used widely in the evaluation of integrity and barrier function of the intestinal epithelium (McCormick, Colgan, Delp-Archer, Miller, & Madara, 1993; Nataro, Hicks, Phillips, Vial, & Sears, 1996). The current study evaluated the individual effect of selected commensal intestinal bacteria on infected T84 cells.

2.2 Effect of co-infection on invasiveness of *S. flexneri* on human intestinal epithelial (HIE) cells

Bacterial co-infections of the gastrointestinal tract have recently been recognized in surveillance and case control studies (Kotloff *et al.,* 2013; Nair *et al.,* 2010). However, the effect of this co-infection has not been established. In these infections, the presence of one microorganism can predispose the host to colonization by other microorganisms, or two or more non-pathogenic microorganisms together can cause disease (Peltola, Boyd, McAuley, Rehg, & McCullers, 2006).

Previous studies have indicated antagonistic and synergistic forms of interaction among the enteric bacteria. Protective bacteria can enhance the barrier function of naïve epithelial cells not exposed to any pathogen. As previously reported, exposure of cell monolayers to live *Streptococcus thermophilus* and *Lactobacillus acidophilus* significantly limited adhesion, invasion, and physiological dysfunction induced by EIEC hence protect intestinal epithelial cells from the deleterious effect of EIEC (Resta-Lenert & Barrett, 2003). Similarly, in an interaction study between representative strains of four predominant resident bacteria of the human colon, potent unilateral antagonism against common enteropathogens was evident (Ushijima & Ozaki, 1986). These forms of microbial interference whereby the presence of one microorganism generates an environment within the host that suppresses the colonization of other microorganisms has also been observed in humans who develop pseudomembranous colitis due to *Clostridium difficile* after prolonged treatment with antibiotics (Spencer, 1998). In mixed intestinal infection, where such interactions are possible, the combination could influence

the outcome of *Shigella* infection. These findings subsequently can be used to determine how the interaction affects the outcome of enteric bacterial infection.

In a recent analysis of stool from Global Enteric Multi-Centre Study (GEMS), in a case-control study of diarrhoea (Kotloff *et al.*, 2013), it was observed that co-infection in infants across all participating sites was common. These data suggested the association of certain other pathogens and commensal bacteria with diarrhoea and more aggressive *Shigella* infection. However, *in vitro* studies confirming the effect of co-infection observed in case-control studies remains non-performed.

Shigella flexneri is an invasive pathogen that causes both secretory and bloody diarrhoea (bacillary dysentery). *S. flexneri* directs its own uptake into the colonic mucosa through membrane ruffling via plasmid-encoded Mxi/Spa apparatus and secreted effector proteins that induce endocytosis of *Shigellae* by colonic M cells (Sansonetti, Tran Van Nhieu, & Egile, 1999). M cells allow intact *Shigellae* to transverse into the underlying subepithelial pocket where macrophages reside. The bacteria are then engulfed by macrophages and induce their apoptotic death (Zychlinsky, Prevost, & Sansonetti, 1992), and concurrent release of interleukin-1β (IL-1β). This process allows the bacteria to invade the epithelial cells via their basolateral pole and subsequently spread laterally from one enterocyte to another, a mechanism that activate the production of cytokines and chemokine that causes inflammation, apoptosis, tight junction disruption, and often dysentery (Sansonetti, Tran Van Nhieu, & Egile, 1999). Previous studies demonstrated that distinct mechanisms resulting in the regulation of tight junction-associated proteins are initiated

after either apical or basolateral interactions. The disruption of tight junctions is dependent on direct interaction of living *Shigellae* with intestinal epithelial cells (Sakaguchi, Kohler, Gu, McCormick, & Reinecker, 2002).

Recently, *in vitro* studies have demonstrated molecular basis for microbial interaction which could lead to increased severity of disease *in vivo* in individuals who are co-infected with enteropathogens (Bogaert, De Groot, & Hermans, 2004)As was previously reported, the degree of internalization of *C. jejuni* was significantly induced in the presence of S. Typhimurium (Bukholm & Kapperud, 1987). These co-infections can as well occur between potential pathogens or between pathogens and commensals.

Apoptosis and necrosis are major forms of cell death that occurs in normal and disease pathologies. Induction of apoptosis of the host cells by invading bacteria is recognized as a virulence mechanism that causes tissue damage and facilitates colonization (Navarre W.W and Zychlinsky, 2000). Necrotic cell death evokes inflammatory responses, is closely associated with inflammatory diseases (Komo & Rock, 2008). The major outcome of necrotic cells is permeability of cell membrane which leads to leakage of a cytoplasmic enzyme, lactate dehydrogenase (LDH), from the damaged cells. Co-infection of T84 cells with live enterotoxigenic *E. coli* in combination with enteropathogenic *E. coli* bacteria resulted in enhanced adenosine triphosphate (ATP) release and cell damage confirming mutual enhancement of virulence (Crane, Choudhari, Naeher, & Duffey, 2006).

However, with all the evidences of possible co-infection among enteric bacterial pathogens, the effect of co-infection on invasiveness of *S. flexneri* on human intestinal epithelial (HIE) cells remains unknown. Therefore, the current study assessed the effect of co-infection on invasiveness of *S. flexneri* on human intestinal epithelial (HIE) cells.

2.3 Effect of *S. flexneri* and selected commensal intestinal bacterial on epithelial cell morphology

Cells that line the intestinal mucosal surface of the intestine form a major mechanical barrier that separates the host's internal milieu from the external environment and segregates luminal materials from entering the tissue. Essential components of this epithelial fence are physical intercellular structures termed as tight junction. As described in several reviews (Aijaz, Balda, & Matter, 2006; Guttman & Finlay, 2009; Shin, Fogg, & Margolis, 2006), these junctions use a variety of transmembrane proteins coupled with cytoplasmic adapters, and the actin cytoskeleton, to attach adjacent cells together thereby forming intercellular seals.

Tight junctions also demarcate the boundary between the epical and basolateral membrane domains of a cell and serve as a barrier to intramembrane diffusion of proteins and macromolecules between the apical and basolateral membrane domains. The three main families of transmembrane proteins found in tight junctions are occludin, claudins and junctional adhesion molecules (JAM) (Furuse, Fujita, Hiiragi, Fujimoto, & Tsukita, 1998; Furuse et al., 1993; Shin, Fogg, & Margolis, 2006). Recent studies have implicated JAM proteins in the formation of intercellular junction and in epithelial barrier function

(Liu et al., 2000). Binding of *Shigellae* to intercellular junctions and rounding of a cell surrounded by bacteria and detachment from neighboring cells was evident following infection with *S. flexneri* (Mounier, Vasselon, Hellio, Lesourd, & Sansonetti, 1992) suggesting that bacteria have the capacity to pass through the tight junctions of the cell. Thus, some bacteria can bind to the intercellular junctions on the apical side and cause local disruption of the junctions and progress into the intercellular space where they mediate entry process.

In vitro experiments carried out with various epithelial cell lines have shown that invasion by *Shigellae* is a multistep process. It consists of entry into epithelial cells by induced phagocytosis, escape from the phagocytic vacuole, multiplication and spread within the epithelial cell cytoplasm, passage into adjacent epithelial cells by way of finger-like protrusions from the cell surface, and killing of the host cells particularly the macrophages (Bernardini, Mounier, d'Hauteville, Coquis-Rondon, & Sansonetti, 1989; Labrec, Schneider, Magnani, & Formal, 1964), while epithelial cell remain normal despite presence of numerous electron-translucent vacuoles (Mantis, Prevost, & Sansonetti, 1996).

While other bacteria like *Salmonella spp.* invade directly through the brush border of polarized cells after inducing disruption of the microvilli and cellular cytoskeletal rearrangements known as membrane ruffling (Finlay & Falkow, 1990), and entero-aggregative *E. coli* alters the intestinal cell morphology causing vesiculation and shedding of microvilli (Nataro, Hicks, Phillips, Vial, & Sears, 1996), entry of *S. flexneri*

occurs not through the apical surface of the cells but rather efficiently through exposed basolateral surfaces (Mounier, Vasselon, Hellio, Lesourd, & Sansonetti, 1992). On confluent monolayers, the paracellular junctions must be opened, allowing access of the bacteria to the basolateral surfaces, in order for invasion to occur (Mounier, Vasselon, Hellio, Lesourd, & Sansonetti, 1992). However, the *Shigellae* can become internalized in a loosely associated vacuole upon contact with host cell membrane and secretion of a set of invasion proteins which induces important rearrangements of the cell cytoskeleton (Sansonetti, Tran Van Nhieu, & Egile, 1999). These changes remain localized to the very area of bacterial contact on interaction with non-polarized non-phagocytic cells. Cellular extensions rise up to 10µm over the cell surface, forming a flowerlike structure. These projections eventually merge and engulf the bacterial body, in a process lasting 5–10 minutes (Sansonetti, Tran Van Nhieu, & Egile, 1999).

Given the inability of *S. flexneri* to invade through the intact brush border of mature polarized epithelial cells *in vitro*, the potential routes of entry of this organism *in vivo* may include (i) the apical surfaces of cells with disrupted brush borders; (ii) the apical surfaces of immature crypt cells or M cells, both of which lack brush borders; or (iii) the baselateral surfaces of enterocytes, accessed via the opening of intercellular junctions. Only a few inconclusive observations regarding the actual site of entry *in vivo* are available (Goldberg & Sansonetti, 1993).

Recently, strains of non-pigmented *Serratia marcescens* were shown to produce cell-contact toxins that facilitate bacterial invasion, induce haemolysis, cytotoxicity and apoptosis of host cells (Krzyminska, Ochocka, & Kaznowski, 2012). Likewise,

enteroaggregative *Escherichia coli* strain O42 was found to adhere more on T84 cells and cause time-dependent toxic effect on the cells. The toxic effect caused disruption of the microvillar layer, shedding of the microvilli by vesiculation, vacuolization, rupture and exfoliation of the monolayer (Nataro, Hicks, Phillips, Vial, & Sears, 1996).

With multiple infections with microorganisms which can potentially disrupt the brush borders, create pores or vacuoles on the monolayer, entry of *Shigellae* into the basolateral site is likely to occur and facilitate rapid invasion. However, information on morphological changes exhibited by human intestinal epithelial cells infected with the selected enteric commensal bacteria and whether these can escalate invasion by *Shigella* remains unknown. As such, the current study investigated the effect of *S. flexneri* and selected commensal intestinal bacteria on epithelial cell morphology.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study design

This was a laboratory-based experimental research study and retrospectively mined data on bacteria isolated from previous multi-site diarrheal studies for multiple infections (Kotloff *et al.,* 2013) and diarrheal microbiota (Pop *et al.,* 2014) for co-infections and possible association with diarrhoea. The commensal bacteria species found to be associated with diarrhoea and with *Shigella* co-infection were ordered from American Type Culture Collection (ATCC) and *in vitro* experiments conducted to study the effect of co-infection among the enteric bacterial isolates using T84 cells as a model.

3.2 Bacterial strains and growth conditions

The bacterial strains used for the *in vitro* models included the invasive 1143 strain of 2457T, a wild type strain of *S. flexneri* 2a which is invasive in both HeLa cells and T84 cells (McCormick, Siber, & Maurelli, 1998), *Serratia marcescens* (ATCC® 274™), *Klebsialla oxytoca Citrobacter freundii* (ATCC 6879), and *Enterobacter cloacae* (ATCC 222). *E. coli* HS strain (Levine *et al.,* 1978) which is non-invasive to human intestinal epithelial cells and/or non-invasive strain of *S. flexneri* 1270 were used as negative controls. Bacteria were cultured routinely as follows: the 2457T *S. flexneri* 2a strain were grown on Luria-Bertani (LB) agar (American Bioanalytical, 15 Erie Drive, Natick, MA 01760, USA) with Congo red at 37^{o}C aerobically for 18 – 24 hours. *E. coli* HS strain and other enteric bacteria strains were cultured on LB agar without Congo red under the same conditions as above unless indicated otherwise. A single colony of bacteria was picked to

inoculate 10ml of LB broth and incubated overnight in a shaking incubator. Ten microliters of a stationary phase culture was used to inoculate 10ml of LB broth, and the bacteria grown in a shaking incubator for approximately 3 hours at 37^{o}C aerobically to mid-exponential phase.

3.3 Growth and maintenance of cell culture

Human colonic epithelial (T84) cells (cat 88021101, Sigma-Aldrich Co., 3050 Spruce Street. St. Louis, MO 63103, USA) were seeded into 75cm^2 tissue culture flasks and routinely maintained in Dulbecco's Modified Eagle Medium-F-12 medium (1:1 mix; Cellgro, Herndon, Va.) supplemented with 10% foetal bovine serum (FBS), 1% L-Glutamine, 50U/mL penicillin, and 50mg/mL streptomycin (Sigma-Aldrich Co., 3050 Spruce Street. St. Louis, MO 63103, USA) as described in appendix A. The cultures were kept at 37^{o}C in 5% CO_2 incubator. Fresh medium was replenished every 2 days. When the monolayer reached 80-90% confluent, the cells were detached from the bottom of the flask using 0.25% trypsin-EDTA **(**GIBCO, Life Technologies, 3175 Staley Road, Grand Island, NY 14072, USA) and split 1:10 into new flasks.

3.4 Polarizing T84 cells

The T84 cells between passages 4 and 15 were seeded at a density of 3×10^5 cells/ml onto collagen-coated, 12-mm or 6.5mm polycarbonate Costar Transwell permeable support cell culture inserts of 3.0 μm pore size (Corning Inc, Corning, NY 14831, USA) and grown for 5 to 10 days (Appendix B), during which time fresh medium was replenished every 2 days for both chambers. The transepithelial electrical resistance

(TEER) was monitored with an epithelial tissue Voltohmeter resistance reader (World Precision Instruments, Inc.175 Sarasota Center Blvd.Sarasota, FL 34240, USA). Monolayers were considered polarized when resistance was equal to or greater than 1,000 Ω/cm^2 and not more than 5,000 Ω/cm^2. Background resistance from collagen-coated, cell-free membranes was subtracted from initial resistance values in order to obtain resistance values used in statistical analyses (discussed below).

3.5 Bacterial invasion assays

One day before infection, polarized T84 cells were incubated with DMEM/F12 without antibiotic. Overnight LB broth cultures of the bacteria were standardized in DMEM/F12 without antibiotic to an optical density at 600 nm (OD_{600}) of 0.6 ± 0.02, which is equal to approximately 1×10^9 Colony forming units (CFU)/mL. Bacterial samples at multiplicity of infection (MOI) of 100 in 200 µL of the medium without antibiotic were administered on the apical surface of each T84 cell monolayers grown on transwell inserts. The DMEM/F12 without antibiotic was added to the bottom of the transwell, uninfected wells and the wells with no cells. Two sets of the infection experiment were set and were incubated for 3 hours at 37^{o}C in 5% CO_2, at the end of which time the bacteria were aspirated. Supernatants were collected from the basolateral side of the wells and stored at 4^{o}C and -20^{o}C for LDH cytotoxicity and IL-8 cytokines assays respectively. Thereafter, the cells were washed three times with PBS. One set of the cells was washed three times in sterile 1X PBS and lysed with 500µL of triton X-100. The lysates was diluted 1:1000 in sterile PBS and plated on LB agar medium for CFU count to determine the bacterial association with the cells. On the other hand, fresh DMEM-F-12 with 100µg/ml

Gentamicin or 500µg/ml Amikacin (Sigma-Aldrich Co., 3050 Spruce Street. St. Louis, MO 63103, USA), depending on the sensitivity of the test bacteria, was added to the apical and basolateral chambers of the second set of cells and incubated as above for 1 hour to kill extracellular bacteria (antibiotic protection assay). After extensive wash (three times) to remove the antibiotic, the intracellular bacteria were released by lysis with Triton X-100 as above. The cell lysates were diluted 1:10 in sterile PBS and plated on LB agar medium and incubated overnight at 37^{o}C to grow. The CFU on the plate for each strain were counted to determine invasion. Data were expressed as CFU/mL for each monolayer. Adhesion was defined as: the difference between the number of associating bacterial CFU and the number of internalized bacterial CFU while the percent invasion was defined as: the number of internalized bacterial CFU (invasion) divided by the associating bacterial CFU (bacterial association) expressed as percentage. The results for each experiment were presented as means of an assay performed in triplicate and independently repeated three times.

3.6 Interleukin-8 Enzyme-Linked Immunosorbent Assay (ELISA)

To test for IL-8 release, supernatants from each infection experiment were collected and IL-8 protein levels determined by Enzyme linked immunosorbent assay (ELISA) using human IL-8 ELISA kit (Invitrogen Inc. 542 Flynn Rd, Camarillo, CA, USA) as described in Appendix C. Each sample was assessed in duplicate.

3.7 Cytotoxicity Assay

For cytotoxicity, supernatant of each sample was collected from infected and uninfected (control) cell culture transwell plates and transferred into a microcentrifuge tube. The tube was centrifuged at 12000 rpm for 2 min and the supernatant transferred to another clean tube. The sample was store at 4^{o}C and tested within 24 hours after collection. Cytotoxicity was tested using LDH Cytotoxicity kit (BioVision, Inc., 155 South Milpitas Blvd. Milpitas, California 95035, USA) as described in Appendix D.

3.8 Scanning Electron Microscopy

Bacterial strains at multiplicity of infection of 100 (MOI=100) or no bacteria were cultured for 3 hours with T84 cell monolayers grown on 12-mm glass coverslips. Culture medium was removed, and cells fixed in 0.1 M cacodylate buffer (pH 7.4) containing 2.5% paraformaldehyde and 2% glutaraldehyde (Appendix A), rinsed, post-fixed in 1% osmium in 0.1 M cacodylate buffer, and then dehydrated in a graded series of ethanol mixtures and treated with hexamethyldisilane. After drying, coverslips were coated with gold and examined using a JEOL JSM-6400 scanning electron microscope operated at 15kV or 20kV depending on the resolution.

3.9 Data collection and management

3.9.1 Data entry and storage

Results obtained from commercial kits were interpreted as described in Appendices C and D. All the results were captured in Microsoft Excel spread sheets and exported to the analysis software.

3.9.2 Data analysis

Results were expressed as means and standard deviation (±SD) of individual experiments performed in triplicate *n* times. Comparisons between mean values were performed by one-way analysis of variance (ANOVA) using SAS computer software (Version 9.1, SAS Institute Inc., Cary, NC, USA), and *p* values of less than 0.05 were considered statistically significant.

CHAPTER FOUR

RESULTS

4.1 Effect of selected commensal intestinal bacteria on infected T84 cells

In order to evaluate the individual effect of selected commensal intestinal bacteria compared with *S. flexneri* on infected T84 cells, the following assays were performed; adhesion and invasion, change in TEER to assess the integrity of the tight junctions, IL-8 release for cytokine response and LDH release due to cell destruction.

Adhesion and gentamicin protection (invasion) assays were performed to test whether *S.flexneri* and selected enteric bacteria including *E. coli* HS, *S. marcescens* (ATCC® 274™) and *C. freundii* possesses the ability to adhere to or invade intestinal epithelial cells individually. All the bacterial strains tested yielded some recovery of adherent bacteria from the T84 monolayer. *C. freundii* exhibited higher recovery than *S. flexneri*, *E. coli* HS or *S. marcescens* (Fig. 1A), however, the difference between the strains in adherence to T84 cells was not statistically significant in the single infections except between *C. freundii* and invasive *S. flexneri* (p=0.0218, one-way ANOVA). Of note is the fact that negligible numbers of *K. oxytoca* and *E. Cloacae* were recovered in this assay. On the other hand, significant recovery of *S. marcescens* from intestinal epithelial cells after treatment with gentamicin was observed (Fig. 1B). The recovery was significantly higher compared to that observed for *S. flexneri*, *E. coli* HS or *C. freundii* (p<0.05).

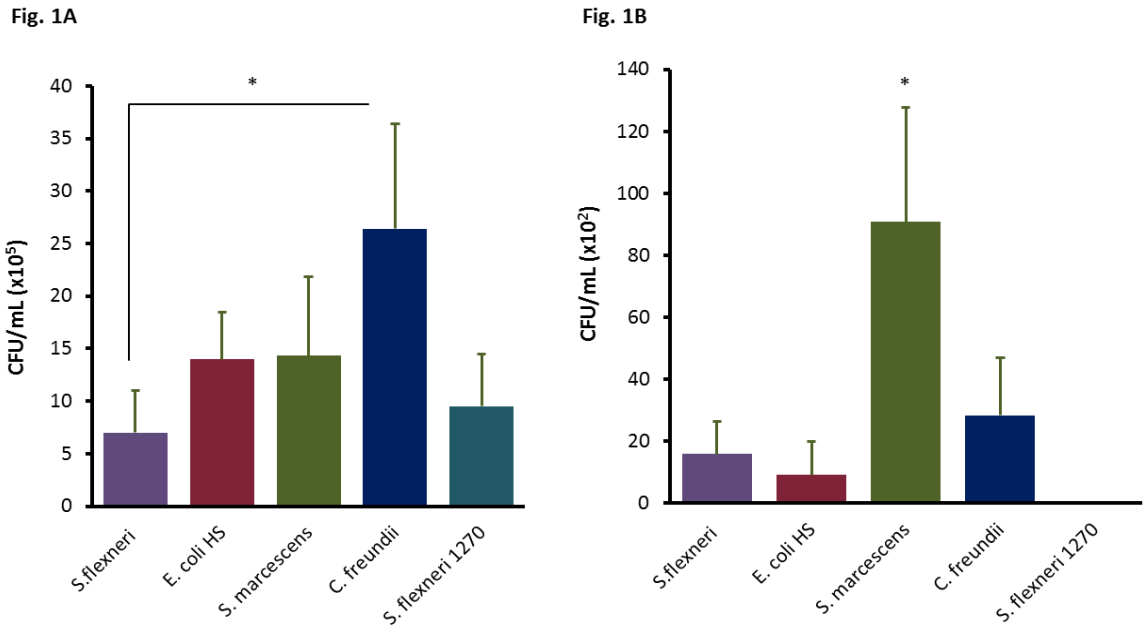

Figure 1: Adhesion and invasion of T84 cells by *S. flexneri* and selected commensal enteric bacteria after 3 hours of exposure. T84 cells were seeded in collagen coated 3.0µm transwell plates and maintained in DMEM-F12 cell culture medium for 5 – 10 days. The cells were infected with invasive strain of *S. flexneri*, Commensal *E. coli* HS, *S. marcescens*, *C. freundii*, or non-invasive strain of *S. flexneri* 1270 for 3 hr. Adhesion (A) and invasion (B) were determined by plating and quantifying CFU as described under materials and methods. The results are compared between the strains. Error bars represent the standard deviation calculated from the means of colony counts estimated from three independent experiments done in triplicate. (*, $p<0.05$, one-way ANOVA).

In order to assess whether or not the infecting bacteria disrupt transepithelial electric resistance (TEER) of a T84 cell monolayer, the TEER of polarized T84 monolayers were assessed before and after addition of bacteria; with experiments comprising 3 hours of infection followed by 1 hour post-infection recovery period. The mean percent change in TEER in T84 monolayers infected with *S. flexneri* alone and other intestinal bacteria was not significant ($p>0.05$, one-way ANOVA). However, TEER in cells infected with *S.*

marcescens dropped by 63% (Figure 2) from the initial TEER value, and was significantly lower than monolayers infected with *S. flexneri*, *E. coli* HS, *K. oxytoca*, *C. freundii*, and *E. cloacae* or compared with uninfected monolayers (p<0.0001, one-way ANOVA).

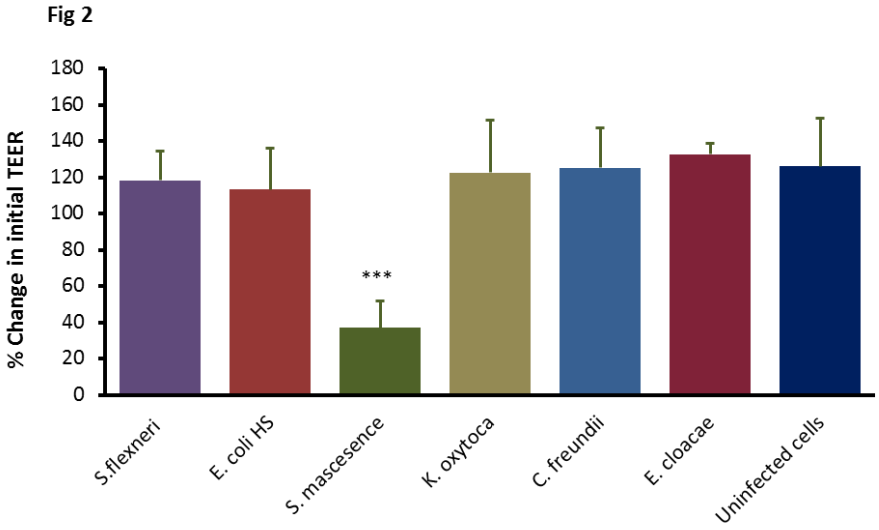

Figure 2: Comparison of the ability of *S. flexneri* and selected enteric bacteria to reduce TEER in polarized T84 cell monolayers in single infection. T84 cells were seeded in collagen coated 3.0µm transwell plates and maintained in DMEM-F12 cell culture medium for 5 – 10 days. The cells were infected with invasive strain of *S. flexneri*, Commensal *E. coli* HS, *S. marcescens*, *K. oxytoca*, *C. freundii*, or *E. cloacae* for 3 hour as described under materials and methods. TEER was measured before infection and after infection using Evom-2 voltohmmeter. Uninfected cells were used as negative control. Data are means percent change ± standard deviation of the means from triplicate readings of three independent experiments. (***, p<0.0001).

In addition, IL-8 secretion by epithelial cells following exposure to *S. flexneri* and other intestinal bacteria was assessed to determine how *S. flexneri* and the selected intestinal commensal bacterial infection induces IL-8 secretion from T84 cells. Basolateral supernatants of T84 cell monolayers were collected after 3 hours of exposure to each

bacterial species and examined for presence of IL-8 cytokines. There was significantly higher secretion of IL-8 (426.6pg/mL and 381pg/mL) in T84 intestinal epithelial monolayers infected with *S. marcescens* and *C. freundii*, respectively (Figure 3A) as compared to each of the five enteric bacteria tested and to the uninfected cell monolayers ($p<0.0001$), thus suggesting that exposure to *S. marcescens* and *C. freundii* induces a greater inflammatory response than that caused by invasive *S. flexneri*.

On the other hand, the release of LDH from eukaryotic cells suggests cell death and lysis. To determine the amount of LDH release from T84 cell monolayers infected with *S. flexneri* and other intestinal commensal bacteria, an LDH assay was utilized. Polarized T84 cell monolayers were infected with *S. flexneri*, *S. marcescens*, *E. coli* HS, *K. oxytoca* *C. freundii,* or *E. cloacae*. Basolateral supernatants were collected after 3 hours post-infection, and LDH was assayed (Figure 3B). The results showed that LDH release from cells infected with *S. marcescens* was more than 3 times greater than that observed for each of the other comparisons ($p<0.0001$, one-way ANOVA).

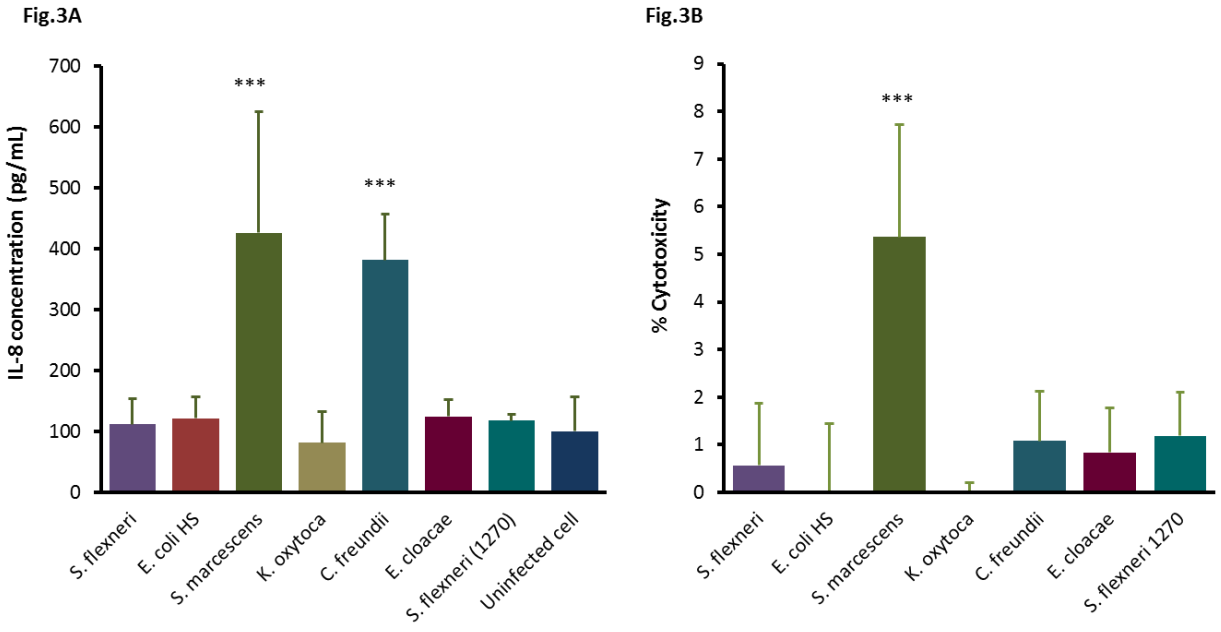

Figure 3: IL-8 secretion and LDH release by T84 cells upon single infection *S. flexneri* and selected intestinal bacteria. T84 cells were infected with invasive strain of *S. flexneri* and commensal *E. coli* HS, *S. marcescens*, *K. oxytoca*, *C. freundii*, *E. cloacae* or *S. flexneri* 1270 for 3 hour and basolateral supernatants collected for pro-inflammatory cytokine IL-8 assay by ELISA (A) and LDH assay (B) as described under materials and methods. Uninfected cells were used as negative control. Data are means ± standard deviation of the means from triplicate readings of three independent experiments (***, $p<0.0001$).

4.2 Effect of co-infection on invasiveness of *S. flexneri* on human intestinal epithelial (HIE) cells

To assess the effect of co-infection on invasiveness of *S. flexneri* on human intestinal epithelial cells, T84 cell monolayers were infected with *S. flexneri* in combination with three intestinal commensal bacteria. The T84 cells were assessed for adhesion and invasion, change in TEER, IL-8 release and LDH release as a measure of cell death. The difference between the strains in adherence to T84 cells was not statistically significant in

co-infections experiments (p>0.05, one-way ANOVA) (Figure 4A). However, there was significant recovery of bacteria in cells co-infected with *S. flexneri* and *S. marcescens* as compared to cell co-infected with *E. coli* HS or *C. freundii* (p<0.05, one-way ANOVA) following treatment with gentamycin for 1 hour (Figure 4B). Of note, there was a synergistic effect when the intestinal epithelial cells were co-infected with *S. flexneri* and *S. marcescens,* and *S. flexneri* and *C. freundii*. However, the internalization seemed to be more efficient in cells co-infected with *S. flexneri* and *C. freundii* as suggested by the percent invasion experiment (Figure 4C). Though these differences were not statistically significant between the bacteria strains tested *(p*>0.05, one-way ANOVA).

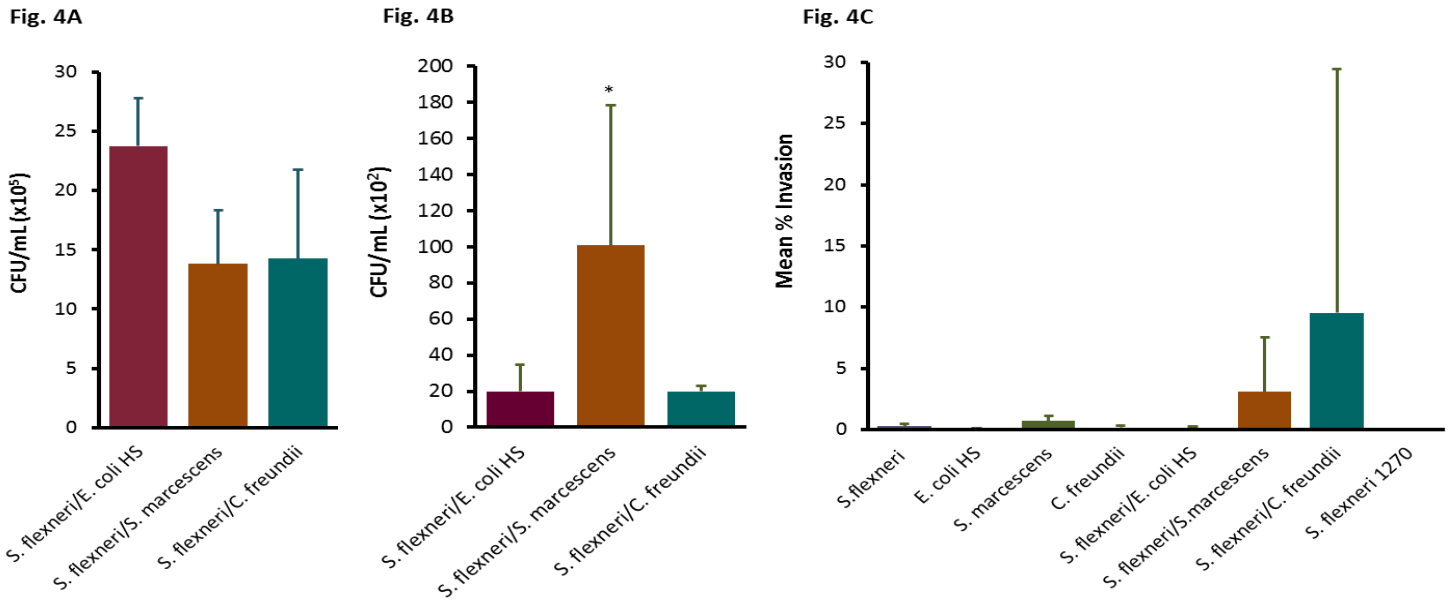

Figure 4: Adhesion and invasion of T84 cells co-infected with *S. flexneri* and selected commensal enteric bacteria after 3 hours of exposure. T84 cells were seeded in collagen coated 3.0µm transwell plates as described in fig.1. The cells were co-infected with invasive strain of *S. flexneri* and either commensal *E. coli* HS, *S. marcescens* or *C. freundii* for 3 hr. Non-invasive strain of *S. flexneri* 1270 was used as a negative control. Adhesion (A) and invasion (B) were determined by plating and quantifying CFU and percent invasion (C) calculated as described under materials and methods. The results were compared between the strains. Error bars represent the standard deviation calculated from the CFU counts obtained from three independent experiments done in triplicate. Adhesion to T84 cells (A) was not significantly different when compared between the strains. The internalization of bacteria was higher in cells co-infected with *S. flexneri* and *S. marcescens* compared to cell co-infected with *E. coli* HS or *C. freundii* ($p<0.05$). Similarly, the percent invasion was significantly higher where cells were co-infected with *S. flexneri* and *S. marcescens* or *S. flexneri* and *C. freundii* compared to *S. flexneri* and *E. coli* HS but was not statistically significant (*, $p<0.05$).

On the tight junctions of the T84 cells, there was a significant drop in TEER in monolayers co-infected with *S. flexneri* and *S. marcescens* compared to other co-infections or with the uninfected cells ($p<0.0001$, one-way ANOVA) (Figure 5A). However, the difference was minimal when compared with single infection with *S. marcescens* ($p=0.9998$, one-way ANOVA).

Similarly, there was a greater IL-8 secretion in epithelial cells following exposure to *S. flexneri* and *S. marcescens,* and *S. flexneri* and *C. freundii* in co-infections (444pg/mL and 508pg/mL, respectively) compared to each of the other three co-infections and with uninfected cells (Figure 5B) ($p<0.0001$, one-way ANOVA). The co-infection with *S. flexneri* and *S. marcescens,* and *S. flexneri* and *C. freundii* presented with a slight increase in IL-8 release as compared to single infections with *S. marcescens* or *C. freundii* but was statistically not significant ($p=1.000$ and $p=0.5606$, respectively, one-way ANOVA). These results demonstrate that *S. marcescens* and *C. freundii* are potential causes of inflammation in the intestinal tract individually or in mixed infection with *S. flexneri* and the inflammation could be higher in co-infection.

In addition, LDH in supernatants from cells co-infected with *S. flexneri* and *S. marcescens* appeared to be much greater than the concentrations measured from co-infection with other enteric bacteria tested ($p<0.001$, one-way ANOVA) (Figure 5C).

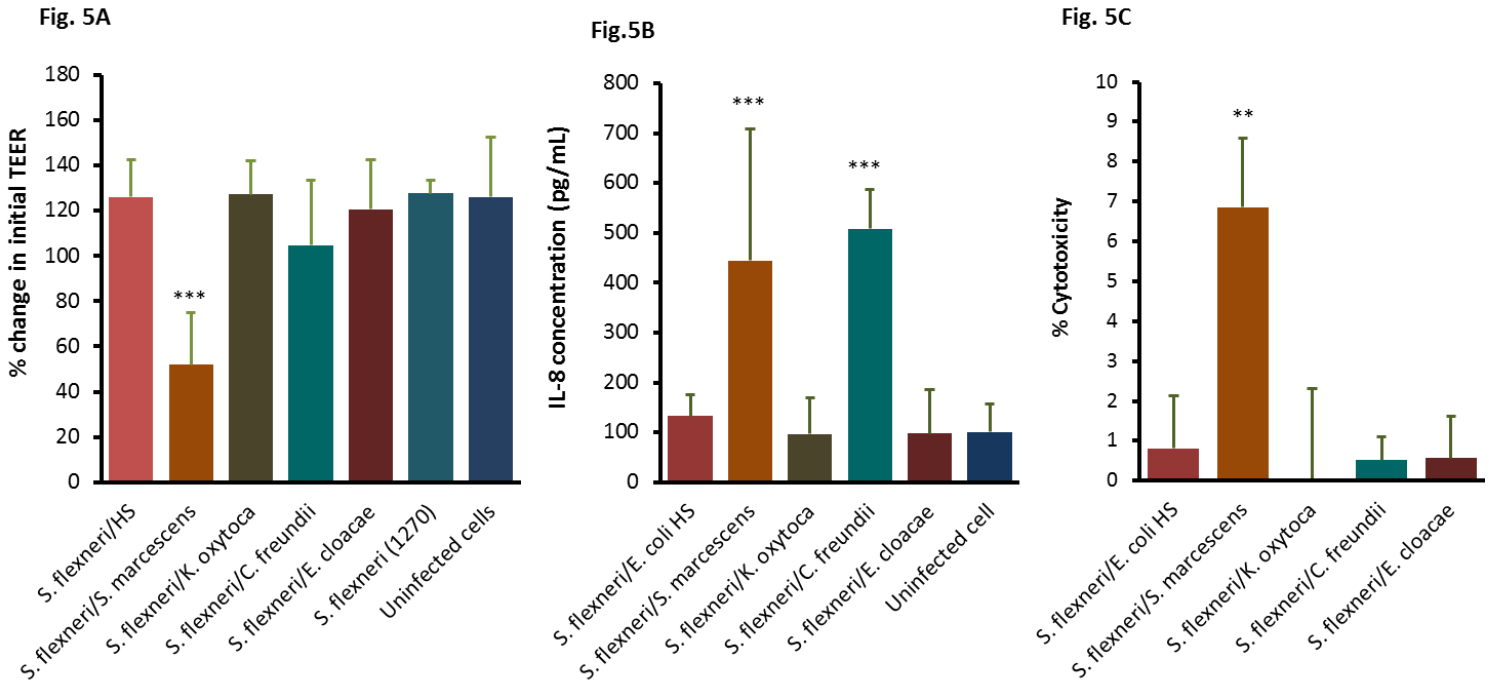

Figure 5: Comparison of TEER, IL-8 secretion and LDH release by T84 cells upon co-infection with *S. flexneri* and selected intestinal bacteria. T84 cells were seeded in collagen coated 3.0µm transwell and co-infected and co-infected with invasive strain of *S. flexneri*, and Commensal *E. coli* HS, *S. marcescens*, *K. oxytoca*, *C. freundii*, or *E. cloacae* for 3 hour as described under materials and methods. TEER was measured before and after infection using Evom-2 voltohmmeter. IL-8 and LDH levels were determined as described under methodology. Uninfected cells and *S flexneri* 1270 were used as negative control. Data were expressed as means ± standard deviation of the means from triplicate readings of three independent experiments (***, $p<0.0001$; **, $p<0.001$).

4.3 Effect of *S. flexneri* and selected commensal enteric bacterial infection on the morphology of T84 cells

To investigate the effect of *S. flexneri* infection on intestinal epithelia cell morphology, non-polarized T84 cell monolayers were infected with *S. flexneri*, *S. marcescens*, *E. coli* HS, or *C. freundii* at a multiplicity of infection of 100 for 3 hours. Uninfected cell monolayers were included in the same experiment for comparison. Uninfected monolayer (Figure 6A), monolayers infected with *E. coli* HS (Figure 6B), *S. flexneri* (Figures 6C and 6D) or *C. freundii* (Figure 6H) exhibited no morphological alteration. However, a remarkable difference in cellular morphology between the cells infected with *S. marcescens* (Figures 6E, 6F and 6G) and cells infected with *S. flexneri* and the other two enteric bacteria was observed. Cell monolayers infected with *S. marcescens* manifested close adherence of bacteria, loss of microvilli and vacuolization (Figures 6E, 6F and 6G) thus confirming cytotoxic effect.

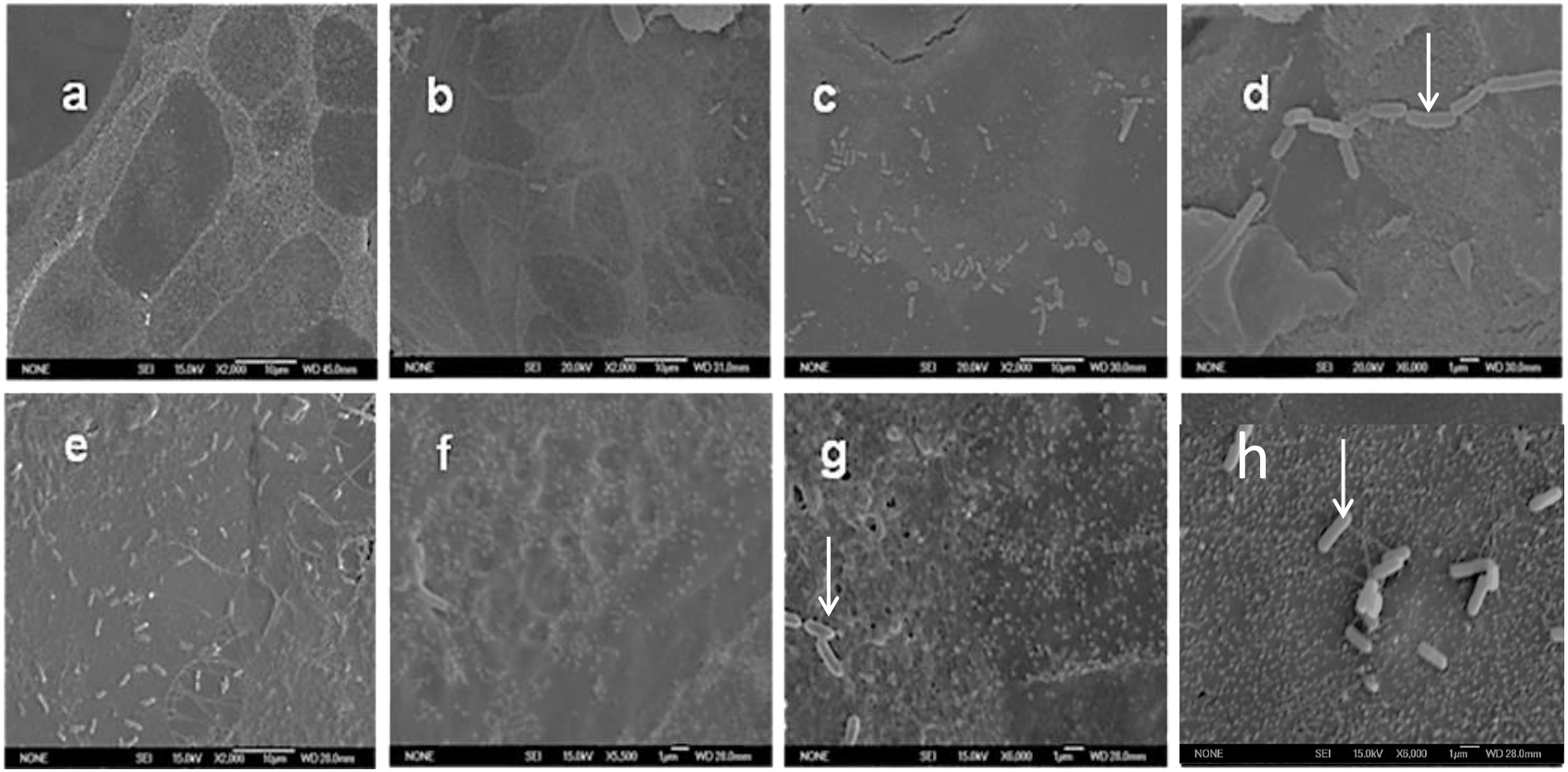

Figure 6: Effect of *S. flexneri* and selected commensal enteric bacteria on the morphology of T84 cells monolayer after 3 hours of infection. T84 cells were infected with bacteria for 3 hour and fixed with paraformaldehide solution overnight at 4^{0}C as described under materials and methods. The cells were prepared and examined using JSM-6400 scanning microscope. (a) Uninfected T84 cells (b) infected with *E. coli* HS, (c and d) infected with *S. flexneri* (e, f and g) infected with *S. marcescens*, (h) infected with *C. freundii.* Cells infected with *S. marcescens* exhibited loss of microvilli (e) and vacuolization (f and g) while cells infected with other bacteria expressed no visible evidence of morphological alteration. The bacteria are marked by arrows.

CHAPTER FIVE

DISCUSSION

Using human-colon derived polarized epithelial (T84) cells, the results demonstrated that *S. flexneri* potential for invasion, cytotoxicity, perturbation of intestinal barrier function, stimulation of release of potent chemotactic agonist IL-8, and alteration of cellular morphology is less efficient when infecting on the apical site of the intestinal epithelial cells. Therefore, the potential routes of entry of *shigella in vivo* may include the apical surfaces of cells with disrupted brush borders, the apical surfaces of immature crypt cells or M cells or the baselateral surfaces of enterocytes, accessed via the opening of intercellular junctions. Thus, entry of *Shigellae* into the baselateral site is likely to occur and facilitate rapid invasion in cases of multiple infections with microorganisms which can potentially disrupt the brush borders or create vacuoles on the monolayer. However, only a few inconclusive observations regarding the actual site of entry in vivo are available (Goldberg & Sansonetti, 1993). The current study shows that interaction between *S. flexneri* and *S. marcescens* or *C. freundii* influences invasion, cytotoxicity and IL-8 production by intestinal epithelial cells *in vitro*.

Adhesion is a critical first step in intestinal colonization (Edwards, Bajaj-Elliott, Klein, Murch, & Phillips, 2011). In this study, both adhesion and invasion of *S. flexneri* in human intestinal epithelial cells using the gentamicin protection assay (Elsinghorst, 1994) and employing T84 cells culture as a model (Nataro, Hicks, Phillips, Vial, & Sears, 1996) was demonstrated in line with the previous studies. Polarized T84 cell monolayers have

been used extensively to study enteric bacterial pathogens. For example, it has been generally established that pathogens reproduce their pathogenic behaviour in this *in vitro* reductionist model (McCormick, Siber, & Maurelli, 1998). Previous studies have demonstrated that *S. flexneri* preferentially entered epithelial cells via the basolateral membrane domain and that attachment to T84-cell basolateral membranes was a necessary component in the signalling cascade for induction of basolateral-to-apical directed transepithelial PMN migration (McCormick, Siber, & Maurelli, 1998). Furthermore, the bacteria that interacted with the apical surface of the cells either bound to the microvilli without causing any detectable alteration or bound at the level of intercellular junction and demonstrated limited invasion (Mounier, Vasselon, Hellio, Lesourd, & Sansonetti, 1992). This is consistent with the current study which demonstrated that *S. flexneri* exhibited less recovery than *E. coli* HS, *C. freundii* or *S. marcescens* in single infection of intestinal epithelial cells. In contrast, significant recovery of *S. marcescens* from intestinal epithelial cells after treatment with gentamicin was observed. The recovery was significantly higher compared to that observed for *S. flexneri, E. coli* HS or *C. freundii.* These results suggest that *S. marcescens* has greater potential to invade the monolayer from the apical surface than *S. flexneri*. As expected, the effect of commensal *E. coli* HS was not statistically significant.

Due to strong cytotoxic effect exhibited by *S. marcescens*, observing the adhesion and invasion phenotypes in intestinal epithelial cells infected with the bacteria proved difficult (Livrelli et al., 1996; Ochieng et al., 2015). It is believed that the number of the invading bacteria might be higher than counted in the current study, considering that

gentamicin is lethal to the bacteria in damaged cells and the fact that the antibiotic was added 3 hours post-infection.

Tight adhesion increases the proximity of the bacteria with the cell, possibly bringing the cell bound cytotoxin in close contact with the cell, thereby enhancing host-cell invasion (Gauthier & Finlay, 1998). The invading bacteria can extensively disrupts the tight junction structure (Sakaguchi, Kohler, Gu, McCormick, & Reinecker, 2002), gain access to the basolateral pole of the epithelium and consequently cause further invasion and secretion of inflammatory cytokines (Jung et al., 1995; Sansonetti, 2002). This study demonstrated that TEER in cells infected with *S. marcescens* dropped by 63% from the initial TEER value, and was significantly lower than monolayers infected with *S. flexneri*, *E. coli* HS, *K. oxytoca*, *C. freundii*, and *E. cloacae*. Similarly, there was significantly higher secretion of IL-8 in T84 intestinal epithelial monolayers infected with *S. marcescens* and *C. freundii*, as compared to each of the five enteric bacteria tested while LDH release from cells infected with *S. marcescens* was more than 3 times greater than that observed for each of the other comparisons. These findings are consistent with previous studies which showed that some strains of *S. marcescens* poses toxin which induces lysis of human bladder epithelial cells and that less adherent strains of *S. marcescens* were less cytotoxic (Hertle & Schwarz, 2004). Likewise, adherent strains of *C. freundii* were implicated in cases diarrheal disease in humans (Bai *et al.*, 2012). In a previous study, the number of invading bacteria and the amount of secreted pro-inflammatory cytokine IL-8 were correlated (Eckmann *et al.*, 1993) which is similar to the observation made from the current study on IL-8 release following individual

bacterial infection experiments that the number of adherent and invading *S. flexneri* was significantly lower compared to *S. marcescens* and *C. freundii* in single infection of intestinal epithelial cells with *S. flexneri* and selected enteric bacteria. These results suggest that exposure to *S. marcescens* and *C. freundii* induces a greater inflammatory response than that caused by invasive *S. flexneri*.

This study further demonstrated the effect of co-infection on intestinal epithelial cells. In *S. flexneri* co-infection with *S. marcescens* or *C. freundii*, more abundant adherence and greater invasiveness for the epithelial cells was observed. The finding from the current study is consistent with previous studies which implicated adherent strains of *C. freundii* with a potential to cause diarrheal disease in humans (Bai *et al.,* 2012) supporting the importance of adhesion in the pathogenesis of *S. marcescens* and *C. freundii* (Bai *et al.*, 2012; Krzyminska, Ochocka, & Kaznowski, 2012).

Similarly, the effect of co-infection with *S. flexneri* and *S. marcescens* on the integrity of intestinal epithelial cells was demonstrated. The integrity of the epithelial monolayer is sustained by tight cell-cell junctions, and many bacterial pathogens target tight junctions by perturbing this structure (Guttman & Finlay, 2009). For some pathogens, previous studies have demonstrated that reduction in TEER is correlated with significant decrease in tight junction protein expression and increased permeability, thus allowing translocation of virus and bacteria across the mucosa (Nazli *et al.,* 2010). Similarly, apical application of bacteria has been shown to induce opening of the paracellular

pathway and transmigration of polymorphonuclear leucocytes, which in turn facilitate pathogen invasion (Perdomo, Gounon, & Sansonetti, 1994).

It was observed in the current study that infection with *S. marcescens* drastically decreased TEER of T84 cell monolayers, suggesting functional impairment of the epithelial cell-cell barrier. Moreover, a similar observation was made in cells co-infected with *S. flexneri* and *S. marcescens* which would possibly facilitate access of the bacteria into the basolateral compartment and enhance invasion by *Shigellae*. Interestingly, the invasive strain of *S. flexneri* alone did not exhibit significant effects on TEER in the current model. Of note, *Shigella* invades intestinal epithelial cell monolayer more efficiently through the basolateral surface (Mounier, Vasselon, Hellio, Lesourd, & Sansonetti, 1992), and assays employed in the current study were of apical infection. Thus the current findings concur with previous reports suggesting that *S. flexneri* interacts with the apical surface of human colonic cells and binds to microvilli of the cells without causing detectable alteration (Mounier, Vasselon, Hellio, Lesourd, & Sansonetti, 1992).

Intestinal epithelial cells act as sentinels in intestinal infection (Waterhouse, Joseph, Winsor, Lacombe, & Stadnyk, 2001), mounting early innate immune responses against foreign substances via pro-inflammatory cytokine production. For example, recent studies suggest that IL-8 produced by the intestinal epithelial cells plays a central role in the initial control of infection by recruiting polymorphonuclear leukocytes, and transmigration of these cells to the epithelial lining. The polymorphonuclear leukocytes

contribute to bacterial killing, often at the expense of tissue destruction (Sansonetti, Arondel, Huerre, Harada, & Matsushima, 1999). Thus IL-8 appears to be an essential chemokine in *Shigella* transepithelial translocation even though the role of intestinal epithelial cells as a source of IL-8 in intestinal bacterial infections remains controversial with other studies showing that infiltrating macrophages and PMNs are the major source of IL-8 (Eckmann *et al.,* 1993).

Therefore, the ability of *S. flexneri* and other co-infecting intestinal bacteria to induce IL-8 responses in intestinal epithelial cell monolayers was investigated. Whereas all the enteric bacteria tested induced secretion of IL-8 from T84 cells at levels less than 100 pg/ml, co-infection with *S. flexneri* and *S. marcescens*, and *S. flexneri* and *C. freundii* induced more than three times the levels of IL-8 secretion suggesting high potential of causing inflammation in the intestinal tract compared to *S. flexneri* alone, and in co-infection with *K. oxytoca*, *E. cloacae*, or E. *coli* HS. Thus, results from the current study are in concurrence with previous co-infection studies which suggested enhancement of pathogenicity among these pathogens (Crane, Choudhari, Naeher, & Duffey, 2006).

S. marcescens has been reported to have a cytotoxin/cytolysin and a type VI secretion system that exports a non-diffusible cytolysin (Hertle, Hilger, Weingardt-Kocher, & Walev, 1999). These effects have currently been demonstrated on intestinal cells and rapid destruction of the epithelial cells. The cytolytic effect may contribute to the observed invasiveness and pro-inflammatory ability in the current study. The overall effect of *S. marcescens* on the intestinal epithelial may potentially open up the

intercellular tight junction and create gaps/holes on the cell through which *Shigellae* can exploit to gain access to the basolateral sites and invade the cells. Cell damage leads to leakage of a cytoplasmic enzyme LDH, a soluble enzyme that is released into extracellular space when the plasma membrane is damaged (Chan *et al.,* 2013). In the current study, LDH in supernatants from cells co-infected with *S. flexneri* and *S. marcescens* appeared to be much greater than the concentrations measured from co-infection with other enteric bacteria tested. These results suggest a synergistic effect in the cytolysis and necrosis of intestinal epithelial cells during co-infection with *S. flexneri* and *S. marcescens*.

Of note, detachment of epithelial cell monolayers and rounding of cells was also observed on cells infected with *S. marcescens*. Observations of infected monolayer by using SEM confirmed these data. The cytolytic effect apparently included disruption of the microvilli, shedding of the microvilli and vacuole formation on the epithelial cells. This is consistent with previous studies which demonstrated pore-formation, extended vacuolation and cell lysis induced by secreted cytolysin ShlA on bladder epithelial cells in single infection experiments (Hertle & Schwarz, 2004).

A similar cytolysin excreted *by Haemophilus ducreyi* was shown to enhance invasion of human epithelial cells and contributes to evasion of immune responses (Wood, Dutro, & Totten, 1999). Thus, the disruption of microvilli layer, vacuolation and possibly lysis of the host-cell elicited by *S. marcescens* in the current study may also enable *Shigellae* to penetrate the tissue layer.

Similarly, previous studies demonstrated that clinical isolates of *K. oxytoca* induce antibiotic-associated haemorrhagic colitis and a high proportion of stool isolates tested were cytotoxin positive (Hoffmann *et al.*, 2010; Joainig *et al.*, 2010). A similar experiment was conducted using *C. freundii* isolates (Bai *et al.*, 2012). The cytotoxin caused rounding, fragmentation and detachment of HEp-2 cells from the substratum which may precede cell death. In the current study the *K. oxytoca* and *E. Cloacae* strains used exhibited no cytotoxicity suggesting that the cytotoxin production might be strain-specific. Likewise, culture filtrates from some clinical isolates of *E. Cloacae* were able to exhibit cytotoxic activity on Vero cell lines whereby the activity was detectable after treatment with 2-ME solution (Krzyminska, Mokracka, Koczura, & Kaznowski, 2009). In contrast, a minimal cytotoxicity was observed in the current study.

CHAPTER SIX

SUMMARY OF FINDINGS, CONCLUSIONS AND RECOMMENDATIONS

6.1 Summary of the findings

Using an *in vitro* model, it was demonstrated that some intestinal bacteria regarded as commensal bacteria have the potential of interacting with intestinal epithelial cells and exhibit pathogenic effects in a manner similar to know pathogenic enteric bacteria. The integrity of the tight junction was disrupted, as determined by drop in TEER, in monolayers infected with *S. marcescens* and in those co-infected with *S. flexneri* and *S. marcescens.* The disruption was accompanied with higher IL-8 response and an elevated increase in LDH concentration in supernatants from cells co-infected with *S. flexneri* and *S. marcescens* suggesting inflammatory responses and increased cytotoxicity. A synergistic effect in invasion and inflammatory response appeared where the intestinal epithelial cells were co-infected with *S. flexneri* and *S. marcescens,* and *S. flexneri* and *C. freundii* even though the difference was not statistically significant between the bacteria strains tested except for the inflammatory responses. Furthermore, the pathologic effects as confirmed by altered cellular morphology, demonstrated by SEM, revealed loss of microvilli and vacuolization in the monolayers following exposure to *S. marcescens.* Interestingly, the invasive strain of *S. flexneri* alone did not exhibit significant pathogenic effects in the current model possibly because *Shigella* invades intestinal epithelial cell monolayer more efficiently through the basolateral surface while this study used apical side of the monolayer in all the experiments.

6.2 Conclusions

1. This current study demonstrated that *S. marcescens* and *C. freundii* previously considered non-pathogenic have the potential to interact with intestinal epithelial cells in culture to induce dramatic alterations similar to those produced by known enteric pathogens.
2. The current study further provides evidence that *S. flexneri* and *S. marcescens* or *C. freundii* can potentially cause deleterious enteric infection through elevated inflammatory responses and epithelial cell destruction.
3. *S. marcescens* manifested significant pathologic effect on epithelial cell morphology demonstrated by loss of microvilli and vacuolization thus confirming cytotoxic effect.

6.3 Recommendations from the current study

1. *S. marcescens* and *C. freundii* demonstrated pathogenic activity *in vitro* suggesting that these are potential enteric pathogens. Therefore, the two species of bacteria should be investigated in clinical diagnosis of diarrheal disease.
2. Laboratory investigations and epidemiologic studies should consider possible association between *S. flexneri* and *S. marcescens*, and *S. flexneri* and *C. freundii* in pathogenic states such as severe diarrhoea.
3. *S. marcescens* can potentially cause severe intestinal tissue damage hence should be investigated in cases of severe inflammatory diarrhoea and necrotizing enterocolitis.

6.4 Recommendations for future research

1. Future studies need to look at the alteration intestinal microbiota in diarrhoea and non-diarrhoea cases to identify potential pathogens and probiotics.
2. The findings from this study were based on *in vitro* experiments and used a few ATCC organisms. Future studies should explore the use of bacteria isolated from clinical specimens, test more potential pathogens and novel pathogens including non-*Enterobacteriaceae* to confirm these findings and their association with diarrhoea.
3. An animal model should be used to reproduce the pathology observed *in vitro* and to provide a better understanding of the pathogenicity of these commensals enteric bacteria.

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APPENDICES

Appendix A: Reagents preparation

1. **Dalbecco's Modified Eagle's Medium nutrient mixture F-12 Han's (DMEM-F-12)** (Cat. No. D8437**,** Sigma-Aldrich Co., 3050 Spruce Street. St. Louis, MO 63103, USA**) complemented**
    a. DMEM-F-12 …….. 500mL
    b. Fetal bovine serum……..… 50mL
    c. L- Glutamine (200mM)…….. 5mL
    d. Streptomycin (10mg/mL)/Penicillin (10,000U)…. 2.5mL

2. **DMEM-F-12 with Amphotericin**
    a. DMEM-F-12 …….. 500mL
    b. Fetal bovine serum……..… 50mL
    c. Amphotericin…….. 5mL

3. **DMEM-F-12 without antibiotic**
    a. DMEM-F-12 …….. 500mL
    b. Fetal bovine serum……..… 50mL
    c. L- Glutamine (200mM)…….. 5mL

4. **Paraformaldehyde Fixative**
    a. Heat 90mL of distilled water in a beaker to boiling
    b. Turn off heat
    c. Add 8gm of paraformaldehyde (Cat. No. 158127, Sigma-Aldrich Co., 3050 Spruce Street. St. Louis, MO 63103, USA) while stirring for 1 minute
    d. Add 1N Sodium hydroxide drop wise with continuous stirring until the solution clears
    e. Bring the final volume to 100mL with distilled water

    NB. Store in a sealed bottle at 4^{o}C if not used immediately

f. Add 6.25mL of paraformaldehide solution in a 25mL tube
g. Add 1.25mL of 50% glutaraldehyde solution (Cat. No. G7651, Sigma-Aldrich Co., 3050 Spruce Street. St. Louis, MO 63103, USA)
h. Add 12.5mL of 0.2M sodium cacodylate buffer pH 7.4 (Cat. No. 11650, Electron Microscopy Sciences, PA, USA)
i. Add 5mL of distilled water for a final volume of 25ml working solution
j. Use fresh after preparation.

Appendix B: Preparation of collagen coated cell culture inserts and seeding with cells to polarize

i. Prepare collagen solution as follows
   a. Dissolve 2.5mg of collagen (Cat No. C7661-10mg, Sigma-Aldrich Co., 3050 Spruce Street. St. Louis, MO 63103, USA) in 5ml of sterile water
   b. Add 28µl of acetic acid and stir for 3 hours to mix
   c. Transfer to a screw top glass vial and overlay with 500µl of chloroform
   d. Keep at 4^oC overnight
   e. Aseptically transfer the top layer into a separate vial and discard the bottom layer

      NB. Keep at 4^oC for use

ii. Add 12µl (about 6µg) inside each cell culture transwell insert membrane (1.1mm diameter)

iii. Using a sterile serological pipette, spread the collagen until it dries evenly on the membrane

iv. Sterilize using UV light overnight with tops off

v. Add 500µl and 1000ml of DMEM/F-12 containing 50µg/ml (10ml/L) amphotericin (Cat. No. A5955, Sigma-Aldrich Co., 3050 Spruce Street. St. Louis, MO 63103, USA) inside the insert and at the bottom respectively to wash the inserts then aspirate

vi. Seed 500µl of $3x10^5$ in the transwell plate and add 1000ml DMEM/F-12 with penicillin and streptomycin at the bottom of each transwell. Two well are filled with medium without cells (unseeded) for control

vii. Incubate at 37^oC in a 5% CO_2 incubater

viii. Cells are grown for 10 – 14 days changing medium after every 2 days and monitoring monolayer resistance using an EVOM ohmmeter (World Precision Instrument, Inc)

ix. Monolayer is considered polarized when resistance is equal to or greater than $1000\Omega/cm^2$

Appendix C: *Human* IL-8 ELISA

(Catalog # KHC0081)

(a). PURPOSE

IL-8 (also known as NAP-1 for Neutrophil-Activating Peptide) is a chettractant protein for neutrophils. This cytokine belongs to a new family of chemotactic peptides called "chemokines". This proinflammatory mediator is secreted by different cells such as monocytes, neutrophils, endothelial cells, fibroblasts after activation, and by mitogen-stimulated T lymphocytes. IL-8 is a key cytokine that has been found in scales of psoriasis patients and in synovial fluid of patients suffering from rheumatoid arthritis or gout. A role for IL-8 in the recruitment of neutrophils in the lung during adult respiratory distress syndrome (ARDS) has been suggested. IL-8 levels in septic shock patients correlates with mortality. In acute graft liver rejection the IL-8 serum levels are markedly increased. The level of IL-8 in these and other conditions may prove to have prognostic significance in these disease conditions. The Invitrogen Human Interleukin–8 (Hu IL-8) ELISA is to be used for the quantitative determination of Hu IL-8 in human serum, plasma, buffered solution, or cell culture medium.

The assay recognizes both natural and recombinant forms of Hu IL-8.

(b). PRINCIPLE OF THE METHOD

The Invitrogen Hu IL-8 kit is a solid phase sandwich Enzyme Linked-Immuno-Sorbent Assay (ELISA). A monoclonal antibody specific for Hu IL-8 has been coated onto the wells of the microtiter strips provided. Samples, including standards of known Hu IL-8

content, control specimens, and unknowns, are pipetted into these wells followed by the addition of a second biotinylated monoclonal antibody. During the first incubation, the Hu IL-8 antigen binds to the immobilized (capture) antibody on one site and to the solution phase biotinylated antibody on a second site. After removal of excess second antibody, Streptavidin-Peroxidase (enzyme) is added. This binds to the biotinylated antibody to complete the four-member sandwich. After a second incubation and washing to remove all the unbound enzyme, a substrate solution is added, which is acted upon by the bound enzyme to produce color. The intensity of this coloured product is directly proportional to the concentration of Hu IL-8 present in the original specimen.

(c). REAGENTS PROVIDED

- *Hu IL-8 Standard*, recombinant Hu *IL-8*. (Refer to vial label for quantity and reconstitution volume).
- *Standard Diluent Buffer*. Contains 8 mM sodium azide; 25 mL per bottle.
- *Hu IL-8 Antibody-Coated Wells*, 96 wells per plate.
- *Hu IL-8 Biotin Conjugate* (Biotin-labeled anti-*IL-8*). Contains 8 mM sodium azide; 6 mL per bottle.
- *Streptavidin-Peroxidase (HRP),* (100x) concentrate. Contains 1.3 mM thymol; 0.125 mL per vial.
- *Streptavidin-Peroxidase (HRP) Diluent.*Contains 0.05% Proclin® 300 ; 25 mL per bottle. *Wash Buffer Concentrate (25X);* 100 mL per bottle.
- *Stabilized Chromogen, Tetramethylbenzidine (TMB);* 25 mL perbottle.
- *Stop Solution*; 25 mL per bottle. 1 bottle 1 bottle 3 bottles

(d). REAGENT PREPARATION AND STORAGE

1. Reconstitution and Dilution of Hu IL-8 Standard Note: Either glass or plastic tubes may be used for standard dilutions.

i. Reconstitute standard to 10.0 ng/mL with Standard Diluent Buffer. Refer to standard vial label for instructions. Swirl or mix gently and allow to sit for 10 minutes to ensure complete reconstitution. Use standard within 1 hour of reconstitution.

ii. Add 0.100 mL of the reconstituted standard to a tube containing 0.900 mL Standard Diluent Buffer. Label as 1000 pg/mL Hu IL-8. Mix.

iii. Add 0.300 mL of Standard Diluent Buffer to each of 6 tubes labeled 500, 250, 125, 62.5, 31.2, and 15.6 pg/mL Hu IL-8.

iv. Make serial dilutions of the standard as described in the following dilution table. Mix thoroughly between steps.

2. Dilution of Hu IL-8 Standard

Standard	Add	Into
1000 pg/mL	Prepare as described in Step 2.	
500 pg/mL	0.300 mL of the 1000 pg/mL std.	0.300 mL of the Diluent Buffer
250 pg/mL	0.300 mL of the 500 pg/mL std.	0.300 mL of the Diluent Buffer
125 pg/mL	0.300 mL of the 250 pg/mL std	0.300 mL of the Diluent Buffer
62.5 pg/mL	0.300 mL of the 125 pg/mL std.	0.300 mL of the Diluent Buffer
31.2 pg/mL	0.300 mL of the 62.5 pg/mL std.	0.300 mL of the Diluent Buffer
15.6 pg/mL	0.300 mL of the 31.2 pg/mL std	0.300 mL of the Diluent Buffer
0 pg/mL	0.300 mL of the Diluent Buffer	An empty tube

Discard all remaining reconstituted and diluted standards after completing the assay. Return the *Standard Diluent Buffer* to the refrigerator.

3. Storage and Final Dilution of Streptavidin-HRP

i. Dilute 10 µL of this 100x concentrated solution with 1 mL of *Streptavidin-HRP Diluent* for each 8-well strip used in the assay. Label as Streptavidin-HRP Working Solution.

For Example:

# of 8-Well Strips	**Volume of Streptavidin-HRP Concentrate**	**Volume of Diluent**
2	20 µL solution	2 mL
4	40 µL solution	4 mL
6	60 µL solution	6 mL
8	80 µL solution	8 mL
10	100 µL solution	10 mL
12	120 µL solution	12 mL

ii. Return the unused *Streptavidin-HRP* concentrate to the refrigerator.

4. Dilution of Wash Buffer

Allow the *Wash Buffer Concentrate (25X)* to reach room temperature and mix to ensure that any precipitated salts have dissolved. Dilute 1 volume of the *Wash Buffer Concentrate (25X)* with 24 volumes of deionized water (e.g., 50 mL may be diluted up to 1.25 liters, 100 mL may be diluted up to 2.5 liters). Label as Working Wash Buffer.

(e). ASSAY METHOD: PROCEDURE AND CALCULATIONS

Allow all reagents to reach room temperature before use. Gently mix all liquid reagents prior to use. Note: A standard curve must be run with each assay.

i. Determine the number of 8-well strips needed for the assay. Insert these in the frame(s) for current use. (Re-bag extra strips and frame. Store these in the refrigerator for future use.)

ii. Add 50 µL of the Standard Diluent Buffer to zero wells. Well(s) reserved for chromogen blank should be left empty.

iii. Add 50 µL of standards, samples or controls to the appropriate microtiter wells.

iv. Pipette 50 µL of biotinylated anti-IL-8 (Biotin Conjugate) solution into each well except the chromogen blank(s). Tap gently on the side of the plate to mix.

v. Cover plate with a plate cover and incubate for 1 hour and 30 minutes at room temperature.

vi. Thoroughly aspirate or decant solution from wells and discard the liquid. Wash wells 4 times.

vii. Add 100 µL Streptavidin-HRP Working Solution to each well except the chromogen blank(s). (Prepare the working dilution as described in reagent preparation and storage)

viii. Cover plate with a *plate cover* and incubate for 30 minutes at room temperature.

ix. Thoroughly aspirate or decant solution from wells and discard the liquid. Wash wells 4 times.

x. Add 100 µL of *Stabilized Chromogen* to each well. The liquid in the wells will begin to turn blue.

xi. Incubate for 30 minutes at room temperature and in the dark. *Please Note*: Do not cover the plate with aluminum foil or metalized mylar. The incubation time for chromogen substrate is often determined by the microtiter plate reader used. Many plate readers have the capacity to record a maximum optical density (O.D.) of 2.0. The O.D. values should be monitored and the substrate reaction stopped before the O.D. of the positive wells exceeds the limits of the instrument. The O.D. values at 450 nm can only be read after the *Stop Solution* has been added to each well. If using a reader that records only to 2.0 O.D., stopping the assay after 20 to 25 minutes is suggested.

xii. Add 100 µL of *Stop Solution* to each well. Tap side of plate gently to mix. The solution in the wells should change from blue to yellow.

xiii. Read the absorbance of each well at 450 nm having blanked the plate reader against a chromogen blank composed of 100 µL each of *Stabilized Chromogen* and *Stop Solution*. Read the plate within 2 hours after adding the *Stop Solution*.

xiv. Plot on graph paper the absorbance of the standards against the standard concentration. (Optimally, the background absorbance may be subtracted from all data points, including standards, unknowns and controls, prior to plotting.) Draw the best smooth curve through these points to construct the standard curve. If using curve fitting software, the four parameter algorithm provides the best curve fit.

xv. Read the Hu IL-8 concentrations for unknown samples and controls from the standard curve plotted in step 14. (Samples producing signals greater than that of the highest standard (1000 pg/mL) should be diluted in Standard Diluent Buffer and reanalyzed, multiplying the concentration found by the appropriate dilution factor.)

(f). SENSITIVITY

The minimum detectable dose of Hu IL-8 is <5.0 pg/mL. This was determined by adding two standard deviations to the mean O.D. obtained when the zero standard was assayed 20 times.

Appendix D: LDH-Cytotoxicity Assay

LDH-Cytotoxicity Assay Kit (Catalog #K311-400)

(a). Introduction:

Cell death or cytotoxicity is classically evaluated by the quantification of plasma membrane damage. **The LDH-Cytotoxicity Assay Kit** provides a fast and simple method for quantitating cytotoxicity based on the measurement of activity of lactate dehydrogenase (LDH) released from damaged cells. Unlike many other cytoplasmic enzymes which exist in many cells either in low amount (e.g., alkaline and acid phosphatase) or unstable, LDH is a stable cytoplasmic enzyme present in all cells and rapidly released into the cell culture supernatant upon damage of the plasma membrane. LDH activity can be determined by a coupled enzymatic reaction: LDH oxidizes lactate to pyruvate which then reacts with tetrazolium salt INT to form formazan. The increase in the amount of formazan produced in culture supernatant directly correlates to the increase in the number of lysed cells. The formazan dye is water-soluble and can be detected by spectrophotometer at 500 nm. The LDH-cytotoxicity assay is sensitive, convenient, and precise, and is applicable to a variety of cytotoxicity studies. Assay takes ~0.5–1 hr.

(b). Kit Contents:

Components	**K311-400 (400 assays)**	**Part Number**
Catalyst, Lyophilized	1 vial	K311-400-1
Dye Solution	45 mL	K311-400-2

(c). Preparation of Working Solutions:

- Reconstitute the Catalyst in 1 mL double distilled water for 10 min and mix thoroughly. The catalyst solution is stable for several weeks at $4^{o}C$.
- After thaw, the Dye Solution is stable for several weeks at $4^{o}C$. Avoid freeze/thaw cycles.
- Preparation of reaction mixture: For 100 assays mix 250 µL of catalyst solution with 11.25 mL of dye solution. The mixture solution should be prepared immediately before use.

(d). LDH-Cytotoxicity Assay Protocol:

i. Collect cells and wash 1X with assay medium (e.g., medium containing 1% serum or 1% BSA).

ii. Preparing the following samples individually in a 96-well plate:

Background Control: Add 200-µl medium/well into triplicate wells. The background value has to be subtracted from all other values.

Low Control: Add 1-2 x 10^4 cells/well in 200-µL assay medium into triplicate wells.

High Control: Add 1-2 x 10^4 cells/well in 200-µL assay medium containing 1% Triton X-100 into triplicate wells.

Test Sample: Add 1-2 x 10^4 cells/well in 200-µL assay medium containing test substance into triplicate wells.

iii. Incubate cells in an incubator (5% CO2, 90% humidity, $37^{o}C$) for the appropriate time of treatment determined for test substance.

iv. Centrifuge the cells at 250 g for 10 min.

v. Transfer 100 µL/well supernatant carefully into corresponding wells of an optically clear 96-well plate.

vi. Add 100 µL reaction mixture to each well and incubate for up to 30 min at room temperature. Protect the plate from light.

vii. Measure the absorbance of all samples at 490-500 nm using a microtiter plate reader. The reference wavelength should be more than 600 nm.

(e). Calculation of the Percentage Cytotoxicity:

$$\textbf{Cytotoxicity (\%)} = \frac{\text{(Test Sample - Low Control)}}{\text{(High Control - Low Control)}} \times 100$$