# Development of a bacterial adhesin into a next generation antimicrobial agent

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## Abstract

For most pathogenic bacteria, the adherence to host cells is a crucial step for infection. Therefore, targeting bacterial attachment could be a potential way for treating bacterial infections. This thesis focuses on the interactions between the adhesin Multivalent Adhesion Molecule (MAM7) from the commensal *Escherichia coli* strain HS with host receptors, aiming to understand the molecular basis of these interactions. Targeting the interactions between MAM7 and host cells was one of this study's aims towards developing anti-bacterial agents. In this study, HSMAM7 based adhesion inhibitors- beads coupled to HSMAM7 and an engineered bacterium expressing HSMAM7- were designed and their role as anti–adhesion agents against pathogenic bacteria was investigated. This study demonstrates that HSMAM7 based inhibitors have the ability to displace pathogenic bacteria from the host cells.

Dissecting the binding of HSMAM7 to host receptors was the second part of this study. The findings revealed that HSMAM7 binds to sulfatide, several components of the extracellular matrix (ECM) and mucin. Further investigation of the interaction between HSMAM and host receptors revealed that HSMAM7 binds to mucin and the host lipid sulfatides via specific recognition of a shared 3-O-sulfo-galactosyl moiety in a sulfation dependent manner.

Sulfatase-producing *Bacteroides thetaiotaomicron* affect the binding of HSMAM7 to mucin. HSMAM7 binding to mucin was inhibited in the presence of *B. thetaiotaomicron* and that means the commensal *E. coli* would be free and able to attach to the host epithelial cells, a finding of potential relevance for inflammatory bowel diseases (IBDs).

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# Table of Contents

## List of Figures

## List of Tables

## List of Abbreviations

| | |
|---|---|
| *B. theta* | *Bacteroides thetaiotaomicron* |
| bp | Base pair |
| BHI | Brain heart infusion |
| BSA | Bovine serum albumin |
| CFU | Colony forming unit |
| DMEM | Dulbecco's modified Eagle's medium |
| dH2O | Distilled water |
| DNA | Deoxyribonucleic acid |
| dNTP | Deoxynucleotide triphosphate |
| ECL | Enhanced chemiluminescence |
| ECM | Extracellular matrix |
| EDTA | Ethylenediaminetetraacetic acid |
| ELISA | Enzyme-linked immunosorbent assay |
| FITC | Fluorescein isothiocyanate |
| g | Gram |
| GST | Glutathione S-transferase |
| HIFBS | Heat inactivated fetal bovine serum |
| HRP | Horseradish peroxidase |
| IPTG | Isopropyl β-D-1-thiogalactopyranoside |
| ICC | immunocytochemistry |

| | |
|---|---|
| KDa | Kilodalton |
| IBD | Inflammatory bowel disease |
| LB | Luria Bertani |
| LDH | Lactate dehydrogenase |
| µg | Microgram |
| µl | Microlitre |
| µM | Micromolar |
| µm | Micrometer |
| MAM | Multivalent adhesion molecule |
| MOI | Multiplicity of infection |
| nm | Nanometer |
| OD | Optical density |
| PBS | Phosphate buffer saline |
| PFA | Paraformaldehyde |
| rpm | Revolution per minute |
| v/v | Volume/Volume |

# Chapter 1. Introduction

## 1.1 Bacterial attachment to host cells: principles and prevention

### 1.1.1 Bacterial attachment to host cells

Bacterial attachment to host cells is an important step towards bacterial colonization and infections (Ribet and Cossart, 2015). Pathogenic bacteria express a wide range of bacterial adhesion factors that enable them to achieve tight interactions with host cells. Initial tight attachment of bacterial cells to the host cell surface is prerequisite for translocation of effector proteins, disruption of host actin cytoskeleton or induction of host cellular signalling that aids bacterial transmigration (Stones and Krachler, 2016). The adherence is important for bacterial pathogenicity due to several factors. First, the bacterial cell is able to attach to the target cells in the host. In addition, the attachment aids the bacterium to not be removed by the host secretions or killed by host defences (Lewis et. al., 2016). Thus, successful binding to host cells enhances bacterial ability to survive within the host and cause infections (Ribet and Cossart, 2015). Varieties of infectious diseases can be caused due to bacterial adherence such as oral, respiratory, urinary, gastrointestinal tracts and wound infections (Bosch et. al., 2013; Kim et. al., 2015; Cantlon et. al., 2006).

## 1.1.2 Molecular basis of host-bacterial interactions

Bacterial attachment to host cells involves the interactions between specialized structures on both host cells and bacterial cells. Based on the nature of these structures, three types of adhesin-receptor interactions have been described. Hydrophobin-protein interactions, lectin-carbohydrate interactions and protein-protein interactions (Ofek et. al., 2003a).

To achieve tight adherence between bacteria and host cells, bacteria need to overcome the repulsive forces that result from the similar charges found on both host cells and bacterial cell surface. Brownian motion, electrostatic forces, Van der Waals forces, hydrophobic interactions (Gottenbos et. al., 2002) and complementary interactions result in bacterial attachment that involves two steps. First, non-specific weak reversible interactions between hydrophobin on bacterial surface and hydrophobic moieties on the host cell surface (Krasowska and Sigler, 2014). These interactions take place during early stages of bacterial host contact. Subsequently, specific irreversible interactions occur between bacterial adhesins and host cell receptors (Bank et. al., 2011; Seneviratne et. al., 2011; Umeda et. al., 2012). These interactions include lectin-carbohydrate interactions or protein-protein interactions. Lectin-carbohydrate interactions occur when lectins on the bacterial cell surface interact with the sugar groups on the host cell membrane such as those found in glycoproteins, glycolipids or proteoglycans (Esko and Sharon, 2009; Ofek et. al., 2003a). An example includes the binding of FimH adhesin from uropathogenic *E. coli* to mannose-containing host glycoprotein receptors (Lewis et. al., 2016). In addition, these interactions involve the binding of the host cell surface lectins such as mannose receptors on macrophages, to the surface with bacterial lipopolysaccharides, capsular polysaccharides or peptidoglycan (Targosz et. al., 2006; Sahly et. al., 2008).

Protein–protein interactions occur between proteins on both bacterial cells and host cells. These interactions usually involve the binding of bacterial proteinaceous adhesins to extracellular matrix (ECM) proteins, integrins and cadherins. Examples include *S. aureus* fibronectin binding protein A (FnBPA) which bind to the extracellular matrix fibronectin. This interaction facilitates bacterial binding to host cell surface by exploiting fibronectins binding to the host cell integrin $\alpha_5\beta_1$. The binding of *S. aureus* FnBPA to integrin $\alpha_5\beta_1$ via fibronectin has been shown to aid bacterial uptake into host cells (Sinha, et. al.1999). Adhesin of collagen from *E. faecalis* (Ace) that binds to collagen Type IV, collagen Type I and laminin (Nallapareddy et. al., 2006). In addition, invasin, a surface adhesin on *Yersinia pseudotuberculosis* that binds to β integrins to invade mammalian cells (Isberg and Leong, 1990), and internalin, a surface protein on *Listeria monocytogenes*, binds to host cells E- cadherin to get into host epithelial cells (Bonazzi,et. al., 2009).

### 1.1.3 Mechanisms of bacterial attachment

Studying and understanding the mechanisms used for attachment by pathogenic bacteria is important for development of novel antibacterial therapies. Bacterial aderence to host is critical for the infection and mainly mediated by specialized structures expressed on bacterial cell surface commonly known as adhesins (Bank et. al., 2011; Seneviratne et. al., 2011; Umeda et. al., 2012). Adhesins can be made up from proteins or polysaccharides. Proteinaceous adhesins are organized either as appendages extended from bacterial surface as hair-like structures known as fimbria or pili or a single

protein present directly on bacterial cell surface and serve as adhesion factors (Craig et. al., 2004; Chagnot et. al., 2012). The interaction between fimbria or pili with host receptors occur via fimbrial tips. Polysaccharide adhesins are commonly constituents of bacterial cell membrane, cell wall or capsule (Wilson et. al., 2002). These adhesins are able to recognize and bind to host receptors (Heilmann, 2011). Due to the role of adhesins in bacterial binding that provide bacterium the resistance to the clearance by mucosal secretions (Kline, et.al., 2009). In addition, the formation of bacterial biofilm that depend on bacterial adherence to host cell increase bacterial resistance to the antibiotics. Furthermore, bacterial binding result in changes in host signalling that aids bacterial spread and avoid the host defence (Stones and Krachler, 2015a). Thus, bacterial adhesins are considered as a critical virulence mechanisms for variety of pathogenic bacteria (Bosch et. al., 2013).

### 1.1.3.1 Adhesion mechanisms of *Staphylococcus aureus*

*Staphylococcus aureus* is an important pathogenic bacterium due to the ability to cause serious infections and the resistance to antibiotics (Schito, 2006). *S. aureus* can cause different infections to the host such as pneumonia, meningitis, bacteraemia, toxic shock syndrome and wound infections. The interaction with the host ECM components is the first stage towards biofilm formation (Otto, 2008). *S. aureus* possess different proteins to interact with the host ECM. These proteins are known as microbial surface components recognizing adhesive matrix molecules (MSCRAMMs), (Clarke and Foster, 2006; Chagnot et. al., 2012). MSCRAMMs are covalently anchored to peptidoglycan via sortase A, an

enzyme that recognizes LPXTG motif at C- terminal of protein and cleaves between the threonine and the glycine. Deletion of the *srtA* gene in *S. aureus* leads to failure to anchor surface proteins and thus inhibition of bacterial infections (Weidenmaier et. al., 2008).

Fibronectin binding proteins (FnBPs) are a major family of MSCRAMMs that mediates the interaction of most clinical isolates of *S. aureus* with fibronectin (Peacock et. al., 2000). It has been shown that biofilm formation in methicillin resistant *S. aureus (*MRSA) is promoted by FnBPA and FnBPB (O'Neill et. al., 2008). Clumping factors (ClfS) ClfA and ClfB are another group of MSCRAMMs. ClfA binds to fibrinogen protein (Scully, et. al., 2015) and ClfB, in addition to fibrinogen, binds to type I cytokeratin 10 on squamous epithelial cells, that results in bacterial colonization of the nasopharynx (O'Brien et. al., 2002). Collagen binding proteins (Cna) (Patti et.al., 1992), elastin binding protein(Ebps) (Downer et.al., 2002) and Staphylococcal protein A (spa) are further MSCRAMMs that allow the bacteria to avoid the host immune response (Votintseva et. al.,2014).

Another group of proteinaceous adhesins is the secretable expanded repertoire adhesive molecules (SERAMs) (Chavakis et. al., 2005). These proteins include extracellular adherence protein (Eap), extracellular fibrinogen binding protein (Efb) (Lee et. al, 2004) and Coagulase (Coa) which binds to collagen, fibronectin, laminin and fibrinogen (Watanabe, et. al., 2005). In addition, the family of autolysin/adhesinscontains proteins that have both enzymatic and adhesive properties, such as autolysin AtI which binds to fibronectin (Hirschhausen, 2010) and autolysin/adhesin from *S. aureus* Aaa that mediates the adherence to fibronectin, fibrinogen and vitronectin (Heilmann et. al., 2005). *S. aureus* also produce non–proteinaceous adhesion factors such as polysaccharide

intercellular adhesin (PIA), teichoic and lipoteichoic acids (Heilmann, 2011; Lin et. al., 2015).

### 1.1.3.2 Adhesion mechanisms of *Enterococcus faecalis*

*Enterococcus faecalis* is an opportunistic Gram positive pathogenic bacterium that has been shown to be a causative agent of nosocomial infections with resistance to antibiotics (Vilela et. al., 2006). *E. faecalis* can cause a wide array of diseases such as urinary tract infections, bacteraemia, endocarditis and wound infections (Fowler et. al., 2005; Olawale et. al., 2011). *E. faecalis* possess surface proteins associated with bacterial adherence to host ECM proteins (Nallapareddy and Murray, 2006). It has been shown that *E. faecalis* has a family of putative MSCRAMMs (Sillanpaa et. al., 2004) and pilus subunits (Sillanpaa et. al., 2013). Fibronectin-binding protein (EfbA) mediates the binding to fibronectin, collagen I, and collagen V (Singh et. al., 2015). In addition, the adhesin of collagen from *E. faecalis* (Ace) mediates the binding to collagen Type IV, collagen Type I and laminin (Nallapareddy et. al., 2006a). Ace plays an important role in the *E. faecialis* infections including endocarditis. It has been shown that deletion of genes encoding Ace attenuates bacterial colonization of the heart valve (Singh et. al., 2011). In addition, *E. faecalis* express the endocarditis and biofilm-associated pilus (Ebp) that shows a significant role in bacterial pathogenicity (Montealegre et. al., 2015). Ebp is an extracellular hair-like structure consisting of three subunits EbpA, EbpB and EbpC, and adhesion to host ECM proteins occurs via the subunit EbpA (Schembri et. al., 2015). Ebp interacts with ECM proteins and human platelets, and it is not known whether Ebp pili bind directly to platelet

receptors or indirectly via ECM proteins (Nallapareddy et. al., 2011a). The Ebp pilus contributes to bacterial infections including urinary tract infections and endocarditis (Nallapareddy et. al., 2006b; Nallapareddy et. al., 2011b; Singh et. al., 2007). Disruption of genes encoding Ebp attenuates bacterial infections (Nallapareddy et. al., 2006b).

### 1.1.3.3 Adhesion mechanisms of *Pseudomonas aeruginosa*

*Pseudomonas aeruginosa* is a Gram negative opportunistic pathogen which can cause a wide range of infections especially in immunocompromised patients (Sadikot et. al., 2005). It causes bacteraemia, respiratory system infections, urinary tract infections and wound infections (Alharbi and Zayed, 2014; Kim et. al., 2015; Li et. al., 2005). *P. aeruginosa* expresses different structures that mediate the binding to the host cells such as flagella, type IV pili, lipopolysaccharide (LPS), surface polysaccharide (alginate) and lectins (Sadikot et.al., 2005; Chemani et.al., 2009).

The major adhesins in *P. aeruginosa* are flagella and type IV pili (Jyot et. al., 2007; Lillehoj et. al., 2002). The single polar flagellum is involved in motility, adhesion of the bacteria to host cells and biofilm formation (Bucior et. al., 2012). The major flagellar protein is flagellin that interacts with Muc1 mucin (Lillehoj et. al., 2002). In addition, flagellin is detected via Toll-like receptor (TLR5) at the cell surface (Raoust et. al., 2009). It has been found that deleting flagellum genes such as flagellar cap *fliD* leads to disruption of motility and adherence (Arora et.al., 1998).

Type IV pili are composed of pilin subunit PilA, which is produced in the cytoplasm and translocated by Sec apparatus, then assembled in the outer membrane (Leighton et. al.,

2015; Lee and Schneewind, 2001). Many studies have shown that pili play a significant role in the bacterial binding and colonization of mucosal epithelial cells. Type IV pili bind to glycolipids on the epithelial surfaces (Craig et. al., 2004).

The glycolipid LPS is anchored in the bacterial outer membrane and comprises of lipid A, core oligosaccharide and O antigen (Pier, 2007), recognized by TLRs such as TLR2 and TLR4 which effectively defend against *P. aeruginosa* infections (Mc Isaac et. al., 2012; Raoust et. al., 2009). In addition LPS mediates binding to the glycolipid Asialo GM1 (Gupta et.al., 1994). Exopolysaccharide (alginate) mediates the binding to mucin on tracheal cells (Ramphal et. al., 1987; Lovewell et.al., 2014).

The adhesion of *P. aeruginosa* is also mediated by carbohydrate binding lectins, such as LecA and LecB. Lectins are present on the outer membrane and recognize glycoconjugates on the epithelial cells (Chemani et. al., 2009; Tielker et. al., 2005). Blocking lectins may prevent *P. aeruginosa* infections (Sharon, 2006; Thomas and Brooks, 2004; Grishin et. al., 2015). It has been revealed that using lectin inhibiting carbohydrates (methyl derivatives of galactose and fucose) on *P. aeruginosa* lung infection, both *in vitro* using lung epithelial cell line and *in vivo* using a murine model, results in reduction of bacterial cytotoxicity and lung infections (Chemani et. al., 2009).

### 1.1.3.4 Multivalent adhesion molecules (MAMs)

Recently, it has been reported that many Gram negative bacteria have outer membrane proteins known as Multivalent Adhesion Molecules (MAMs) which facilitate bacterial attachment to host cells throughout the initial steps of host infection (Krachler et.al., 2011). MAMs consist of six or seven mammalian cell entry (Mce) domains headed by an N-terminal hydrophobic region (Krachler and Orth, 2011), (Figure 1.1). However, proteins comprising of a single mce domain are found in *Mycobacterium tuberculosis* (Arruda et. al., 1993), pathogenic Leptospira strains (Zhang et. al., 2012), and higher plants (Lu and Benning, 2009).

The first description of mammalian cell entry domains was in *M. tuberculosis*. *M. tuberculosis* contains four mce operons, known as *mce* 1, *mce* 2, *mce* 3 and *mce* 4. The mce loci consists of two *yrbE* (*yrbEA* and *yrbEB*) genes encoding two integral membrane proteins, homology to ATP binding cassette (ABC) transporter permeases which responsible for translocation of substrates through membranes, and six mce genes (*mceA*, *mceB*, *mceC*, *mceD*, *mceE*, and *mceF*) as substrate binding proteins (Arruda et.al., 1993; Casali and Riley, 2007). The physiological function of these is thought to be in lipid metabolism (Santangelo et. al., 2009; Casali and Riley, 2007), in addition to the role in *M. tuberculosis* virulence (Chitale, 2001). It has been found that expressing *M. tubercolusis* mce1A in non-pathogenic *E. coli* allows this bacterium to enter and persist inside epithelial cells (Arruda et.al., 1993).

Pathogenic strains of Leptospira species possess an Mce protein, a surface-exposed outer membrane protein, which use α5β1 and αvβ3 integrins as host receptors (Zhang et. al., 2012). The leptospiral Mce protein has a potential role in bacterial pathogenesis, and

disruption of mce gene attenuates the ability of the bacterium to enter to host cells (Zhang et.al., 2012; Cole et.al., 1998).

Plants such as Arabidopsis contain a TGD2 gene that encodes for TGD2, a chloroplast protein which consists of a predicted transit peptide, a transmembrane domain and an mce domain similar to the mce domains of mycobacteria, and it binds to phosphatidic acid (Lu and Benning, 2009).

Ekiert et. al., reported that *E. coli* contain three mammalian cell entry (Mce) proteins; MlaD, PqiB and YebT. MlaD is a peripasmic hexameric ring, PqiB comprises of three MCE domains, whereas YebT (known as MAM7) contains seven MCE domains. The Mce proteins involved in the carrying of lipids between bacterial inner and outer membranes (Ekiert et. al., 2017).

The well characterized *V. parahaemolyticus* MAM7 is an outer membrane protein and consists of an N- terminal region of 44 amino acids for MAM localization on the bacterial outer membrane and seven mce domains that are exposed extracellularly and responsible for bacterial binding to host cells. Removal of the *N*-terminal region of MAM7 results in cytoplasmic retention of the protein (Krachler et. al., 2011). The attachment of MAM7 to host cells occurs via recognition of the host receptors, fibronectin and phosphatidic acid (Krachler and Orth, 2011). *V. parahaemolyticus* MAM7 uses fibronectin as a co–receptor to facilitate the initial binding with host cells. Biochemical analysis of this interaction revealed that MAM7 binds to the 30-kDa *N*-terminal fragment of fibronectin and at least five mce domains of MAM7 are required to achieve stable binding between MAM7 and fibronectin. The interaction between *V. parahaemolyticus* MAM7 and phoshatidic acids on the host membrane induces host signalling that involves the activation of the GTPase

RhoA and actin rearrangements in host cells leading to disruption of the epithelial barrier and finally help bacteria to arrive at the underlying tissue (Lim et.al., 2014; Stones and Krachler, 2015b). MAM7 contributes to bacterial virulence due to its function in bacterial attachment, a key step towards infection. It is known that many bacterial virulence factors are regulated and their expression is induced in response to a change in invironmemtal conditions during infection (Pizarro-Cerda and Cossart, 2006). However, MAM7 adhesin is constitutively expressed and thus is able to mediate initial attachment to host cells early during infection (Krachler and Orth, 2011), therefore facilitates the translocation of effectors into host cells via secretion systems (Filloux et. al., 2008; Zhang and Orth, 2013; Cambronne and Roy, 2006).

Using a nematode infection model, it was demonstrated that lethal effects mediated by T3SSs resulted from feeding *Caenorhabditis elegans* worms with *V. parahaemolyticus* RIMD 2210633 or *V. parahaemolyticus* POR1. However, the ingestion of mutant strains lacking MAM7 leads to reduced mortality (Krachler et. al., 2011). Similar results of a MAM7 dependent reduction in mortality were obtained when *Galleria mellonella* larvae were injected with *Shigella sonnei* wild type or MAM7 deletion strains (Mahmoud et. al., 2016). Moreover, in a guinea pig model, keratoconjunctivitis was caused due to the infection with *Shigella sonnei* wild type compared to a MAM7 deletion strain which did not cause infection (Mahmoud et. al., 2016). In addition, using mammalian cell models, it has been shown that disruption of MAM7 gene in pathogenic bacteria such as *V. parahaemolyticus, Y. pseudotuberculosis*, *V. cholerae*, enteropathogenic *E. coli* or *Shigella sonnei* results in decreased bacterial attachment and cytotoxicity (Krachler et. al., 2011; Mahmoud et. al., 2016). MAM7 confers the adherence properties to the non-

adherent bacteria. It has been revealed that expressing MAM7 in the non-adherent *E. coli* BL21 enables them for attachment and competition with pathogens for binding sites (Krachler et. al., 2011). The competition between BL21 expressing MAM7 with pathogenic bacteria results in inhibited bacterial adherence and cytotoxicity towards host cells. Targeting MAM7 or using MAM7 based tools is a promising way towards developing antibacterial therapies. In tissue culture models, *V. parahaemolyticus* MAM7 based inhibitors were used to inhibit the infections caused by multi-drug resistant pathogens such as *P. aeruginosa, E. coli, Acinetobacter baumannii-calcoaceticus* complex and *Klebsiella pneumoniae* (Krachler et. al., 2012). In addition, a MAM7 based inhibitor blocked *S. aureus* (MRSA) infection without interfering with host cell functions (Hawley et. al., 2013). Recently, MAM7 based inhibitor, recombinant GST-MAM7 coupled to beads, has been used *in vivo* to heal burn wound infections caused by multi -drug resistant *P. aeruginosa* (Huebinger et. al., 2016).

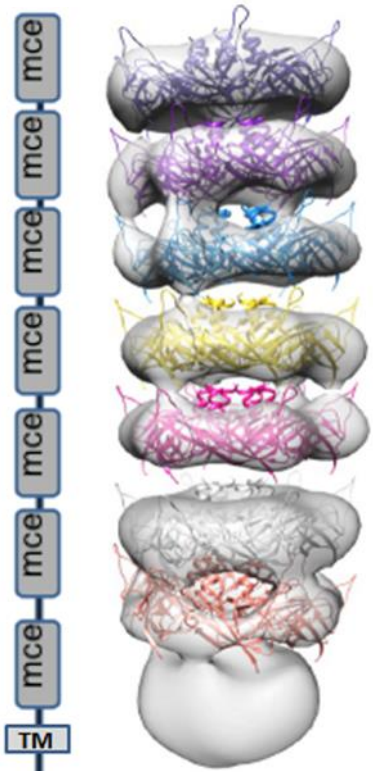


**Figure 1.1 Diagram of MAM7 protein architecture.** The N-terminal transmembrane region headed by seven MCE domains. Figure adapted from (Ekiert et. al., 2017).

### 1.1.4 Ways to prevent bacterial attachment

Considering that bacterial attachment is a key step towards infections, targeting bacterial attachment is a promising approach to inhibit bacterial colonization and infection at an early stage. Antibiotics prevent bacterial infections by killing or inhibiting bacterial growth, by interfering with essential bacterial functions, and hence, bacteria have developed mechanisms to resist antibiotics and survive. In contrast, anti–adhesion therapy prevents bacterial infections by targeting a virulence-specific function, which in theory gives less selection pressure and therefore would result in less resistance. It works by blocking bacterial adherence to host cells without killing them (Cozens, 2012; Krachler and Orth, 2013). A variety of ways can be used to inhibit bacterial adherence and thus prevent infections, such as disruption of adhesin biosynthesis (Larsson et. al., 2005; Jahanshahi et. al., 2010), receptor analogs (Ofek et. al., 2003b; Kouki et. al., 2013), anti-adhesive vaccines (Langermann and Ballou, 2003; Kisiela et. al., 2015), probiotics and engineered probiotics (Asahara et. al., 2011;Paton et. al., 2006) and adhesin analogs (Krachler et. al., 2012; Daep et. al., 2011), (Figure 1.2). These strategies will be covered in detail in the next sections.

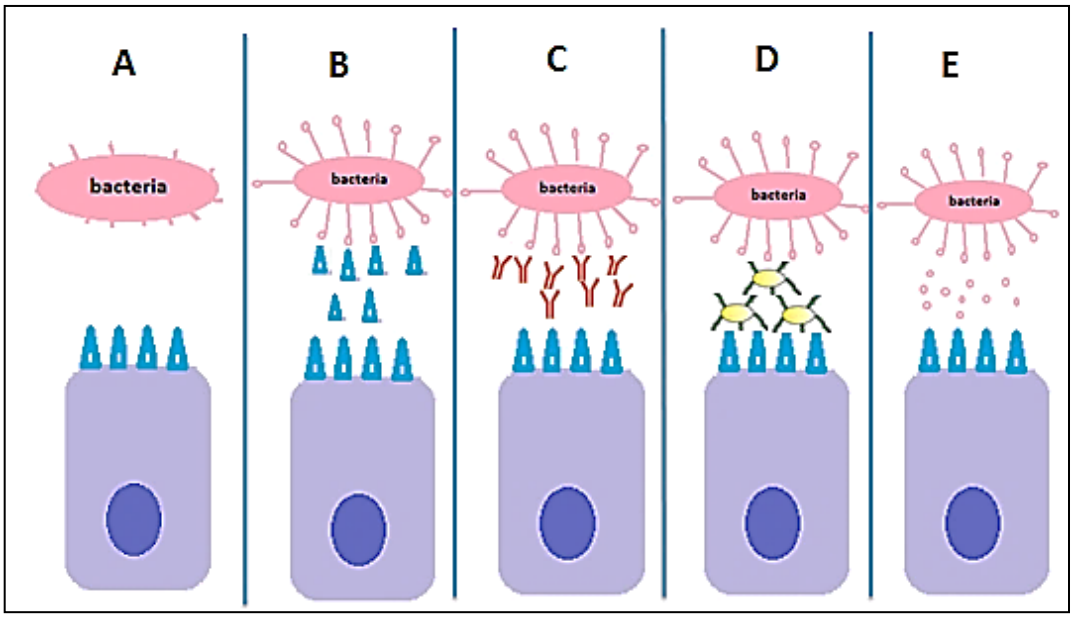


**Figure 1.2 Different strategies to prevent bacterial adhesion.** A: disruption of adhesin biosynthesis. B: receptors analogs. C: anti-adhesive vaccines. D: probiotics and engineered probiotics. E: adhesin analogs. Figure adapted from (Krachler and Orth, 2013).

### 1.1.4.1 Disrupting adhesin biosynthesis

The ability of bacterial cells to attach to host cells could be inhibited by the exposure to sub inhibitory concentrations of antibiotics. Morphological changes could cause bacteria to lose the ability to adhere (Wagner and Hensel, 2011). It was found that using sub inhibitory concentrations of ciprofloxacin induce morphological changes such as formation of filaments in *Escherichia coli, Klebsiella oxytoca, Enterobacter cloacae* and *Acinetobacter calcoaceticus* biotype anitratus (Wojnicz et. al., 2007). *P. aeruginosa* adherence was inhibited when sub inhibitory concentrations of piperacillin and tazobactam were used due to the changes that occurred in the bacterial morphology and hydrophobicity (Fonseca et. al., 2004). Using sub inhibitory concentrations of trimethoprim, sulphiazine and sulphamethaxazole resulted in prevention of the binding of *E. coli to* host cells. In general, these antibiotics at sub inhibitory concentrations inhibit protein synthesis and bacterial fimbriae display (Jahanshahi et. al., 2010).

In addition, disruption of pili could be accomplished via the interference with fimbriae assembly by the chaperone–usher pathway. This pathway involves the delivery of the pilus subunits by chaperone FimC, which are translocated by Sec apparatus in the inner membrane, to the outer membrane where the subunits are separated from chaperone FimC and assembled via the usher FimD (Pizarro-Cerda and Cossart, 2006). It has been reported that using pilicides, bicyclic 2-pyridones inhibited the attachment of *E. coli* as a result of the binding of pilicides to the surface fragment of the chaperone that contributes to the interaction with the usher protein (Larsson et. al., 2005; Pinkner et. al., 2006; Aberg and Almqvist, 2007). In addition, the adherence could be inhibited via disruption of curli

biosynthesis using curlicides, a group of bicyclic2-pyridone derivatives (Cegelski et. al., 2009).

### 1.1.4.2 Inhibition using receptor analogs

Characterization of host receptors helps in designing and developing adhesion inhibitors. Receptor analogs are polysaccharides that are similar to those on host receptors and competitively block bacterial adherence to the host cells surface (Ofek et. al., 2003b). Usually, bacterial adhesion factors target the host receptors containing sugar groups such as glycoproteins, glycolipids or proteoglycans. Thus, using soluble oligosaccharides as adhesion inhibitors may inhibit pathogens that bind to host cells by lectins (carbohydrate binding adhesins) (Ofek et. al., 2003b; Kouki et. al., 2013). Receptor analogs can be obtained from a range of natural and synthetic sources. The naturally sourced analogs involve milk and plant extracts. Oligosaccharides, glycoproteins and glycolipids in human milk contain sugar groups similar to the host glycans. Therefore, milk glycans competitively bind to pathogens and inhibit infections (Peterson et. al., 2013). Milk glycans have protective effects against infant diarrhoea (Morrow et. al, 2004), in addition to preventing the binding of a variety of enteropathogenic bacteria to Caco2 cells (Salcedo et. al., 2013). Plants such as Cranberry, Ginger, Cayenne and green tea are attractive sources of adhesion inhibitors (Stapleton et. al., 2012; Kontiokari et. al., 2001; Bonetta et. al., 2012; Bensch et. al., 2011). Cranberry extract prevents *Helicobacter pylori* binding to human red blood cells and mucus (Burger et. al., 2000), and inhibits the binding of *E. coli* to uroepithelial cells (Gupta et. al., 2007). Green tea (*Camellia sinensis*) inhibits the

attachment of *Propionibacterium acnes, H. pylori* and *S. aureus* due to it containing acidic polysaccharides (Lee et. al., 2006).

Studying the interactions between bacterial adhesins and host receptors provides information which helps to design synthetic receptor anologs to inhibit bacterial attachment (Almant et. al., 2011; Salminen et. al., 2007). For example, the mannosylated uroplakin Ia (UPIa) on the urothelial cells serve as a binding site for FimH present at the tip of type 1 pili on the uropathogenic *E. coli* cell surface (Zhou et.al., 2001; Min et.al., 2002). It was found that the adherence of *E. coli* mediated by type 1 pili was reduced using multimeric heptyl-mannosides in a murine model (Gouin et. al., 2009). Uropathogenic P-fimbriated *E. coli* recognize Galα1, 4Gal motif in the host glycolipids. Thus, the trisaccharide globotriose (Galα1, 4Galα1, 4Glc) inhibited bacterial colonization of mouse bladder (Leach et. al., 2005). Furthermore, various strains of *Streptococcus suis* bind to host glycolipids via Galα1-4Gal-containing oligosaccharides. Therefore, using a multivalent adhesion inhibitor containing Galα1-4Gal- may result in the inhibition of bacterial adherence (Kouki et. al., 2013).

### 1.1.4.3 Anti adhesin vaccine

Immunization of the host to produce adhesin -specific antibodies is an attractive way to protect the host from infections (Kisiela et. al., 2015). Protection can be achieved by active or passive immunization. Active protection involves the application of adhesin directly to the mucosal surface which leads to secretion of antibodies. Passive protection involves

treating the host with antibodies prepared in another host. For example, the infection of enterotoxigenic *Escherichia coli* (ETEC) in pigs is mediated by K88 fimbria. The binding of K88 with glycosphingolipids on enterocytes aids bacterial colonization and release of enterotoxins that cause diarrhoeal disease (Dubreuil et. al., 2016). It has been shown that the immunization of piglets with vaccines based on K88 fimbriae prevent diarrhoeal disease (Melkebeek et. al., 2013).

The FimH adhesin mediates *Escherichia coli* adherence to bladder cells. It has been shown that anti FimH antibodies inhibit the adherence of *E. coli* to bladder cells *in vitro* (Palaszynski et.al., 1998). In addition, the immunization with FimH adhesin protects against uropathogenic *E. coli* infections in mice and monkeys (Palaszynski et. al., 1998; Langermann and Ballou 2003; Langermann et. al., 2000; Kisiela et. al., 2015). An anti-adhesin vaccine was used to inhibit *P. aeruginosa* infection. It was reported that a small peptide in *P. aeruginosa* strain K (PAK) pili can bind to epithelial cells. The immunization of a murine model with a vaccine based on this peptide leads to inhibition of *P. aeruginosa* infections (Cachia and Hodges, 2003). *Streptococcus mutans* cause tooth decay and its binding is mediated by the surface protein known as streptococcal antigen I/II (SA I/II). It has been revealed that *S. mutans* colonization was delayed when SA I, II adhesin antibodies were placed directly on the healthy volunteers' teeth (Ma and Lehner, 1990).

### 1.1.4.4 Probiotics and an engineered probiotics

Probiotics are live microorganisms, which when administered in adequate amounts, confer beneficial effects to the host. The most used probiotics are *Lactobacillus* and *Bifidobacterium,* in addition to *Bacillus, Pediococcus* and yeasts (Soccol et. al., 2010). Many studies showed the antagonistic role of probiotics against enteropathogenic bacteria via the production of antimicrobial agents, competition for nutrients and competition for the binding sites on mucosal epithelial cells (Soccol et. al., 2010; Abedi et. al., 2013). The anti-adherence properties of probiotics have been investigated and revealed their effıciency in blocking pathogenic bacterial adhesion (Banerjee et. al., 2009). Using a murine model, it has been reported that colonization by *Bifidobacterium breve* strain Yakult and *Bifidobacterium pseudocatenulatum* DSM 20439 prevents the production of shiga toxin and protects mice from Shiga toxin-producing *Escherichia coli* (STEC) infections (Asahara et. al., 2004). The competition of *Lactobacillus delbrueckii* subsp bulgaricus with *Escherichia coli* reduced *E. coli* attachment to Caco-2 cells (Abedi et. al., 2013). Another study revealed that the attachment of *V. parahaemolyticus* to HT-29 cells was reduced using *L. plantarum* AS1 (Satish Kumar et. al., 2011). Furthermore, *Lactobacillus salivarius* was able to inhibit *Salmonella enteritidis* colonization in chickens (Pascual et.al., 1999). Another *in vivo* study using a murine model showed that the infection of *Salmonella enterica* serovar Typhimurium DT104 (DT104) was inhibited using the *Lactobacillus casei* Shirota strain (Asahara et. al., 2011).

Although probiotics effectively prevent enteropathogenic infections, they are non- specific in action and their activity may differ from host to host. In addition, many factors affect their efficacy such as the exact strain used, the dose and method of administration, and

the formulation as a probiotic product (Morrow et. al., 2008). Therefore, engineered probiotics have been generated. The designer microbes can be engineered to express lipopolysaccharides mimicking host receptors. It has been shown that the probiotic *Escherichia coli* was engineered to express lipopolysaccharide similar to host receptor (ganglioside) for enterotoxogenic *E. coli* or cholera toxin therefore prevent diarrheal infections (Paton et. al., 2006). In addition, probiotics could be designed to express specific bacterial adhesin which bind to host mucosal surface and compete with pathogens for the binding sites. It was found that the pre incubation of Caco-2 cell with the recombinant *Lactobacillus paracasei* expressing *Listeria* adhesion protein (LAP) results in inhibition of *L. monocytogenes* infection (Koo et.al., 2012). In addition, the colonization of pig intestine by ETEC was inhibited using engineered *L. acidophilus* expressing ETEC K99 fimbriae (Chu et. al., 2005).

### 1.1.4.5 Inhibition using adhesin analogs

This strategy relies on isolated bacterial adhesins or synthetic or recombinant adhesin fragments binding to host receptors and thus competing with bacterial adhesins and preventing their binding to host cells (Shoaf and Hutkins, 2009). Several investigates have shown the potential role of adhesin analogs in inhibiting pathogens adherence. Daep et. al., showed that the initial colonization of the oral cavity by the anaerobic bacterium *Porphyromonas gingivalis* occurs via the interaction with *Streptococcus gordonii* by a specific region of the streptococcal SspB polypeptide (BAR) on the *S. gordonii* surface. The authors reported that a synthetic peptide containing the BAR sequence effectively

prevents the binding of *P. gingivalis* to *S. gordonii* and thus inhibits *P. gingivalis* to form biofilms (Daep et. al., 2006; Daep et. al., 2011). Another study revealed that a synthetic peptide mimicking the *S. mutans* adhesin AgI/II (p1025) sequence reduced Streptococci adherence to the immobilized salivary receptor (Kelly et. al., 1999). Furthermore, *Streptococcus* colonization was reduced when lipoteichoic acid was used as inhibitor to block bacterial adherence to the host receptor (Cox, 1982). Moreover, in a tissue culture model, the recombinant MAM7 from *V. parahaemolyticus* was used to prevent the adherence of a range of multidrug-resistant pathogens (Krachler et. al., 2012). In addition, MAM7 coupled to beads competed with *S. aureus* (MRSA) for the binding site without interfering with host cell functions (Hawley et. al., 2013). In experiments using a rat model, a MAM7 based inhibitor has been used to treat burn wound infections caused by *P. aeruginosa* (Huebinger et. al., 2016).

## 1.2 Host receptors

### 1.2.1 The extracellular matrix

Extracellular matrix (ECM) is a collection of macromolecules secreted by cells and fills the intercellular space. The extracellular matrix is made up of two main classes of components: proteoglycans or glycosaminoglycans (GAG), and fibrous proteins such as collagen, laminin, elastin, vitronectin, fibrinogen and fibronectin (Jarvelainen et.al, 2009; Schaefer and Schaefer, 2010). The glycosaminoglycans provide a gel-like structure and resistance to compressive forces. However, the strength and flexibility of the matrix is

provided by the fibrous proteins. ECM provides cells with structural support, in addition to other functions such as mediating cell proliferation, adhesion, migration and differentiation (Westerlund and Korhoner, 1993; Hynes, 2009).

The ECM constituents are exploited by pathogenic bacteria for adherence and stimulation of inflammatory responses, which result in colonization and invasion of the host. In addition, they are targeted by pathogen proteases that degrade them and increase tissue damage (Singh et. al., 2012). This review will cover several extracellular components and examples of bacterial attachment to ECM components.

#### 1.2.1.1 Proteoglycans and glycosaminoglycans

Proteoglycans are macromolecules found on the cell-surface and as part of the extracellular matrix. They consist of a core protein coupled to glycosaminoglycan (GAG) chains (Kjellen and Lindahl, 1991; Schaefer and Schaefer, 2010). Glycosaminoglycans are linear polysaccharides containing repeats of disaccharide units. Based on core disaccharides structures, glycosaminoglycans are divided into several categories: Heparan sulfate (HS)/heparin, Keratan sulfate (KS), Chondroitin sulfate (CS)/dermatan sulfate (DS), and Hyaluronan (Schaefer and Schaefer, 2010).

Heparan sulfate proteoglycans (HSPGs) consist of heparan sulfate (HS) chains covalently bound to a variable core protein. HS chains are linear polysaccharide chains with repeating sulphated disaccharide units. Based on their location, HSPGs can be categorized into to three families: the cell surface HSPGs such as glypicans and

syndecans, the ECM HSPGs such as perlecan, agrin and type XVII collagen, and HSPGs inside intracellular vesicles such as serglycin (Sarrazin et. al., 2011). HSPGs contribute to the organization of the ECM by the interactions with ECM constituents such as vitronectin, laminin and fibronectin (Bernfield et. al., 1999; Sarrazin et. al., 2011).

HSPGs bind to several ligands such as chemokines, cytokines and growth factors, and this binding confers protection against proteolysis (Sarrazin et. al., 2011).

HSPGs are attractive targets for bacterial adherence, colonization and invasion (Garcia et. al., 2016). It has been shown that *N. meningitidis* binds to HSPGs via the adhesin OpaC to invade epithelial cells (de Vries et. al., 1998; Bos et. al., 2002). Heparan sulfate (HS) proteoglycans mediate the binding and invasion of *L. monocytogenes* to host cells via the surface protein ActA (Alvarez-Dominguez et. al., 1997). In addition, HPSGs mediate *P. aeruginosa* (Plotkowski et. al., 2001; Bucior, et. al., 2012), *S. aureus* (Garcia et. al., 2014) and *S. pneumoniae* (Tonnaer et. al., 2006) attachment to host cells.

#### 1.2.1.2 Fibronectin

Fibronectin is a high molecular weight glycoprotein that is important in several cellular functions such as cell adhesion, migration, differentiation (Yamada and Clark, 1996; Hocking et.al., 2000), and wound healing (Cho et. al., 2006).

Generally, fibronectin comprises of two similar subunits of approximately 250 kDa, linked by two disulfide bonds. Each subunit encompasses three types of repeating units: type I, type II and type III and three regions: extra domains A and B (EDA and EDB) and a

variable region (Pankov and Yamada, 2002). Two types of fibronectin exist; soluble plasma fibronectin and insoluble cellular fibronectin (To and Midwood, 2011). Plasma fibronectin is synthesized by hepatocytes and secreted into the blood in a soluble form and incorporated into fibrin clots during wound healing (Cho et. al., 2006). It does not contain the extra domains A and B (EDA and EDB). Insoluble cellular fibronectin is synthesized by fibroblasts and other cells and deposited locally and organized as a network of fibrils on the cell surface via the binding with cell surface receptors (integrins) (Singh et. al., 2010).

Bacteria exploit fibronectin and use it for the adherence to host cells. It has been shown that a variety of both Gram negative and Gram positive bacteria have fibronectin binding proteins (Henderson et. al., 2011). Examples for fibronectin binding proteins are FnBPA and FnBPB in *S. aureus* (O'Neill et. al., 2008), surface protein F1 in *S. pyogenes* (Hyland et. al., 2007), curli in *E. coli* (Oh et. al., 2016) and the surface adhesin FlpA on *Campylobacter jejuni* (Konkel et.al, 2010).

### 1.2.1.3 Laminins

The laminins are abundant constituents of the extracellular matrix and play an important role in the architecture and stability of basement membranes (Li et. al., 2002; Durbeej, 2010). Generally, laminins are high molecular weight glycoproteins (400–900 kDa), consisting of three different polypeptide chains; an alpha-chain, a beta-chain, and a gamma-chain, that are joined together through the coiled-coil region to make a cross-like form (Aumailley et. al., 2005). To date, five alpha-chains, three beta-chains, and three

gamma-chains are known in mammals (Miner and Yurchenco, 2004). Laminins bind to each other to form self-assembling networks of basement membranes; in addition they contain binding sites for ECM proteins such as entactin and they contain binding sites for cell surface receptors such as integrins and dystroglycan and other receptors (Yu and Talts, 2003). Through these interactions laminins influence cell adhesion, proliferation, migration and differentiation (Colognato and Yurchenco, 2000; Durbeej, 2010).

Laminins are targeted by a variety of pathogenic bacteria for the adherence and invasion of host cells (Lemichez et. al., 2010). It has been revealed that *Streptococcus pyogenes,* a causative agent of bacterial tonsillitis and skin infections, recognize laminin for adhesion to epithelial cells and dermal cells using Laminin-binding protein (Linke et. al., 2009). In addition, *Moraxella catarrhalis* bind to laminin through surface adhesins UspA1 and UspA2 (Tan et. al., 2006). Furthermore, leptospiral surface adhesin (Lsa27) mediates the binding of *Leptospira interrogans* to laminin (Longhi et. al., 2009).

### 1.2.1.4 Type IV collagen

Collagens are the main components of the extracellular matrix that play a significant role in the stability, organization and shape of tissue, in addition to their role in cell proliferation, differentiation and migration (Gelse et. al., 2003; Kadler et. al., 2007). To date, collagens consist of 28 different types (Ricard-Blum, 2011). They comprise of a triple-helix assembly which consists of three polypeptides with Gly-X-Y repeats, where X is any amino acid, and Y is proline or hydroxyproline (Gelse et. al., 2003).

Type IV collagen is the major constituent of the basement membrane. Six α-chains (α1–α 6) have been characterized which form three different promoters: α1.α1.α2 (IV), α3.α4.α5 (IV) and α5.α5.α6 (IV) (Hudson et. al., 2003). Each α-chain comprises of three domains: a 7S domain, a triple-helical domain, and a non-collagenous NC1 domain. Three α- chains of collagen IV gather at the NC1 domain to form triple-helical molecule known as promotor. Further interactions between promotors form supramolecular networks which interact with the laminin network to form basement membrane scaffolds (Kalluri, 2003).

Bacteria use collagens to bind to and enter host tissues. It has been found that *M. catarrhalis* bind to collagen types I, II, and III, collagens IV and VI using ubiquitous surface protein A2 (UspA2 and UspA2H) (Singh et. al.,2016). The adhesin of collagen from *E. faecalis* (Ace) mediates bacterial binding to the Type IV collagen and the Type I collagen (Nallapareddy et. al., 2006). In addition, collagen IV is targeted by *S. pyogenes* M1 adhesin (Bober et. al., 2010).

### 1.2.2 Sulfatide

Sulfatide (3-O-sulfogalactosylceramide) is a type of glycosphingolipid that contains a sulfate group. It consists of the ceramide and one monosaccharide, galactose. The synthesis of sulfatide is started at the endoplasmic reticulum where a galactose from UDP-galactose is added to ceramides to make GalCer (galactocerebroside), a reaction catalysed by galactose transferase (Schaeren-Wiemers et.al., 1995). The product GalCer (galactocerebroside) is then transported into the Golgi apparatus to react with 3'-phosphoadenosine-5'-phosphosulfate (PAPS) to make sulfatide. This reaction is carried

out by cerebroside sulfotransferase (Honke et.al., 1997). The degradation of sulfatide occurrs in the lysosome, where Arylsulfatase A (ASA) hydrolyzes the sulfate group in the presence of saponin B (Kolter and Sandhoff, 2005). Sulfatide is abundantly expressed in the gastrointestinal tract, trachea, kidney, and particularly the central nervous system, accounting for for 4% of total membrane lipids (Ishizuka, 1997). Cellular sulfatide is mostly localized in the Golgi apparatus, cellular membrane, and lysosomes in cytosol (Takahashi and Suzuki, 2015; Takahashi and Suzuki, 2012; Hirahara et. al., 2000). Sulfatide contributes to a wide range of biological processes such as cell adhesion, aggregation and protein trafficking (Xiao et. al., 2013). Furthermore, sulfatide is involved in immune system function, cancer, insulin secretion and microbial infection (Takahashi and Suzuki, 2012). Sulfatide has a role in the pathogenesis of Alzheimer's disease. In a study on human brain tissues revealed that a significant decrease in sulfatides is associated with Alzheimer's disease (Han et. al., 2002).

For microbial infections, sulfatide is known to be a receptor for the adherence of many pathogenic bacteria (Piao et. al., 2011; Pantzar et. al., 2006; Hartmann et. al., 2001; Yagci et. al., 2007). It has been shown that *H. pylori* bind to the sulfatide via Hsp 70 on its surface (Kamisago et. al., 1996; Kobayashi et.al., 2009). In addition, *P. aeruginosa* binds to sulfatide on human pharyngeal epithelial cells (Xia et. al., 2007). *Bordetella pertussis* use Filamentous hemagglutinatinin adhesin (FHA) to bind to sulfatide on the host cells (Julie et. al., 2007). Enterotoxigenic *Escherichia coli* (ETEC) binds to sulfatide via coli surface antigen 6 (CS6) (Jansson et. al., 2009). Moreover, sulfatide is a receptor for Heat-stable toxin b (STb) produced by Enterotoxigenic *Escherichia coli* (ETEC) (Labrie, et.al. 2001).

### 1.2.3 Mucus and Mucins

Mucus is a highly hydrated viscous secretion that covers epithelial cell surfaces. It acts as a barrier to protect epithelial surfaces such as respiratory, gastrointestinal, reproductive, urinary tracts and the eye from environmental stresses (Derrien et. al., 2010). The key function of mucus is to protect against dehydration and damage that could be caused by drugs, toxins, acids and pathogens. Mucus thickness and composition varies depending on its location in the body. In the oral cavity, salivary glands produced saliva with a thickness of 70-100 μm. Saliva consists of water, mucin and other proteins to protect teeth and oral soft tissue (Collins and Dawes, 1987). The stomach is lined with a thick mucus layer of around 300 μm that protects the gastric epithelium from the damage resulting from either ingested compounds or the enzymes and the acidic pH in the stomach (Atuma et. al., 2001). In the intestinal tract, submucosal glands and goblet epithelial cells produce the intestinal mucus layer, a continuous layer that consists of a firm inner cell-attached layer and a loose outer non-attached layer (Atuma et. al., 2001). In the small intestine, the mucus layer is a discontinuous layer with a thickness around 150–400 μm (Atuma et. al., 2001; Johansson et. al., 2011). Colonic mucus layer thickness reaches to 800-900 μm in the distal colon. This thickness is essential for the protection of the underlying tissue from the interactions with pathogens (Derrien et. al., 2010). 95% of the mucus composition is water and the remainder is mucins and other components such as proteins, salts and lipids (Allen, 1981). In addition, mucus contains a variety of antimicrobial proteins and peptides involved in host defence, such as immunoglobulins, defensins and others (Phillipson et. al., 2008).

The main constituents of the mucus are mucins that play a significant role in the interactions between host epithelial surfaces and microorganisms (Linden et. al., 2004; Johansson et. al., 2011). Even with the diversity among mucins, they are similar in their structures. Mucins are large glycoproteins with a high molecular weight of several million Daltons (Derrien et. al., 2010). They contain one or more glycosylated protein domains. The major constituents of these glycoproteins are carbohydrate (80% of the weight is carbohydrate) (Thornton and Sheehan, 2004). The domains have repeats of the amino acids serine and threonine, which work as the attachment point for N-acetylgalactosamine. In addition, these domains may contain N-acetylglucosamine, sialic acids, fucose and galactose, and the sugars may be further modified by sulphate or phosphate. The attachment of oligossacharides to the polypeptides results in the enlargement of the molecule, which contributes to mucin functions such as filling the space and gel like properties (Jentoft, 1990; Thornton and Sheehan, 2004). In addition, the presence of oligosaccharides results in bacterial binding to the mucin which finally facilitates their removal by mucin shedding and inhibits the colonization of epithelial cells (Thornton and Sheehan, 2004). In addition to the high molecular weight and high carbohydrate contents, mucins have a low isoelectric point (are acidic under physiological conditions) because of their sialic acid and sulphate content (Rose, 1992). Currently, two types of mucins are known; secreted and cell surface attached mucins. Secreted mucins can be divided into secreted gel forming mucins such as MUC2, MUC5AC, MUC5B, MUC6, MUC19 and secreted non gel forming mucins such as MUC7. Whereas, cell surface mucins include MUC1, MUC3A/B, MUC4, MUC12, MUC13, MUC15, MUC16, MUC17, and MUC20 (Linden et. al., 2004). Secreted gel forming mucins are the main

components of mucus and they are responsible for the viscoelasticity of the mucus by forming disulphide bond via cysteine rich domains existing at the N- and C-termini (Perez-Vilar et. al., 1998).

### 1.2.3.1 Mucus- bacterial interactions

Mucus provides a barrier to protect the underlying epithelium from colonization with pathogens and commensals. Intestinal mucus comprises of two layers; an outer layer and an inner layer. The outer layer provides nutrients for the commensals that aid in the protection against pathogens. It is well known that commensals do not enter the inner mucus layer and do not colonize the healthy mucosal surface. In the colon, commensal bacteria live in symbiotic relationship with the host. They contribute in processing indigestible glycoconjugates and provide the host with vitamins. Commensals also participate to limit pathogenic colonization and invasion of epithelial cells via the competition for nutrients, competition for binding sites and development of the mucosal immune response (Hudault et. al., 2001; Lu and Walker, 2001; Kamada et. al., 2013). In addition they release antimicrobial peptides known as bacteriocins that inhibit bacterial growth of other strains or species (Cunliffe and Mahida, 2001). Alteration of the microbiota composition results in an increase of pathogenic bacteria that cause ulcerative colitis such as *Clostridium difficile* (Leffler and Lamont, 2015) and enteroaggregative *E. coli* (EAEC) (Watanabe et. al., 2012).

Mucin is an attractive niche for bacterial colonization (Tailford et. al., 2015). Bacteria use mucin carbohydrates as a source of carbon and nitrogen. In addition, mucin

carbohydrates provide binding sites for bacteria. Those sites are similar to the binding sites present on the cell surface. Therefore, mucins inhibit pathogens adhesion to underlying cells (Derrien et. al., 2010). In addition, mucins carry a negative charge which provides protection against bacterial attack. This protection based on the repulsion forces between mucin and bacterial cell surface (Wiggins et. al., 2001)

The importance of mucin has been reported in many studies. It has been shown that binding of Muc1 from human breast milk to pathogenic bacteria inhibits their adherence to the infant gastrointestinal tract (Ruiz-Palacios et. al., 2003). MUC2 is the main mucin found in the intestinal tract. Infection of mice with a deficiency in MUC2 with *Citrobacter rodentium* resulted in increased intestinal inflammation (Van der Sluis et. al., 2006), and more severe disease (Bergstrom et. al., 2010).

A variety of strategies have been developed by pathogens in order to go through the mucus and infect the underlying epithelial cells. Pathogens trigger inflammation which results in altered microbiota composition and end the competition with commensals (Pedron and Sansonetti, 2008). In addition, commensals are affected by the increase in the amount of antimicrobial peptides (Raffatellu et. al., 2009; Stecher and Hardt, 2011). Furthermore, the production of specific compounds during the inflammation process can be used by pathogens for their growth such as glycosylated proteins or tetrathionate (Winter et. al., 2010; Stecher et. al., 2008). It has been revealed that tetrathionate is used by *Salmonella typhimurium* as a terminal electron acceptor during anaerobic respiration, thus this bacterium competes with other fermenting commensals in the infected region (Winter et. al., 2010). Another strategy used by pathogens to go through mucus is motility.

Pathogens use flagella to penetrate the mucus barrier. It has been shown that *H. pylori* decrease the mucus viscosity by changing the surrounding pH to increase their motility (Celli et. al., 2009). *V. cholerae* use its single polar flagellum to swim through the mucus and colonize the epithelial cells (Liu et. al., 2008). In addition, the curved shape of *V. cholerae* cells facilitates the movement through the mucus (Bartlett et. al., 2017).

Mucin degradation is also used by pathogens to reduce the viscosity and dilute mucus compounds. Pathogenic bacteria produce a variety of enzymes such as glycosidases and proteases to degrade mucin (Roberton et. al., 2005). The enteric pathogenic bacteria use this strategy to reach the host epithelial cells. It has been shown that enterotoxigenic *Escherichia coli* (ETEC) secrete metalloprotease that degrade intestinal mucins, including MUC2 and MUC3 to aid bacterial access to enterocytes (Luo et. al., 2014).

### 1.2.3.2 Mucin desulfation

Mucin carbohydrates are heavily sulfated in the regions that are rich in microorganisms such as colon and mouth (Derrien et. al., 2010). This physically protects the carbohydrates and other substrates from enzymatic degradation (Tsai et.al., 1991). Sulfation of mucin oligosacharides occurs in the linkage to N-acetylglucoseamine, galactose and, rarely, N-acetylgalactosamine (Brockhausen et.al., 2009; Kawasaki, et. al., 2001; Lo-Guidice et.al., 1994). However, to overcome this protection, microorganisms develop mechanisms to degrade mucin and infect epithelial cells. Some bacteria have sulfatase that catalyse the hydrolysis and release of sulfate groups from substrates such

as sulfomucins, sulfoglycoproteins and sulfoglycolipids. Therefore, sulfatases play a role in mucin desulfation and thus increase the degradation by glycosidases and proteinases (Rho et. al., 2005). Desulfation results in providing bacteria with sulphur and release carbohydrates and amino acids used as substrates for bacterial growth (Rho et. al., 2005). Several pathogenic bacteria secrete sulfatases, such as *Proteus rettgeri* (Fitzgerald and Milazzo, 1970), S*almonella enterica* Serovar Typhimurium (Das et. al., 2013), *Salmonella enterica* Enteritidis (Ganguly and Joerger, 2017), *Klebsiella aerogenes* (Murooka et.al., 1990), and *Serratia marcescens* (Murooka et.al., 1980).

In addition, sulfatase is secreted by anaerobic commensals, such as *Prevotella* strain RS2, that has been shown to have a mucin-desulfating sulfatase (MdsA), and sulfoglycosidase (SGL) in the bacterial periplasm (Wright et. al., 2000). The sulfoglycosidase (SGL) from *Prevotella* strain RS2 desulfates mucin by removal of a 6-SO3-β-GlcNAc residue from sulphated mucin to be a substrate for MdsA. The reasons behind removing the 6-SO3-β-GlcNAc residue could be to create or remove binding sites or to allow further mucin degradation (Rho et. al., 2005). The commensal *Bacteroides thetaiotaomicron* secretes sulfatase to remove sulphate groups and allow glycosidase enzymes to degrade mucins (Tsai et. al., 1991; Benjdia et. al., 2011).

*Bacteroides thetaiotaomicron* will be covered in more detail in the following section.

#### 1.2.3.2.1 *Bacteroides thetaiotaomicron*

One of the commensal bacteria present in the human gut is *Bacteroides thetaiotaomicron* which makes up about 6% of the gut microbiota in healthy individuals (Murphy et.al, 2011). *B. thetaiotaomicron* is an obligate anaerobic Gram-negative bacterium and belongs to the Bacteroidetes phylum. In the gut, *B. thetaiotaomicron* releases carbohydrate degrading enzymes that provide availability of nutrients for other commensals (Sonnenburg et. al., 2005; Xu et. al., 2003). In addition, it aids the human host in the metabolism of carbohydrates like sucrose, starch and lactose in the gastrointestinal tract because humans are able to metabolize only a limited range of dietary carbohydrates (Lammerts van Bueren et. al., 2015).

Although *B. thetaiotaomicron* is a commensal bacterium, it can be an opportunistic pathogen. *B. thetaiotaomicron* produce two virulence factors that have been involved in the pathogenicity of this bacterium; LPS and capsular polysaccharide (CPS) (Rokosz et.al., 1999). It can cause infections such as meningitis (Feuillet et. al., 2005), bacteraemia and intraabdominal sepsis (Aldridgeand O'Brien, 2002; Goldstein, 1996; Redondo et.al., 1995). In addition to the pathogenicity, the resistance of *B. thetaiotaomicron* to antibiotics such as cephalosporins and clindamycin has been reported (Aldridgeand O'Brien, 2002; Snydman, 1996; Tanaka-Bandoh et.al., 1995).

*B. thetaiotaomicron* dedicates a large number of its genome to carbohydrate metabolism (Martens et.al, 2009). In addition to dietary sources, *B. thetaiotaomicron* is capable of using mucin and mucopolysaccharides as carbon sources (Sonnenburg et. al, 2005). *B. thetaiotaomicron* uses mucosal glycan for degradation when the dietary sources of glycans are unavailable (Benjdia et. al., 2011). The ability to forage both dietary

polysaccharides and the mucosal glycans is a strategy by which *B. thetaiotamicron* is able to adapt and compete with other microorganisms in the gut.

*B. thetaiotamicron* has carbohydrate active enzymes (CAZymes) that are implicated in the metabolism of carbohydrates (Hehemann et. al., 2012). In addition, *B. thetaiotaomicron* genome contains 28 putative sulfatase genes and an anaerobic sulfatase-maturating enzyme (anSME) (BT0238) which is necessary for the activity of all sulfatases (Benjdia et. al., 2011).

*B. thetaiotaomicron* enzymes may have a role in gut infectious disease. *B. thetaiotaomicron* harvests sugars on mucins which become available for both commensals and pathogenic bacteria. It has been shown that *B. thetaiotaomicron* releases fucose which is used by *S. typhimurium* as a source of carbon (Ng et. al., 2013), and also it is used by EHEC to regulate the expression of virulence factors (Pacheco et. al., 2012).

In addition, mucin degradation by *B. thetaiotaomicron* sialidase results in release the sialic acid that supplying pathogenic bacteria such as *Clostridium difficile* and *Salmonella enterica* with sugars (Ng et. al., 2013). The contact between the outer membrane vesicles in *B. thetaiotaomicron* containing sulfatase and host macrophages results in gut inflammation, and deleting the anaerobic sulfatase maturating enzyme (anSME) from *B. thetaiotaomicron* prevent colitis in dnKO mice (Hickey et. al., 2015).

## 1.3 The commensal *Escherichia coli*

*Escherichia coli* are Gram negative, non-sporulating rod shaped bacteria, facultative anaerobes, and belong to the family Enterobacteriaceae. Commensal *E. coli* exist on the mucus layer of the human gastrointestinal tract in a symbiotic relationship with the host (Gill et. al., 2006). In addition to providing the host with vitamins, commensals *E. coli* enhance tight junctions between epithelial cells, stimulate the mucosal immune response and compete with pathogens and inhibit infections (Hudault et. al., 2001; Lu and Walker, 2001). The inhibition of pathogenic bacteria by *E. coli* is the result of the competition for the binding sites, the competition for nutrients and the production of antibacterial agents (Cunliffe and Mahida, 2001). For example, it has been reported that the adhesion of pathogenic *E. coli* was inhibited when *E. coli* strain Nissle 1917 was co cultured with invasive *E. coli* using intestinal epithelial cell models (Boudeau et. al., 2003). In addition, using mouse models, it has been revealed that the establishment of the commensal *E. coli* JM105 strain and EM0 strain results in inhibition of *S. typhimurium* infection (Hudault et. al., 2001). Furthermore, the commensal *E. coli* strain HS competes with pathogenic *E. coli* O157:H7 for nutrients that are important for bacterial colonization and inhibit infections (Maltby et. al., 2013). In the current study, the human isolate *E. coli* strain HS was used to characterize its MAM7 homolog. *E. coli* HS was isolated from a healthy scientist at the Walter Reed Army Institute of Research, and it has the ability to inhabit the human gastrointestinal tract using pili or fimbria without causing illness (Rasko et. al., 2008).

## 1.4 Aims

The overall aim of the work performed for this thesis was to evaluate the potential role of adhesion inhibitors based on MAM7 adhesin from the human commensal *E. coli* strain HS to inhibit bacterial infection.

Towards this aim:

1- Characterize MAM7 protein from the human commensal *E. coli* strain HS.

2- Design an adhesion inhibitors based on MAM7 protein from the commensal *E. coli* strain HS.

A- An engineered bacterium expressing HSMAM7 protein on the surface (BL21-HSMAM7).

B- Polymer beads coupled to the purified recombinant GST- HSMAM7 protein.

3- Evaluate the potential role of HSMAM7 based inhibitors against pathogenic bacteria.

4- Dissect the biochemical interactions of HSMAM7 protein and its host cell receptors: lipids and proteins.

# Chapter 2. Materials and Methods

## 2.1 Bacterial strains and growth conditions

The bacterial strains used were *Escherichia coli* strain HS (GenBank accession number ABV06236.1; GI 157066981) *Pseudomonas aeruginosa* PAO1, *Enterococcus faecalis* ATCC 49452, *Staphylococcus aureus* Newman and *E. coli* BL21. These strains were grown in LB medium at 37 °C. *Bacteroides thetaiotaomicron* (wild type strain VPI-5482) and a derivative strain lacking the anaerobic sulfatase-maturating enzyme (anSME), Δ BT0238, was grown in Brain Heart infusion (BHI) medium under anaerobic conditions at 37 °C .

## 2.2 Cell lines and growth conditions

Hela cells, Caco-2 cells and HT-29MTX cells were grown in a 75 $cm^2$ flask and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum (HIFBS), 5 mM L-glutamine, 50 µg/ml streptomycin and penicillin in an atmosphere containing 5% $CO_2$ at 37 °C. After confluent growth, cells were washed with sterile PBS (phosphate buffer saline) and treated with Trypsin EDTA to prepare the required cell concentration.

## 2.3 Molecular techniques

### 2.3.1 Extraction of Genomic DNA

Genomic DNA was extracted from *E. coli* strain HS using Gen Elute Bacterial Genomic DNA Kit (Sigma). The extraction process was done according to the manufacture's instructions. Briefly, 1.5 ml of an overnight culture growth was centrifuged at 12000 xg for 2 minutes and the pellet was re suspended in 180 µl lysis solution T. 20 µl of proteinase K solution were added to the sample, mixed well and incubated at 55 °C for 30 minutes. Followed by adding 200 µl of lysis solution C mixed gently and incubated at 55 °C for 10 minutes. 200 µl of ethanol (95%) were added to the lysate and mixed. Then 500 µl of the column preparation solution were added to column, and centrifuged at 12000 xg for 1 minute. The entire contents were transformed into binding column and centrifuged at 12000 xg for 1 minute. The collection tube containing the flow through was discarded and the column was placed into new collection tube. Then 500 µl of wash solution 1 were added to the column and centrifuged at 12000 xg for 1 minute. 500 µl of wash solution were added to the column, centrifuged at 12000 xg for 3 minutes. 200 µl of the elution solution was pipetted into the centre of the column and incubated for 5 minutes at room temperature, then centrifuged at 12000 xg for 1 minute. The concentration of the purified DNA was measured. In addition, gel electrophoresis was run to test the quality of the DNA. Then the DNA was stored at -20 °C.

## 2.3.2 Polymerase Chain Reaction (PCR)

The *E. coli* HSMAM7 gene was amplified using the primers designed for the PCR reaction (table 2.1). 50 µl of PCR mixture containing 10 µl of 5x phusion buffer, 1 µl of 10 mM dNTPs, 2.5 µl of 10 mM Forward primer, 2.5 µl of 10 mM Reverse primer, 0.5 µl of phusion polymerase, 0.5 µl of DNA template and the DNase free $dH_2O$. The amplification conditions are shown in table 2.2.

**Table 2.1 Primers pairs used for the amplification of HSMAM7 gene**

| Primer description | Sequence |
|---|---|
| *E. coli* HS(ΔTM)MAM7 FOR | 5'CAGTGGATCCGACAGTTATCAGGACCGGG'3 |
| *E. coli* HS(ΔTM)MAM7 RE | 5'CAGTCTCGAGTTATTTGGGAAGCGCAGTACC'3 |
| *E. coli* HS (Full)MAM7 FOR | 5'CAGTCCATGGGACACATGAGTCAGGAAACG'3 |
| *E. coli* HS (Full )MAM7 RE | 5'CAGTAAGCTTTTATTTGGGAAGCGCAGTACC'3 |

Table 2.2 The PCR conditions used to amplify HSMAM7 gene

| Condition | Temperature | Time | No. of cycles |
|---|---|---|---|
| Initial denaturation | 95℃ | 2min | 1 |
| Denaturation | 95℃ | 30sec | 30 |
| Annealing | 61℃ | 30sec | |
| Extension | 72℃ | 3min | |
| Final extension | 72℃ | 5 min | 1 |
| Hold | 4℃ | ∞ | |

## 2.3.3 Agarose gel electrophoresis

The agarose gel was prepared as following:

A 0.8% agarose was made by mixing 0.8 g agarose with 100 ml of 1X TBE buffer and heated in microwave oven. Then, 2 µl Sybr safe stain was added, gently mixed and poured into a rack containing comb. After gel solidification, the comb was removed, and the gel was soaked into a chamber containing 1X TBE buffer. The loading dye was added to the DNA samples, they were loaded alongside to the DNA ladder. The gel was run at 87 volts for 45-60 minutes.

## 2.3.4 Agarose gel purification

The DNA fragment was cut out the agarose gel and weighed in a 1.5 ml Eppendorf tube. Gene JET Gel Extraction Kit (Ferments) was used for DNA purification as following:

1:1 volume of the binding buffer was added to the gel slice and left at 55 °C for 10 minutes. 800 µl of the solution was moved into the column and centrifuged at 12000 xg for 1 minute. Then, 700 µl of wash buffer was added to the column and centrifuged at 12000 xg for 1 minute. The flow through was discarded and the empty column was centrifuged for 1 minute. The column was transferred into 1.5 ml micro centrifuge tube, and 50 µl of elution buffer were added to the column, incubated at room temperature for 2 minutes, and then centrifuged at 12000 xg for 1 minute.

## 2.3.5 Digestion of DNA with restriction enzymes

pGEX 4T-3 and pBAD/Myc-His plasmids were used in this study. The plasmids and the DNA amplicons were digested with the appropriate restriction enzymes as shown in tables 2.3 and 2.4. The reaction mixtures were incubated at 37 °C for 1 hour.

Table 2.3 Digestion of the insert (ΔTM MAM7) and pGEX 4T-3 vector

| **Reagents** | **insert (ΔTM MAM7)** | **pGEX4T-3** |
|---|---|---|
| DNA | 30 µl | 15 µl |
| BamHI buffer | 10 µl | 10µl |
| BamHI enzyme | 1 µl | 1µl |
| XhoI enzyme | 2 µl | 2µl |
| d H2O | 7 µl | 22µl |

Table 2.4 Digestion of the insert (Full length HSMAM7) and pBAD /Myc-His vector

| **Reagents** | **Insert (Full length MAM7)** | **pBAD /Myc-His** |
|---|---|---|
| DNA | 30 µl | 15 µl |
| Tungo buffer | 10 µl | 10 µl |
| Hind III enzyme | 1 µl | 1 µl |
| NCOI enzyme | 2 µl | 2 µl |
| d H2O | 7 µl | 22 µl |

## 2.3.6 DNA ligation

T4 DNA Ligase was used to ligate the digested inserts into the indigested vectors .The reaction mixture contained 2 µl of 10x Ligation Buffer, 1 µl of purified digested vector ,16 µl of purified digested DNA (insert) and 1µl of T4 DNA Ligase. The ligation mixture was incubated at room temperature overnight.

## 2.3.7 Transformation

Heat shock method was used to transform *E. coli* with the recombinant DNA as following: 5 µl of ligation mixture was added to 50 µl of competent *E. coli* DH5α cells and incubated on ice for 30 minutes. The cells were heat-shocked at 42 °C for 30 seconds. Followed by incubation on ice for 5 minutes. 950 µl of SOC broth were added and incubated at 37 °C for an hour with shaking at 225 rpm. LB agar plate containing an appropriate antibiotic was inoculated with100 µl of the transformation mixture and incubated at 37 °C overnight.

## 2.3.8 Purification of plasmid DNA

The recombinant plasmids were purified using Gen Elute™ plasmid mini prep kit (Sigma). 1.5 ml of bacterial overnight growth was centrifuged at 12000 xg for 1 minute. The pellet was re suspended in 250 µl of re suspension solution. Then 250 µl of lysis solution was added and mixed gently. 350 µl of neutralizing solution was added; the tube was inverted 4-6 times and centrifuged at 12000 xg for 5 minutes. The lysates were transferred to the Gen Elute mini prep column and centrifuged at 12000 xg for 1 minute. 700 µl of wash

solution was added and centrifuged at 12000 xg for 1 minute. The flow through was discarded, and these steps were repeated. The column was centrifuged to remove the ethanol. The column was transferred into a clean tube and 50 µl of elution buffer were added and incubated at room temperature for 2 minutes then centrifuged at 12000 xg for 1 minute. The purified plasmids were stored at - 20 °C.

### 2.3.9 DNA Sequencing

The purified recombinant plasmids were sent to the Functional Genomic Service at the School of Bioscience at Birmingham University (http://www.brimingham.ac.uk/facilities/genomics/) for sequencing using the primers listed in table 2.5. In two separate micro centrifuge tubes, the reaction mixture contains 1 µl of the recombinant DNA (40-100 ng), 1 µl of fw or rev primer (3.2 pmol) and 8 µl of PCR water.

**Table 2.5 Primers pairs used for sequencing of the recombinant plasmids pGEX 4T3-ΔTMHSMAM7 and pBAD-Myc-His HSMAM7**

| Primer description | Sequence |
|---|---|
| pGEX-4T3 Forward | 5'GGGCTGGCAAGCCACGTTTGGTG3' |
| pGEX- 4T3 Reverse | 5'CCGGGAGCTGCATGTGTCAGAGG3' |
| pBAD Forward | 5' ATGCCATAGCATTTTTATCC 3' |
| pBAD Reverse | 5' GATTTAATCTGTATCAGG 3' |

## 2.4 Protein expression and purification

### 2.4.1 Protein expression

IPTG induction method was used to produce the recombinant proteins GST-HSMAM7 and GST. 10 ml of LB containing 100 µg/ml ampicillin was inoculated with a colony of *E. coli* BL21 containing the recombinant plasmid (pGEX 4T-3 ΔTMMAM7) for production of GST-HSMAM7 protein or *E. coli* BL21 containing pGEX 4T-3 plasmid for production of GST protein and incubated at 37◦C overnight with shaking at 225 rpm. 500 ml of LB broth containing 100 µg/ml of ampicillin was inoculated with 10 ml of the bacterial growth and incubated at 37 °C with shaking at 225 rpm until the optical density reached 0.6-0.7. IPTG was added at a final concentration of 1 mM to the cultures and incubated at 37 °C for 4 hours with shaking at 225 rpm. Cultures were centrifuged at 6,000 rpm for 10 minutes. The pellets were frozen at -20 °C until further purification.

### 2.4.2 Protein purification using GST- affinity chromatography

The frozen bacterial pellets were defrosted on ice and resuspended in 25 ml of GST lysis buffer (25 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, protease inhibitors, lysozyme, DNAse I) and 20 mM Na-cholate was added, and then incubated at 4 ℃ overnight. The suspension was sonicated on ice twice for 60 seconds each time (5 sec on, 5 sec off; 60% output) and centrifuged at 10000 rpm for 30 min at 4 ℃. The supernatants were filtered through a 0.45 µm filter. 1 ml of washed glutathione beads was added to the supernatant and rotated at 4 ℃ for 2 hours. 20 ml of binding buffer (25 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA) were added to the column containing the beads. This step was repeated

twice. 1.5 ml of elution buffer (10 mM reduced glutathione/ 50 mM Tris-HCl pH 8.0) was added to beads and incubated for 15 minutes and drained. This step was repeated twice. 1.5 ml of elution buffer (100 mM reduced glutathione) was added to beads and incubated for 15 minutes, then drained. This step was repeated three times. Glutathione was removed from the protein eluate by dialysis against PBS at 4 °C overnight.

### 2.4.3 Polyacrylamide gels

12 % Polyacrylamide Gel was used in the experiments. The concentration of separating gel was12 %, stacking gel was 6 %. The recipe for 12 % Polyacrylamide Gel is shown below:

| 32 ml of 12% Separating Gel | | 20 ml of 6% Stacking Gel | |
|---|---|---|---|
| 10.5 ml | $ddH_2O$ | 10.6 ml | ddHO |
| 12.8 ml | 30% Acrylamide | 4 ml | 30% Acrylamide |
| 8 ml | 1.5 M Tris pH 8.8 | 5 ml | 0.5 M Tris pH 6.8 |
| 320 µl | 10% SDS | 200 µl | 10% SDS |
| 320 µl | 10% APS | 200 µl | 10% APS |
| 32 µl | TEMED | 20 µl | TEMED |

Separation gel was prepared and poured into the cast and isopropanol was added to layer the gel. Following the polymerization of gel, the isopropanol was removed and the gel was washed with water. The stacking gel was prepared and poured, and a comb was inserted into the stacking gel and left to polymerize.

### 2.4.4 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

20 µl of samples were boiled in 5 µl of 5x SDS buffer for 10 minutes at 100 ℃ and were loaded alongside protein markers. The samples were separated in 1x SDS buffer at 120 V for 15 minutes then180-200 V for 30-45 minutes.

### 2.4.5 Coomassie blue staining

Coomassie blue stain was used to stain the protein bands following SDS- PAGE. Staining steps involved adding Coomassie brilliant blue stain (0.1% Coomassie Brillant Blue R250, 50% methanol and 10% glacial acetic acid) to the gel for 15 min with shaking. Followed by washing the gel with the destaining solution (10% methanol, 10% glacial acetic acid) for 10 minutes with shaking. The destaining step was repeated several times to visualize the bands.

## 2.5 Coupling of recombinant GST- HSMAM7 protein to the beads

Fluorescent beads of 2 µm diameter (Sigma) were used to be coupled to the recombinant GST-HSMAM7 protein or GST protein (control). 12 µl of beads were suspended into a tube containing 1 ml PBS and pelleted by spinning for 2 minutes at 16000 xg. The supernatant was dicarded and the pellet was re suspended in 1 ml of sterile PBS. The washing step was repeated three times followed by removing the supernatant. Beads were re suspended in 800 µl PBS and incubated with 200 µl of 10 mM sulfo-SMPB on a

rotating wheel at 25 ℃ for 1 hour. The activated beads were centrifuged for 2 minutes at 16000 xg and the pellet was washed with 1 ml of sterile PBS. Then the pellet was re suspended in 1 ml of 6 μM of protein solution and incubated for 2 hours at 25 ℃ on a rotating wheel. Cysteine was added to give a final concentration of 50 mM and incubated for 30 minutes at 25 ℃ on rotating wheel. Beads were washed twice with PBS and re suspended in 1 ml of PBS to give the final product. In order to verify the coupling efficiency, the concentration of coupled protein was measured and calculated depending on the following the formula: [Coupled protein] = [Initial protein] – [Supernatant protein].

## 2.6 Attachment of protein-coupled beads to host cells

Hela cells were seeded on cover slips at a concentration of 150000 cells/ml the day before the experiment. 10% v/v beads coupled to protein in colourless DMEM was added and incubated at 37 ℃ for 4 hours. The medium was removed and Hela cells were washed three times with sterile PBS. 4% paraformaldehyde in PBS was used to fix the cells for 15 minutes at room temperature. The cells were washed with PBS three times and 0.1% Triton X-100 in PBS was used for 5 minutes. In a dark place, cells were incubated with Alexa Fluor 488-phalloidin for 10 minutes. The coverslips were washed three times with PBS. The coverslips were mounted using ProLong Gold anti fade medium and imaged using a Nikon Ti Eclipse microscope. Images were used to quantify the attached beads.

## 2.7 Attachment of BL21-HSMAM7 to host cells

*E. coli* BL21 was transformed with the recombinant plasmid (pBAD/Myc-His-HSMAM7) (BL21-HSMAM7) or transformed with empty pBAD (control) and grown in the LB broth containing kanamycin at a concentration of 50 µg /ml the day before experiment. For the attachment experiment, *E. coli* BL21-HSMAM7 was grown in LB broth and when the OD600 reached 0.6, protein expression was induced by adding arabinose to 0.05% and incubated at 37 ℃ for 4 hours. Hela cells were seeded into a 24-well plate at a concentration of 150000 cells/ml, the day before the experiment. Hela cells were washed three times using sterile PBS and BL21-HSMAM7 or BL21 (control) was added at an MOI of 100 and the plates were incubated at 37 ℃ for 1 hour. DMEM was removed and Hela cells were washed three times with PBS to remove non-attached bacterial cells, and 0.5% Triton X-100 was added to lyse Hela cells, then serial dilutions were prepared and cultured on LB agar and incubated at 37 ℃ for 24 hours followed by counting the number of colonies. To visualize the attachment, both BL21-HSMAM7 and BL21 (control) were co transformed with pDP151 plasmid for expressing mCherry. The infected cells were then stained with Alexa Fluor 488-phalloidin to stain actin and Hoechst to stain DNA. Samples were imaged using Zeiss Axio Observer. Images were used to visualize and quantify the attached bacteria.

## 2.8 Bacterial attachment

Hela cells were seeded at a concentration of 150000 cells/ml into a 24-well plate, in triplicate. Hela cells were washed with PBS three times and infected with pathogenic bacteria at an MOI of 10 and incubated at 37 ℃ for 1 hour and 4 hours. After bacterial infection, DMEM medium was removed and Hela cells were washed three times with and 0.5% Triton X-100 was added to lyse Hela cells, then serial dilutions were prepared and cultured on LB agar and incubated at 37 ℃ for 20 hours followed by counting the number of colonies.

## 2.9 Cytotoxicity measurement

Lactate dehydrogenase release (LDH) assay was used to measure lactate dehydrogenase released into the medium using LDH cytotoxicity detection kit (Takara). Hela cells were seeded at a concentration of 150000 cells/ml into a 24 well –plate. In triplicate, Hela cells were infected with bacteria. In addition, a triplicate set of wells for untreated Hela cells (negative control) and a triplicate set of wells (1% Triton X-100) for complete cell lysis, were prepared. In triplicate, 200 µl of supernatant from each well were removed and transferred into a 96 well -plate and centrifuged at 22 ℃, 1000 xg for 5 minutes, then 100 µl from each well were transferred into a fresh 96 well plate. 100 µl of the reagents Mix was added to each well and incubated at room temperature. The absorbance was measured at 490 nm. The percentage of cytotoxicity was calculated

using the formula: % cytotoxicity=100 x ([OD490 for experimental release - OD490 for spontaneous release]/ [OD490 for maximum release– OD490 for spontaneous release]).

## 2.10 Competition experiments

The day before the experiment, Hela cells were seeded at a concentration of 150000 cells/ml into a 24 well plate, in triplicate. A triplicate set of wells for untreated Hela cells (negative control) and a triplicate of wells for complete cell lysis (positive control) were also prepared. Bacteria were grown in LB broth. On the day of the experiment, *E. coli* BL21 -HSMAM7 was grown in LB broth and when the OD 600 was 0.6, protein expression was induced by adding arabinose to 0.05% and incubated at 37 ℃ for 4 hours. Hela cells were washed with PBS three times prior to the addition of *E. coli* BL21 HSMAM7 or *E. coli* BL21 (control) in colorless DMEM without antibiotics at an MOI of 100 and incubated for 1 hour, followed by infection with either *S. aureus*, *E. faecalis* or *P. aeruginosa* at an MOI 10 and the plates were incubated at 37 ℃ for 4 hours. For the attachment competition, DMEM medium was removed and Hela cells were washed three times with PBS and 0.5% Triton X-100 was added to lyse Hela cells, and then serial dilutions were prepared and cultured on the selective media, cetrimide agar for *P. aeruginosa,* mannitol salt agar for *S. aureus* and enterococcosel agar for *E. faecalis.* The plates were incubated at 37 ℃ for 20 hours followed by counting the number of colonies. For measuring the cytotoxic effects caused by infection, Lactate dehydrogenase (LDH) release assay was used. When beads were used in competition experiments, Hela cells were infected with infection medium containing *S. aureus*, *P. aeruginosa* or *E. faecalis* at an MOI 10 with10% v/v GST-

HSMAM7 coupled beads or GST coupled beads (control) and incubated for 4 hours. This was followed by attachment assays and cytotoxicity measurements.

## 2.11 Immunostaining for Fluorescence Microscopy

Following removal of the supernatants, cells were washed three times with sterile PBS. For fixation, 4% paraformaldehyde in PBS was used for 15 minutes at room temperature. Followed by washing the cells with PBS and 0.1% Triton X-100 in PBS was used for 5 minutes to permeabilize the cells. In a dark place, cells were incubated with Hoechst and rhodamine-phalloidin or Alexa Fluor 488-phalloidin for 10 minutes. The coverslips were washed with PBS three times, then mounted and imaged using a Nikon Ti Eclipse microscope.

## 2.12 The interactions between HSMAM7 and host receptors

### 2.12 .1 Lipid overlay assays

PIP-Strip™ and Sphingo-Strips (Echelon biosciences) were used to test the lipid binding property of HS MAM7. The membrane was blocked with blocking buffer (1X PBS, 0.1% v/v Tween 20, 5 % milk) for 1 hour at room temperature with gentle shaking. 10 μM of protein were added and incubated for 1 hour with shaking at room temperature. Followed by washing with washing buffer (1X PBS, 0.1% v/v Tween 20) for 10 minutes, this step was repeated 3 times. Anti GST–mouse antibody (1:1000) was applied in blocking buffer and incubated for 1 hour at room temperature with shaking. The membrane was washed

with washing buffer for 10 minutes; this step was repeated three times. The secondary anti mouse IgG -HRP antibody (1:5000) in blocking buffer was added and incubated for 1 hour at room temperature with shaking. Followed by washing with washing buffer for 10 minutes; this step was repeated three times. The antibodies were detected using chemiluminescence reagents A (Clarity ™ western ECL substrate) and B (peroxide solution). The positive control PIP-Grip ™ was used.

### 2.12.2 Protein-lipid interaction plate assays

Protein binding to lipids was measured by a modified version of an indirect quantitative ELISA as follows: 1-C24 mono-sulfagalactosyl (B) ceramide(d 18:1/24:0), C24 ceramide (d 18:1/24:0) and phosphatidic acid (Avanti Polar Lipids) were dissolved in chloroform: methanol: water (C: M: W) at a ratio of 2:1:0.1. 50 µl of lipids at a concentration of 200 µg/ml were immobilized in 96-well glass microtiter plates and left at room temperature for 20 hours for solvent evaporation. Control wells contained only solvents but no lipid. 150 µl/well of blocking buffer (1% Bovine serum albumin in PBS) was added, sealed and incubated at room temperutre for an hour. Following washing the wells, 100 µl/well of the recombinant GST-HSMAM7 or GST protein at a concentration of 500, 250, 125, 62.5, 31, 15.5 and 7.75 µg/ml was added, plates were sealed and incubated for 2 hours at room temperature with gentle shaking. The wells were washed with washing buffer (0.05% Tween 20 in PBS) three times. 100 µl/well of GST-antibody (1:1000 in blocking buffer) were added, plates were sealed and incubated for an hour at room temperature with gentle shaking. Wells were washed three times with washing buffer, and 100 µl/well of

secondary anti-mouse IgG-HRP antibody (1:5000 in blocking buffer) were added, plates sealed and incubated for 1 hour at room temperature with gentle shaking. The wells were washed three times and 100 µl/well of Clarity ECL™ Western substrate was added for detection. Bioluminescence was visualized on a BioRad Imaging system and quantified in a FLUOStar Omega plate reader.

### 2.12.3 Protein-protein interactions using plate assays

Mucin from porcine stomach type II, mucin from porcine stomach type III and mucin from bovine submaxillary glands (Sigma) were used to study the binding between HSMAM7 and mucin. Mucin was dissolved in PBS at 4 °C for 20 hours with gentle shacking. For mucin immobolization, 100 µl of mucin at a concentration of 50 µg/ml was added into 96-well high-binding microtiter plates, plates were sealed and incubated at 4 °C for 20 hours. Plates were washed once with PBS, and 150 µl/well of blocking buffer (1% BSA in PBS) was added, plates were sealed and incubated for an hour at room temperature with gentle shaking. Following washing the wells, 100 µl/well of the recombinant GST-HSMAM7 protein or GST protein at a concentration of 500, 250, 125, 62.5, 31, 15.5 and 7.75 µg/ml was added, plates were sealed and incubated for 2 hours at room temperature with gentle shaking. The wells were washed with washing buffer (0.05% Tween 20 in PBS) three times. 100 µl/well of GST-antibody (1:1000 in blocking buffer) were added, plates were sealed and incubated for an hour at room temperature with gentle shaking. Wells were washed three times with washing buffer, and 100 µl/well of secondary anti-mouse IgG-HRP antibody (1:5000 in blocking buffer) were added, plates sealed and incubated for 1

hour at room temperature with gentle shaking. The wells were washed three times and 100 µl/well of Clarity ECL™ Western substrate was added for detection. Bioluminescence was visualized on a BioRad Imaging system and quantified in a FLUOStar Omega plate reader.

## 2.12 .4 Protein -desulfated mucin interaction using plate assay

### 2.12.4.1 Desulfation of mucin using pure sulfatase

Mucin from porcine stomach type II was dissolved in PBS at 4 °C for 20 hours with gentle shaking. 100 µl/well of mucin at a concentration of 50 µg/ml was immobilized into 96 well high-binding microtiter plates, plates were sealed and incubated at 4 ℃ for 20 hours.100 µl/well sulfatase from *Helix promatia* (Sigma) at concentrations of 0.1-3 U/ml in 20 mM sodium posphate buffer pH 5 was added and incubated at room temperature for 24 hrs with gentle shaking. The wells were washed three times with washing buffer. 150 µl /well of blocking buffer was added, sealed and incubated at room temp for 1 hour. The wells were washed three times with washing buffer. 100 µl/well of GST-HSMAM7 protein at a concentration of 125 µg/ml was added, sealed and incubated for 2 hours at room temperature. The wells were washed three times with washing buffer. Protein binding was done as described above for protein-lipids interaction plate assays.

### 2.12.4.1.1 Measurement of sulfate released from mucin

100 µl/well of mucin (mucin from porcine stomach type II) at a concentration of 50 µg/ml was immobilized into the 96-well microplate, the plate was sealed and incubated at 4 ℃ overnight. 100 µl/well sulfatase from *Helix promatia* (Sigma) at concentrations of 0.1- 3 U/ml in 20 mM sodium posphate buffer pH 5 was added and incubated at room temperature for 24 hours with gentle shaking. In triplicates, 100 µl from each well was transferred into a new 96-well plate and 100 µl of 20% barium chloride solution was added to each well. Barium sulfate was measured at OD 600 nm, and converted into sulfate concentration using a standard curve of $Na_2SO_4$. The standard curve was prepared by dissolving $Na_2SO_4$ in distilled water to prepare different concentrations 200, 100, 75, 60, 50, 25, 12.5, 6.3 and 3 µM. Then, in triplicate, 100 µl of 20% barium chloride was added to 100 µl of each concentration of $Na_2SO_4$ in 96 well-plate, and the optical density ($OD_{600}$) was measured.

### 2.12.4.2 Desulfation of mucin using live *B. theta*

Mucin from porcine stomach type II was dissolved in PBS at 4 °C for 20 hours with gentle shaking. 100 µl/well of mucin at a concentration of 50 µg/ml was immobilized into 96 well high-binding microtiter plates, plates were sealed and incubated at 4 ℃ for 20 hours. 50 µl, 100 µl and 200 µl of *B. theta* wt or *B. theta* anSME mutant at OD 0.6 was added to the immobilized mucin and incubated at 37 ℃ for 24, 48 or 72 hours under anaerobic conditions. Bacteria were discarded and the wells were washed once with washing buffer. Then 150 µl/well of blocking buffer (1% BSA in PBS) was added, the plate was sealed and

incubated at room temperature for an hour. The wells were washed with washing buffer three times. Then 100 µl/well of GST-HSMAM7 protein at a concentration of 125 µg/ml was added, the plate was sealed and incubated for 2 hours at room temperature. The wells were washed three times with washing buffer. Protein binding was done as described above for protein-protein interaction plate assays.

### 2.12.5 Protein pull-down experiments and protein identification

Hela cells in confluent growth were collected using a cell scraper and centrifuged at 1000 xg, at 22 °C for 5 minutes. The pellet was washed in ice cold buffer (20 mM Hepes-KOH pH 7.3, 110 mM KAc, 2 mM MgAc, 1 mM EGTA ), and 1 ml of lysis buffer (10 mM Hepes-KOH pH 7.3, 10 mM KAc, 2 mM MgAc, Roche protease inhibitor cocktail) was added and left on ice for 10 minutes. Cells lysate was centrifuged at 12000 xg for 12 minutes. 200 µg GST-HSMAM or GST (control) was added to the cell lysate and incubated for 5 minutes at room temperature. DSP cross-linker in DMSO was added to give a final concentration of 100 µg/ml and incubated at room temperature for 30 minutes. Tris-HCl pH 8.0 was added to 10 mM to quench the reaction and the solution was added to 25 µl glutathione sepharose beads, incubated at room temperature for two hours and then 20 hours at 4 °C. The suspension was centrifuged and the pellet was washed 4 times with washing buffer (20 mM Hepes-KOH pH 7.3, 110 mM KAc, 2 mM MgAc, 1 mM EGTA, 2 mM DTT, 0.1% Tween-20,150-500 mM NaCl). Proteins were eluted into SDS sample loading buffer, boiled and resolved by SDS-PAGE. Gels were stained with Coomassie staine and bands were sliced and sent for protein tryptic digest and identification of peptides by LC MS/MS

on an Thermo Orbitrap Elite system coupled to a Dionex Nano LC (University of Birmingham Advanced Mass Spectrometry Core).

## 2.13 Inhibition of bacterial attachment

Caco2 cells at a concentration of 7.5 x $10^4$ cell/well were seeded on coverslip two days before the experiments or Hela cells at a concentration of 15 x $10^4$ cell/well were seeded the day before the experiment. In triplicate, cells were infected with colourless DMEM containing non -pathogenic *E. coli* BL21 expressing HSMAM7 at an MOI of 100 with 50 µg/ml mucin, desulphated mucin and 200 µM lactose-3-sulfate, lactose, N-acetyl glucosamine 6-sulfate, N-acetyl glucosamine, galactose-6-sulfate or galactose and incubated for 1 hour. After bacterial infection, DMEM medium was removed and cells were washed three times with PBS to remove unattached bacterial cells, and 0.5% Triton X-100 was added to lyse cells, then serial dilutions were prepared and cultured on LB agar and incubated at 37 ℃ for 24 hours to count bacterial colonies. To visualize bacterial attachment to cells, immunostaining was done as mentioned in section 2.8.

## 2.14 Immunocytochemistry (ICC)

HT-29MTX cells at a concentration of 2 x$10^4$ cells/ml were seeded on coverslips in a 24 well plate and incubated for three weeks. Cells were fed with fresh media every two days. 1 ml 4% paraformaldehyde was used to fix cells for 20 minutes. Then cells were washed with PBS three times and 1 ml of 0.1% Triton X-100 was added for 15 minutes. After

washing with PBS, 1 ml of 1% BSA was added for an hour. Anti muc 2 antibody produced in mouse was added in blocking buffer (1:400) for an hour. Following washing cells with blocking buffer three times, the secondary antibody (anti mouse IgG FITC) (1:1000) was added for 1 hour in the dark. Cells were washed with PBS three times, followed by adding concanavalin A (25 µg/ml) for 20 minutes. Cells were washed three times with PBS and the coverslips were mounted using anti fade reagent. Slides were viewed using the microscope Zeiss Axio Observer.

## 2.15 Alcian blue staining

1 gram of Alcian blue was added to 100 ml of 3% glacial acetic acid mixed and filtered and the pH adjusted to 1 using glacial acetic acid. HT-29MTX cells at a concentration of 2 $x10^4$ cell/ml were seeded on a coverslips in 24-well plate and incubated for three weeks. Cells were fed every two days. The medium was removed and 1 ml 4 % paraformaldehyde was added for 10 minutes. Cells were washed with PBS three times. Then 1ml Alcian blue solution was added for 30 minutes. Cells were washed and mounted using anti- fade reagent.

## 2.16 Attachment of bacteria to HT-29MTX cells

To visualize bacterial attachment to HT-29MTX cells, HT-29MTX cells were infected with *E. coli* BL21 -HSMAM7 or *E. coli* BL21 (control) at an MOI 100 for 1 hour. After bacterial infection, DMEM medium was removed and HT-29MTX cells were washed three times. ICC staining was done as mentioned in section 2.14.

## 2.17 Measurement of sulfatase activity produced by *B. theta*

*B. theta* sulfatase activity was measured using an enzymatic assay (sigma) as follows: *B. theta* wt and *B. theta* anSME mutant were cultured in BHI broth containing 2.5 mg/ml mucin from porcine stomach type II and incubated at 37 ℃ for 48 hours anaerobically. The supernatant was collected by centrifugation at 13000 xg for 5 minutes. Serial dilutions of the supernatant were prepared using 0.2% NaCl and incubated with 500 µl of 200 mM Sodium Acetate Buffer and 400 µl of 6.25 mM p-Nitrocatechol Sulfate Solution at 37 ℃ for 30 minutes. Followed by adding 5 ml of 1 N NaOH. The absorbance at 515 nm was detected using a Jen Way 3600 UV-visible spectrophotometer. Sulfatase from *Helix promatia* (Sigma) at concentrations of 5, 4.5, 4, 3, 3.5, 3, 2.5, 2, 1.5, 1, 0.5, 0.25, 0.125, 0.062, 0.031, 0.015, 0.0075, 0.0035 and 0.0017 U/ml was used to prepare the standard curve.

## 2.18 Mucin transmigration assays

50 µl of 10 mg/ml mucin from porcine stomach type II were added to trans-well filters (24-well thincert, 3.0 µm pore diameter, Greiner Bio-One) and incubated at 4 °C for 20 hours, and then placed onto 24-well plates containing 600 µl DMEM without phenol red. 100 µl of $10^6$ CFU/ml *E. coli* was added to the top compartment and incubated at 37 °C for 2 hours. Bacterial concentrations in the bottom well were enumerated by dilution plating on LB agar following incubation at 37 °C for 20 hours. To test the effect of *B. theta* sulfatase, *B. theta* was grown in BHI broth containing 2.5 mg/ml mucin and incubated anaerobically at 37 °C for 48 hours. 100 µl of 10 mg/ml mucin was incubated with 100 µl of *B. theta* wt or *B. theta* mutant supernatant at room temperature for 24 hours with gentle shaking. In case of using sulfatase from *Helix promatia*, 100 µl of 1 U/ml sulfatase enzyme was incubated with 100 µl of 10 mg/ml mucin at room temperature for 24 hours with gentle shaking prior to coating the Trans-well filters. Then the treated filters were placed onto 24 well plate containing 600 µl colourless DMEM.

## 2.19 Statistical analysis

Statistical analysis of data was completed using GraphPad Prism version 5. Data were analysed for statistical significance with a two-tailed Student *t* test. All experiments were done at least three times in triplicate. Data are presented as mean ± SEM. *p≤0.05, **p≤ 0.01, ***p≤0.001.

# Chapter 3. Characterization of HSMAM7 protein

Parts of this chapter have been published:

Stones DH, **Al-Saedi F**, Vaz D, Perez-Soto N, Krachler AM. 2015. Biomimetic materials to characterize bacteria-host interactions. J Vis Exp. (105): 53400.

**Al-Saedi, F**, Stones, D, Vaz, DP & Krachler, AM. 2016. Displacement of Pathogens by an Engineered Bacterium Is a Multifactorial Process That Depends on Attachment Competition and Interspecific Antagonism. Infection and Immunity. 84(6):1704-1711.

## 3.1 Introduction

Most Gram negative bacteria express Multivalent Adhesion Molecule7 (MAM7) adhesin (Krachler et. al., 2011). The well characterized *Vibrio parahaemolyticus* MAM7 is an outer membrane protein comprising of seven mammalian cell entry (mce) domains headed by an N-terminal region that is necessary for MAM anchoring on the bacterial outer membrane (Krachler and Orth, 2011). *V. parahaemolyticus* MAM7 aids bacterial binding to host cells via the recognition of two types of host receptors, phosphatidic acid and fibronectin (Krachler and Orth, 2011; Stones and Krachler, 2015a). Due to the role of MAM7 in bacterial attachment, it has been used as an inhibitor to compete with pathogens and inhibit infections (Krachler et. al., 2012). However, the interaction between MAM7 with phosphatidic acid induces host cellular signalling. Krachler and her group investigated host signalling induced due to the interaction between MAM7 and host receptors. They found that binding of MAM7 to fibronectin is not involved in host signalling, while the interaction with phosphatidic acid leads to rearrangements of actin and stimulation of

GTPase Rho A. Additionally, it causes disruption of the epithelial barrier due to breaching of the tight junctions between cells and thus aids bacterial transmigration and infection of the underlying tissue (Lim et.al., 2014). Therefore, the hypothesis of this work is that the MAM7 adhesin from a commensal bacterium may have as good an efficacy as an adhesion inhibitor as *V. parahaemolyticus* MAM7 to inhibit or prevent bacterial infections, but with no ill effects on host cells. The commensal *Escherichia coli* strain HS colonizes the human gut without causing disease and competes with pathogens to inhibit infections, and *E. coli* HS also encodes a putative MAM7 orthologue.

*E. coli* is the predominant facultative anaerobic bacteria in the human gastrointestinal tract, involving a variety of biotypes; commensals and pathogens (Todar, 2004). The commensal *E. coli* include variety strains such as *E. coli* strain HS, *E. coli* strain Nissle 1917, *E. coli* strain MG1655, *E. coli* strain EFC1 and *E. coli* strain EFC2 (Maltby et.al., 2013). The existence of the commensals in the gut confers a protective barrier to inhibit infections by pathogens (Leatham et. al., 2009; Conway and Cohen, 2015). The role of commensals in the protection of the host may occur via the competition for nutrients and binding sites. It has been revealed that feeding mice with the pathogenic *E. coli* strain EDL933 previously colonized with commensal strains such as *E. coli* strain Nissle 1917, *E. coli* strain MG1655 and *E. coli* strain HS, results in inhibition of *E. coli* EDL933 colonization (Leatham et. al., 2009). This inhibition could be a result of the competition for nutrients. Using a mouse model, it has been reported that *E. coli* strain HS and *E. coli* strain Nissle 1917 consume nutrients that are essential for the attachment of pathogenic *E. coli* O157:H7, and thus prevent the first step towards infection (Maltby et. al., 2013). Furthermore, commensal bacteria compete pathogens during biofilm formation. It has

been found that *E. coli* Nissle 1917 outcompete enterohaemorrhagic, enteropathogenic and enterotoxigenic *E. coli* strains during biofilm formation, and this could explain why this commensal is protective against intestinal infections (Hancock et. al., 2010). Moreover, commensals may play a protective role via the production of antibacterial agents such as bacteriocins (Dezfulian et. al., 2008; Kamada, et. al., 2013). *E. coli* is the initial bacterium that colonizes neonatal guts (Nowrouzian et.al., 2003), and the colonization occurs via the expression of adhesins such as P fimbria (Nowrouzian et.al., 2001), type 1 fimbriae (Dhakal et.al., 2008; Rendon et.al., 2007), flagellum (Haiko and Westerlund-Wikstrom, 2013) or the non fimbrial adhesion factor adherence *E. coli* (FdeC) (Nesta et. al., 2012) that facilitate the adherence to colonic epithelial cells.

Dissecting the interactions between host receptors and bacterial adhesins is an important step towards developing antibacterial treatments. Studying the role of particular adhesins in bacterial attachment is difficult due to the interactions of other components in the bacterial cell wall with host receptors (Colino et. al., 2013; Rubinsztein-Dunlop et. al., 2005). Therefore, new methods have been developed to study these interactions, such as using recombinant purified adhesins, polymer beads coupled to adhesins or non - adherent bacteria expressing heterologous adhesins. Recombinant peptides labelled with FITC were used to study the interaction between *Streptococcus pyogenes* surface adhesion (protein F1) with host fibronectin (Ensenberger et. al., 2001). The recombinant purified Fba protein was used to study the binding ability of *Streptococcus pyogenes* surface adhesin Fba to the complement regulatory proteins such as factor H-like protein 1 (FHL-1) and factor H (FH)(Pandiripally et. al., 2003). In addition, the recombinant purified

fibronectin binding protein A (FbpA) was used to investigate the role of FbpA in the adherence of *Streptococcus gordonii* to fibronectin (Christie et. al., 2002).

Polymer beads coupled to bacterial adhesins have been used to investigate the interaction between MAM7 from *V. parahaemolyticus* with host receptors and to treat bacterial infections (Krachler et. al., 2012). Non-pathogenic bacteria expressing a particular adhesin have also been used widely to investigate bacterial adhesin adhering to host cells. For example, it has been shown that expressing NadA adhesin from *Neisseria meningitidis* on the surface of non- adherent *Escherichia coli* BL21 leads to stimulation of bacterial adhesion to host cells (Capecchi et. al., 2005). Chitin binding protein CBP21 secreted by *Serratia marcescens* is a critical factor for bacterial adherence to the colonic epithelial cells. It has been shown that expressing chitin binding protein CBP21 from *S. marcescens* in *E. coli* BL21 increases the binding rate to the colonic epithelial cells (Kawada et. al., 2008).

This chapter describes the identification of a MAM7 ortholog from the commensal *E. coli* strain HS and investigates its role in bacterial adherence using two of the above approaches: an engineered bacterium expressing HSMAM7 (BL21-HSMAM7) as well as recombinant purified GST-HSMAM7 coupled to beads.

## 3.2 Identification of an *E. coli* HSMAM7 ortholog

Multivalent Adhesion Molecules (MAMs) are implicated in early bacterial adherence to host cells and they are widely spread in Gram-negative bacteria, but show a large degree of sequence diversity. *E. coli* HSMAM7 has relatively low sequence similarity with the well characterized *V. parahaemolyticus* MAM7.

MAM7 from *V. parahaemolyticus* has been used to compete with pathogens and inhibit their attachment, but its binding to host lipids results in host signalling that aids bacterial invasion. Choosing the commensal *E. coli* HS to identify MAM7 is based on its capability to mediate bacterial adherence and compete pathogenic bacteria to inhibit their infection. MAM7 from *E. coli* HS would be used as an adhesion inhibitor to inhibit bacterial infections.

The web site (http://www.ncbi.nlm.nih.gov/Genomes/index.html) was used for Basic Local Alignment Search TOOL (BLASTp) search of *E. coli* HSMAM7 protein. BLAST search has been done using *V. parahaemolyticus* MAM7 protein sequence as input. The sequence of *E. coli* HSMAM7 was identified and the results revealed that *E. coli* HS encodes for a MAM7 ortholog comprised of 879 amino acids. HSMAM7 protein consisting of an N-terminal region and seven mammalian cell entry (mce) domains (Figure 3.1).

Clustal Omega was used to assess the percentage homology between HSMAM7 protein and *V. parahaemolyticus* MAM7 protein. The result revealed that HSMAM7 protein has 35.78% sequence homology with *V. parahaemolyticus* MAM7 protein (Figure 3.2).

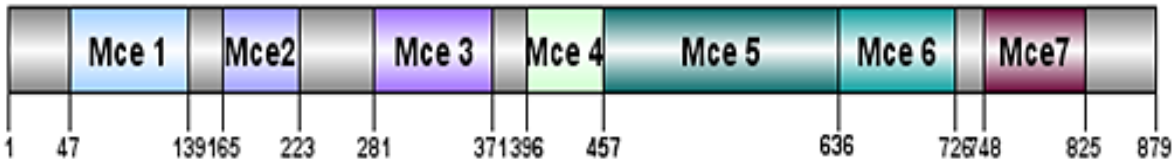


**Figure 3.1 Domain architecture of *E. coli* HSMAM7 protein**. HSMAM7 protein consists of predicted transmembrane region followed by seven mce domains.

```
VibMAM7       -MSDQNNSQTSYVPDVKRSKGISPLWLLPILTMVLAGWLVVKSIHDAGQRVQIYFSDAAG 59
EchsMAM7      MHMSQETPASTTEAQIKNKRRISPFWLLPFIALMIAGWLIWDSYQDRGNTVTIDFMSADG 60
                 .*:.  ::   ::*..: ***:****::::::****: .* :* *: * * * .* *
VibMAM7       LVAGRTTIRYQGLEVGMVRDINLSEDLGSIYVDADIYPEATKLLNDKTRFWLVKPTASLT 119
EchsMAM7      IVPGRTPVRYQGVEVGTVQDISLSDDLRKIEVKVSIKSDMKDALREETQFWLVTPKASLA 120
              :* *** :****:*** *:**.**:** .* *...*  : .. *.::*:****.*.***:
VibMAM7       GVSGLDALVSGNYISIQPGDGQEFETTFHALDSAPTDLRVSQGLNIKLKSRDLGGVSIGS 179
EchsMAM7      GVSGLDALVGGNYIGMMPGKGKEQD-HFVALDTQPKYRLDNGDLMIHLQAPDLGSLNSGS 179
              *********.****.: **.*:* :  * ***: *.    . .* *:*:: ***.:. **
VibMAM7       QIVYKKIPIGAVYSYQLDEDAKSITIQANIQEQYRHIINDRSRFWNVSGIGASIGFEGVD 239
EchsMAM7      LVYFRKIPVGKVYDYAINPNKQGVVIDVLIERRFTDLVKKGSRFWNVSGVDANVSISGAK 239
               : ::***:* **.* :: : :.:.*:. *:.:: .::::. ********:.*.:.:.*..
VibMAM7       VRLESMSALLGGAIAVDSPDDGEPVEENTEFRLYKDLKTAGRGIAIKIALPDDNKVSSEG 299
EchsMAM7      VKLESLAALVNGAIAFDSPEESKPAEAEDTFGLYEDLAHSQRGVIIKLELPSGAGLTADS 299
              *:***::**:.****.***::.:*.* :  * **:**  : **: **: **..  ::::.
VibMAM7       APIMYRGIEIGQVTDLSLSEGREVILASAAIQPAFSDMLTTGTRFVLEEAKVSLSGVENI 359
EchsMAM7      TPLMYQGLEVGQLTKLDLNPGGKVT-GEMTVDPSVVTLLRENTRIELRNPKLSLSDA-NL 357
              :*:**:*:*:**:*.*.*. * :*  .. :::*:.  :*  .**: *.: *:***.. *:
VibMAM7       ANLVRGNFLTIVPGDGERSRRFTAIRKNVFNQQQEKSIAIRLISDNSFGLDSGANVLYKG 419
EchsMAM7      SALLTGKTFELVPGDGEPRKEFVVVPGEKALLHEPDVLTLTLTAPESYGIDAGQPLILHG 417
              : *: *: : :******  :.*..:  :    :: . ::: * : :*:*:*:*  :: :*
VibMAM7       IVVGSIINVGLVDEKKQTKHEVFMDVLIDHEYKHLIKSNNRFYVTGSASAELTESGLSVT 479
EchsMAM7      VQVGQVIDRKLT------SKGVTFTVAIEPQHRELVKGDSKFVVNSRVDVKVGLDGVEFL 471
              : **.:*:  *.      .: * : * *: :::.*:*.:.:* *.. ...::  .*:..
VibMAM7       VPPAKQLLTGSISFVSEGSESIQKEYQLFQNESLAELAQYNKTGSKTLMLFASELPPISK 539
EchsMAM7      GASASEWINGGIRILPGDKGEMKASYPLYANLEKALENSLSDLPTTTVSLSAETLPDVQA 531
                 *.: :.*.* ::  .. .:: .* *: * . *   . ..  :.*: * *. ** :.
VibMAM7       GSPLLYRNLPVGNVSDFHLVDGGVLIKATIENRFAYLVTPQTVFWNRSGIEIDASLSGVS 599
EchsMAM7      GSVVLYRKFEVGEVITVRPRANAFDIDLHIKPEYRNLLTSNSVFWAEGGAKVQLNGSGLT 591
              ** :***:: **:*  .:   ... *.  *: .:  *:* ::*** ..* ::: . **::
VibMAM7       VKAHPLKSLIEGGIAFDSVPGVE—NKVGERWKLYADQQKARKFGRVISLETDGTQEVLK  657
EchsMAM7      VQASPLSRALKGAISFDNLSGASASQRKGDKRILYASETAARAVGGQITLHAFDAGKLAV 651
              *:* **.  ::*.*:**.: *..  :: *::  ***.:  ** .*  *:*.: .: ::
VibMAM7       GMPIEYQGVKVGEVTLVVPNFRRNLVEVTARILPEYVENIAVEGTHFWLTEPEIGLGGVK 717
EchsMAM7      GMPIRYLGIDIGQIQTLDLITARNEVQAKAVLYPEYVQTFARGGTRFSVVTPQISAAGVE 711
              ****.* *:.:*::  :     ** *:..* : ****:.:*  **:* :. *:*. .**:
VibMAM7       NLGALVSKSISVEPGNGKAKFDFQLEKGF----DRVEGVMFTLQSEQRGSVQVGTPVLYR 773
EchsMAM7      HLDTILQPYINVEPGRGNPRRDFELQEATITDSRYLDGLSIIVEAPEAGSLGIGTPVLFR 771
              :*.::::  *.****.*: : **:*::.       ::*: : ::: : **: :*****:*
VibMAM7       QMEVGQVTDVRLGEFADRVVSTIKIKPEYAYLVRQNSVFWNVSGVDVSIGITGANIKAGT 833
EchsMAM7      GLEVGTVTGMTLGTLSDRVMIAMRISKRYQHLVRNNSVFWLASGYSLDFGLTGGVVKTGT 831
               :*** **.: ** ::***: :::*. .* :***:***** .** .:.:*:**. :*:**
VibMAM7       IDSLVRGGIAFSTPEQSQIPPAAKRGHSFYLYPRADESWVQWRTPIPKP 882
EchsMAM7      FNQFIRGGIAFATPPGTPLAPKAQEGKHFLLQESEPKEWREWGTALPK- 879
              ::.::******:**  : : * *:.*: * *     :.* :* * :**
```

**Figure 3.2 Comparison between *V. parahaemolyticus* and *E. coli* HSMAM7 protein.**

Alignment of the amino acid sequences of *V. parahaemolyticus* MAM7 and *E. coli* HS MAM7 using the Clustal Omega program. (*) indicates the identical residues. (:) indicates conserved residues and (.) indicates non-conserved residues.

## 3.3 Cloning of *E.coli* HSMAM7 for expression analysis

### 3.3.1 Extraction of *E. coli* HS genomic DNA

The genomic DNA was extracted from the overnight growth culture of the commensal *E. coli* HS using Gen Elute Bacterial Genomic DNA Kit (Sigma). The purified DNA was analysed by agarose gel electrophoresis to confirm the quality and purity of the DNA. Gel electrophoresis results revealed the presence of a pure band of the *E. coli* HS genomic DNA (Figure 3.3).

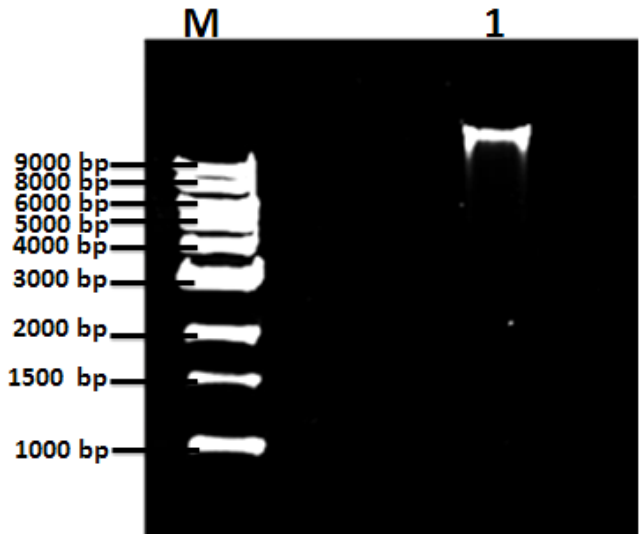


**Figure 3.3 *E.coli* HS genomic DNA.** Lane M: DNA Marker. Lane 1: the extracted *E. coli* HS genomic DNA

### 3.3.2 Amplification of HSMAM7 gene

The extracted genomic DNA from *E. coli* HS was used as a template to amplify the MAM7 gene. The sequence (∆TM MAM7) encoding the soluble portion of the protein was amplified using primers (table 2.1) comprising 5' BamHI and 3' XhoI restriction sites for subsequent cloning into pGEX4T-3 plasmid for recombinant expression. For expression of the full-length HSMAM protein on the surface of non- adherent *E. coli* BL21, the complete gene was amplified using the primers (table 2.1) comprising 5' NcoI and 3'HindIII restriction sites for subsequent cloning into pBADMyc-His plasmid. The PCR products were tested by agarose gel electrophoresis to confirm the presence and the purity of the bands. The results of gel electrophoresis revealed the presence of the expected bands (Figure 3.4), a band size of 2517 bp for ∆TM MAM7 and of 2640 bp for the full-length MAM7. The gel extraction kit was used to purify the PCR products.

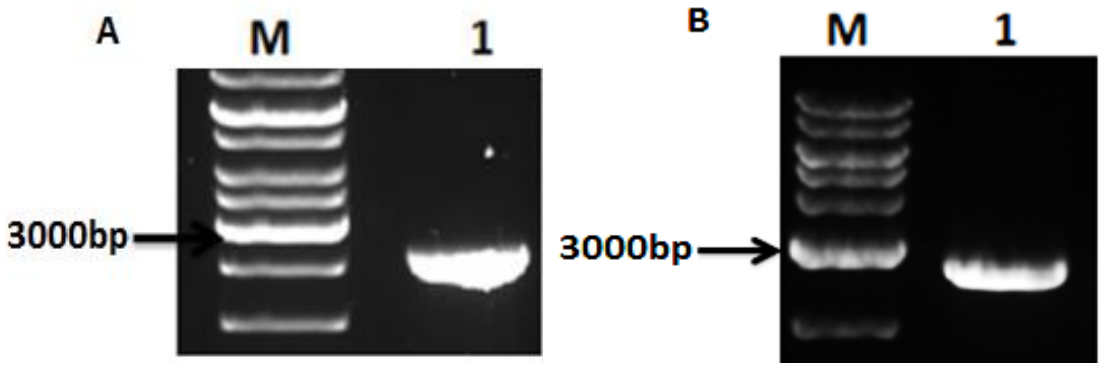


**Figure 3.4: *Escherichia coli* HSMAM7 gene.** Images show the PCR products.**A):** Lane M: DNA Marker.Lane 1**:** ΔTM MAM7 gene (2517 bp). **B):** Lane M: DNA Marker. Lane1: full length MAM7 (2640 bp).

### 3.3.3 Construction of HSMAM7 expression plasmids

In order to construct HSMAM7 expression plasmids, pGEX 4T-3-ΔTM MAM7 and p BAD/Myc-His-HSMAM7, both inserts and plasmids were digested with the restriction enzymes. The insert (ΔTM MAM7) and pGEX 4T-3 plasmid were digested with XhoI and BamHI enzymes. However, the full-length MAM7 insert and pBAD/Myc-His plasmid were digested with NcoI and HindIII restriction enzymes using the conditions mentioned in tables 2.3 and 2.4. The purified digested DNA was ligated using T4 ligase enzyme. The ligation products were transformed into the competent *E. coli* DH5α cells and grown on LB medium containing appropriate antibiotics. The plasmids were extracted from the growth of the picked colonies using the mini prep extraction kit. To prove the success of ligation, the purified recombinant plasmids were digested with the appropriate restriction enzymes and analysed using agarose gel electrophoresis to check the presence of the exact bands. The results revealed the presence of the expected bands of both vectors and inserts. The digested pGEX 4T-3 plasmid showed a band size of 4900 bp and the digested insert a band size of 2517 bp. The digested pBAD/Myc-His had a band size of 4100 bp and the digested full length MAM7 had a band size of 2640 bp (Figure 3.5).

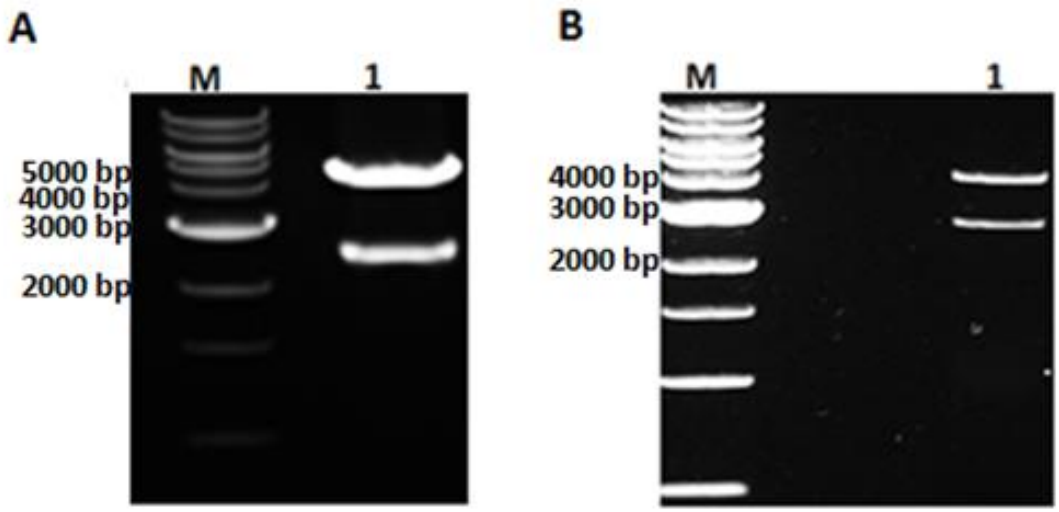


**Figure 3.5 Gel electrophoresis of the digested recombinant plasmids.** A: Lane M: DNA Marker. Lane1: digested recombinant plasmid into pGEX4T-3 (4900 bp) and ΔTM MAM7 (2517 bp). B: Lane M: DNA Marker. Lane 1: digested cloned plasmid into p BAD/Myc-His (4100 bp) and MAM7 (2640 bp).

In addition to the detection of the bands of both inserts and vectors using agarose gel electrophoresis, sequencing analysis was done to confirm the cloning success and to verify the sequence of the recombinant plasmids. The plasmids were sent to the Functional Genomics Service at the School of Bioscience at Birmingham University for the sequencing. The sequence analysis results revealed that the cloning was successful.

## 3.4 Expression and purification of recombinant HSMAM7 protein

For the expression of GST-HSMAM7protein, 10 ml of LB medium containing ampicillin was inoculated with a colony of *E. coli* BL21 transformed with pGEX4T-3 containing the HSMAM construct and incubated with shaking at 37°C overnight. 5 ml of the LB broth culture was added per 500 ml LB broth containing ampicillin and incubated at 37 ℃ until the optical density (OD) reached 0.6-0.7 at 600 nm, then IPTG was added at a final concentration of 1 mM. The cultures were centrifuged and the pellet was collected for the purification using GST affinity chromatography. After the purification process, the eluted proteins were collected and dialyzed in PBS buffer. The samples from the elution were analysed using SDS- PAGE analysis. The SDS- PAGE results showed the presence of the expected size of the recombinant protein (121 kDa). This mass included the GST tag of 26 kDa and the MAM protein of 95 kDa (Figure 3.6). GST protein was expressed using the same method. The *E. coli* BL21 strain was transformed with empty pGEX 4T-3 plasmid. The SDS-PAGE results showed the presence of the GST protein (26 kDa) (Figure 3.7). GST protein was used as a control in following experiments.

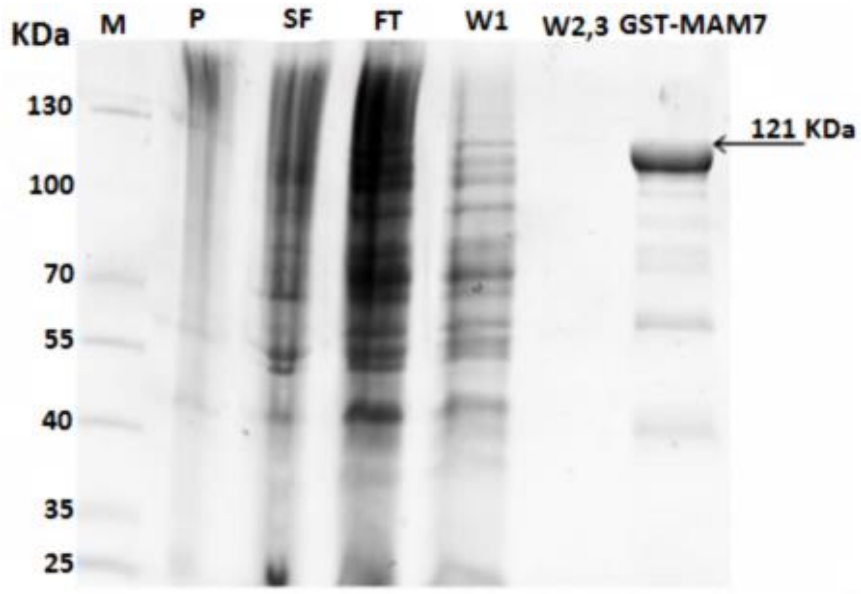


**Figure 3.6 SDS -PAGE analysis of recombinant GST- HSMAM7 protein.** Lane M: marker; Lane P: the pellet; Lane SF: supernatant fraction; Lane FT: Flow through; Lanes W1, W2, W3: washes; Lane GST–MAM7: the purified recombinant protein.

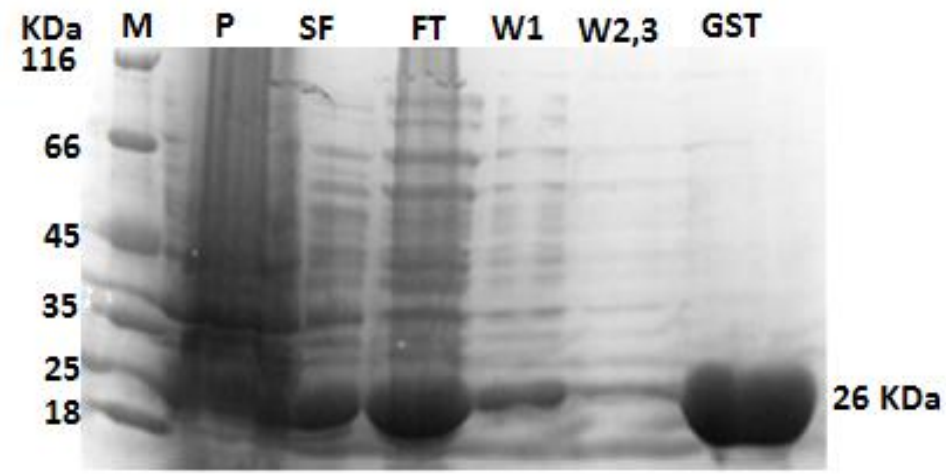


**Figure 3.7 SDS -PAGE analysis of GST protein purification.** Lane M: marker; Lane P: pellet; Lane SF: supernatant fraction; Lane FT: Flow through; Lanes W1, W2, W3: washes; Lane GST: the purified GST-protein.

## 3.5 Coupling of purified proteins to polymer beads

Polymer beads coupled to the purified recombinant protein GST-HSMAM7 were designed to investigate the role of HSMAM7 in bacterial attachment and to be used as a tool to inhibit bacterial infection. Coupling the recombinant GST-HSMAM7 to beads provides a mimic for its display on bacterial surface. Herein, the recombinant purified GST-HSMAM7 protein and polystyrene fluorescent beads of 2 μm diameter were used. The principle of this coupling is that cysteine containing proteins couple to the amine-functionalized polymer beads by using the cross linker Sulfosuccinimidyl4-*p*-maleimidophenyl butyrate (Sulfo-SMPB) (Figure 3.8). Fluorescent beads were incubated with 10 mM Sulfo-SMPB for an hour. Then the activated beads were incubated with the recombinant GST- HSMAM7 protein at a concentration of 6 μM for 2 hours at 25 ℃. Followed by adding cysteine at a final concentration of 50 mM. The beads were pelleted, and then the supernatant was used to evaluate the concentration of the coupled protein. Following washing the beads with strerile PBS twice, they were resuspended in sterile PBS to provide the final product. The same steps were followed to prepare beads coupled to GST protein. This would be used as a control for the subsequent experiments.

The concentration of the coupled protein was calculated based on the following:

[Coupled protein = (Initial protein) – (Supernatant protein)]. The concentration of the coupled protein was 5 μM. The optimized version of this protocol was published (Stones et. al., 2015).

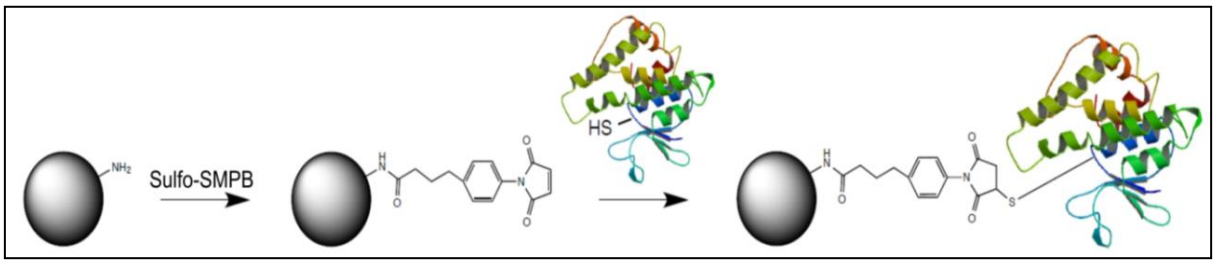


**Figure 3.8 Scheme of bead coupling to purified GST-HSMAM7 protein.** Sulfo-SMPB linker was used to activate the polymer beads which were then coupled to GST- HSMAM7 protein due to the reaction between maleimide group and free cysteine in the GST protein. Figure adapted from (Stones et. al., 2015).

## 3.6 Role of HSMAM7 in bacterial adhesion

### 3.6.1 Beads coupled to HSMAM7 attach to host cells

Since the *E. coli* HSMAM7 ortholog has low sequence similarity with *V. parahaemolyticus* MAM7, the ability of *E. coli* HSMAM7 protein to mediate the attachment to host cells was investigated. The attachment activity of HSMAM7 to host cells was characterized using beads coupled to recombinant protein GST-HSMAM7. Herein, Hela cells were seeded on cover slips at a concentration of 150000 cells/ml the day before the experiment. 10 % v/v beads coupled to GST-HSMAM7 or GST protein (control) in colourless DMEM (2 X $10^{10}$ beads/ml containing protein at a final concentration of 500 nM) were added to Hela cells and incubated at 37 °C for 4 hours. Immunostaining was used to visualize and count the beads attached to Hela cells. The results revealed that GST-HSMAM7 coupled beads adhere to Hela cells (average 767 beads per 100 cells), whereas, no adherence was observed for GST coupled control beads (average of 20 beads per 100 cells, Figure 3.9).

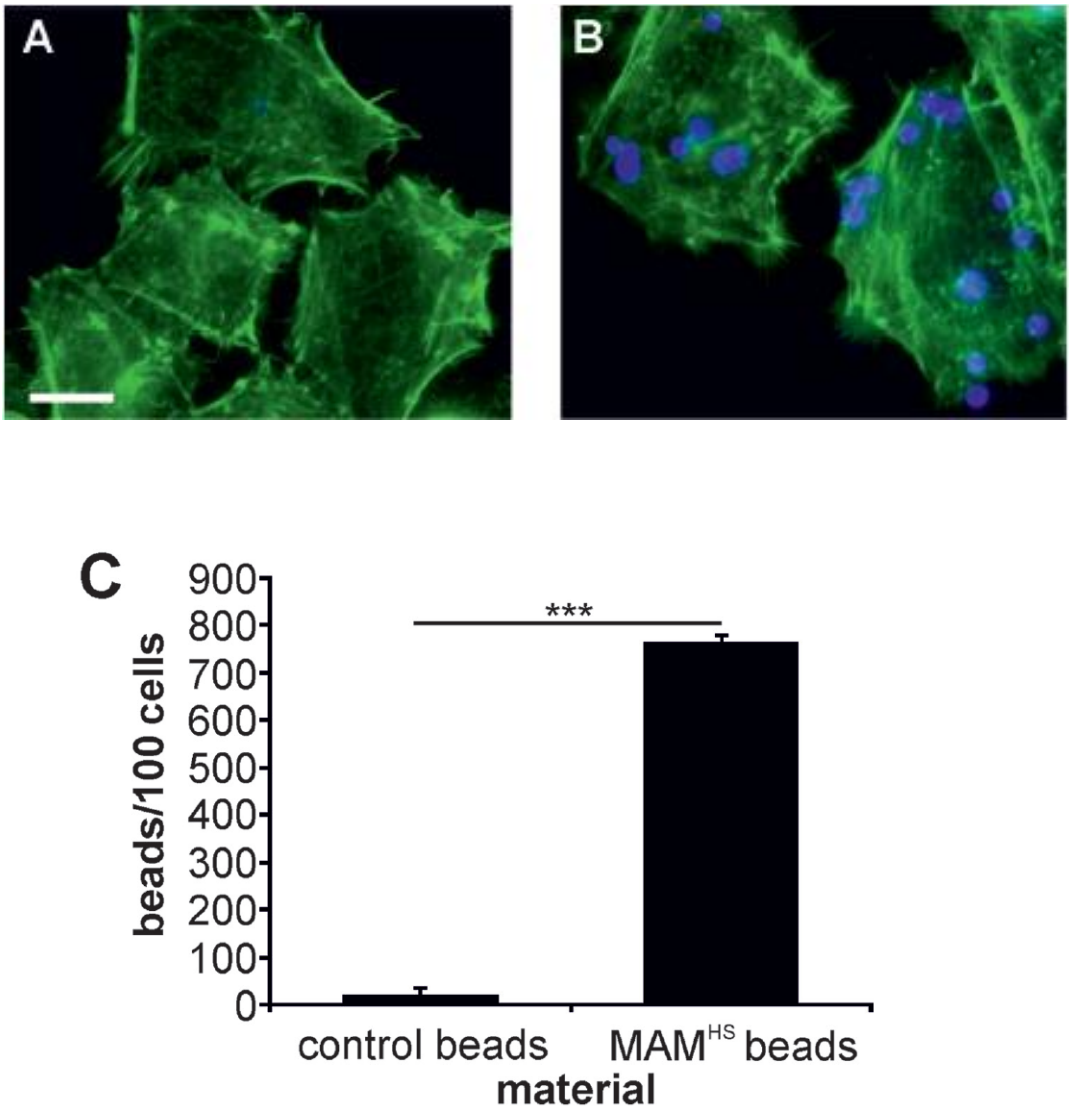


**Figure 3.9 The attachment of HSMAM7- beads to the Hela cells**. Attachment of beads (blue) coupled to GST (A) or GST-HSMAM7 (B) to Hela cells (green), scale bar 10 μm. (C) The numbers of attached beads/100 cells. Values represent the means ± SEM. Significance was determined using two tailed student's t test (*** $p \leq 0.001$).

### 3.6.2 HSMAM7 enhances bacterial binding to host cells

To study the role of HSMAM7 in bacterial adhesion, an engineered bacterium expressing HSMAM7 was generated by transforming *E. coli* BL21 with the expression plasmid pBAD-Myc-His containing the full-length HSMAM7. HSMAM7 was expressed on the surface of *E. coli* BL21 following arabinose induction. The capability of BL21-HSMAM7 to bind to host cells was investigated using Hela cells. Herein, Hela cells were infected with BL21 expressing HSMAM7 or BL21 containing empty vector (control) at an MOI of 100 and incubated for 1 hour. Both BL21-HSMAM7 and BL21 control were co-transformed with a pDP151 plasmid expressing mCherry to visualize the attachment. Immunostaining was used to visualize bacterial attachment and to count the number of attached bacteria. In addition, the number of colony forming units (cfu) was calculated via plating of the bacteria following infection of Hela cells. The results revealed that BL21 expressing HSMAM7 on the surface adhere to Hela cells with an average of 1,070 ± 250 bacteria per 100 host cells, whereas BL21 (control) adhere with an average of 170 ± 20 bacteria per 100 host cells. The number of attached bacteria using dilution plating revealed similar results: 73% of the BL21 bacteria expressing HSMAM7 were attached compared to 19% of the BL21 control (Figure 3.10). These results indicate that HSMAM7 enhances bacterial attachment to host cells.

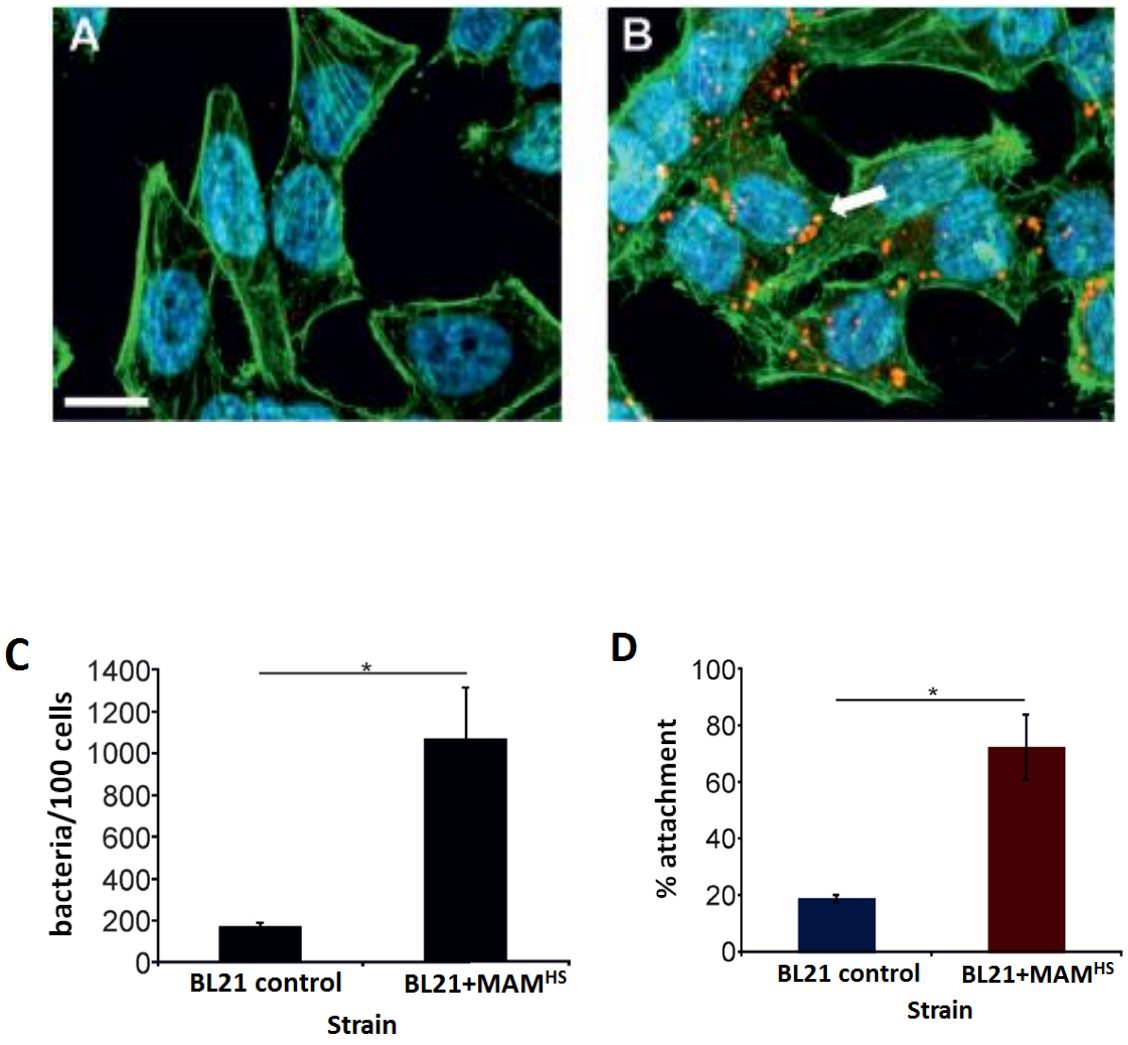


**Figure 3.10 HSMAM7 enhances bacterial attachment to host cells.** Hela cells were infected with BL21 control (A) or BL21 HSMAM7 (B) at an MOI of 100 for 1 hour. Cells were fixed and stained with Hoechst for DNA (blue) and phalloidin for actin (green). Bar 10 µm, and the number of attached BL21 HSMAM7 or BL21 per Hela cells were counted (C). For the bacterial attachment assay, DMEM medium was removed and Hela cells were washed three times with PBS. 0.5% Triton X-100 was added to lyse Hela cells, and then serial dilutions of attached bacteria were cultured on LB agar. The number of colonies was counted after incubation at 37 °C for 24 hrs (D). The values shown are means ±SEM. Significance was determined using two tailed student's t-test (* $p \leq 0.05$).

## 3.7 Discussion

Previously, it has been reported that using inhibitors based on the *V. parahaemolyticus* MAM7 can impede the attachment of a broad range of pathogenic bacteria. However, the binding of MAM7 from *V. parahaemolyticus* to host cells induces host signalling that aids bacterial invasion (Lim et. al., 2014). Therefore, a MAM7 ortholog from the commensal *E. coli* strain HS was characterized for its adhesion properties, to have an alternative protein for the design of future MAM7 based adhesion inhibitors. The commensal *E. coli* strain HS colonizes the human gut without causing disease and has the ability to compete pathogenic bacteria (Maltby et. al., 2013).

This study involves identification and characterization of the MAM7 ortholog from the commensal *E. coli* strain HS. The results revealed that *E. coli* strain HS harbours a MAM7 orthologue consisting of 879 amino acids with an identity of 35.78% to MAM7 from *V. parahaemolyticus*. HSMAM7 is expected to be similar to *V. parahaemolyticus* MAM7, an outer membrane protein comprising an N-terminal region followed by seven MCE domains (Krachler and Orth, 2011). Another study confirmed MAM7 localization and function within the bacterial cell. It has been reported that MAM7 from *Shigella sonnei* is an outer membrane protein comprising seven MCE domains that exposed extracellularly, and this protein contributes in *Shigella sonnei* attachment and invasion of epithelial cells and macrophages (Mahmoud et. al., 2016). MAM7 is not limited just to these pathogens, but other pathogens express it such as enteropathogenic *E. coli*, *Yersinia pseudotubercolusis* and *V. cholerae* (Krachler et. al., 2011).

To study the role of HSMAM7 in bacterial attachment, two modes were designed in this study; firstly, an engineered bacterium expressing HSMAM7 on the surface (BL21-HSMAM7). The ability of BL21-HSMAM7 to attach to host cells was investigated. The results revealed that the expression of HSMAM7 protein on BL21 cell surface enhances bacterial attachment to host cells.

For the second method, polymer beads were coupled to the purified recombinant GST-HSMAM7 protein. The polymer beads have a size similar to bacteria (2 μm), and coupling the recombinant protein to beads provide a mimic for its display on bacterial surface. Thus, studying the role of MAM7 in bacterial attachment to host cell would be independent of other bacterial adhesins. The binding ability of beads coupled to GST-HSMAM7 towards host cells has been investigated. The results showed that HSMAM7 mediates the binding to host cells.

Previous studies with *V. parahamolyticus* MAM7 have shown similar results. It has been revealed that BL21 express MAM7 from the pathogenic bacteria *V. parahamolyticus* and beads coupled to MAM7 have the ability to bind to the host and induce host signalling (Lim et. al., 2014).

The ability of HSMAM7 to bind to host cells has led to the investigation into the role of HSMAM7 as an adhesion inhibitor. In addition to characterize its interaction with host cells in more detail.

# Chapter 4. Displacement of pathogens using HSMAM7 based inhibitors

This chapter has been published:

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## 4.1 Introduction

The increased prevalence of pathogenic bacteria that are resistant to conventional antibiotics has become the most serious problem in treating bacterial infections. Therefore, researchers are trying to find alternative ways to overcome this problem. The early steps of bacterial interaction with host cells involve a variety of virulence factors that are necessary for successful interaction and infection. Therefore, targeting virulence factors could be a successful solution for preventing or treating infectious disease (Allen et. al., 2014). Since bacterial adherence to host cells is an important step towards bacterial colonization and infections (Schluter et. al., 2015), the focus on bacterial adhesion may be a potential way for treating bacterial infections. Anti-adhesion therapy is an alternative way to inhibit bacterial infections due to the interference with bacterial adherence to the host cells as well as to avoid further selection for antibiotic resistant strains. In anti-adhesion treatment, bacteria are not killed by the adhesion inhibitors, but bacteria become non-infective and easily removed by the host. Therefore, no

development of antibiotic resistance will occur. Additionally, if mutants evolve with increased resistance to anti- adhesion treatments, this would deteriorate bacterial ability to attach to the host cells (Krachler and Orth, 2013). Anti-adhesion therapy involves different strategies that result in the inhibition of bacterial adherence to the host such as the interference with adhesin biosynthesis, adhesin or receptor analogs that are structurally similar to adhesins or receptors, or the use of microbes (probiotics and an engineered bacterium) and antibodies (Krachler and Orth, 2013). Studies have revealed the potential of synthetic adhesion inhibitors in preventing bacterial attachment such as using carbohydrate based inhibitors as receptor analogs to block *Streptococcus suis* adherence to host cells (Kouki et. al., 2013). In addition synthetic glycosides were used to inhibit bacterial adhesion to HT29 cells mediated by type 1 fimbriae (Hartmann et. al., 2012).

Polymer beads coupled to particular proteins were used as antimicrobial agents to compete with bacterial adhesins for attachment to host cells (Krachler et. al., 2012).

Probiotic bacteria and recombinant probiotics have been used to compete with pathogens and inhibit infections (Dhanani et. al., 2011; Beltran et. al., 2016). It has been revealed that the adhesion of the pathogenic strains *Escherichia coli* and *Salmonella* spp to the host glycoproteins and Caco2 cells was inhibited using the commensal lactobacilli sp (Lee et.al., 2003). Furthermore, a particular adhesin from lactobacillus was used as adhesion inhibitor such as the purified elongation factor Tu (EF-Tu) from *Lactobacillus plantarum* that used to impede the binding of *E. coli* and *S. typhi* to the gut epithelium (Dhanani et. al., 2011). In addition, the commensal *E. coli* Nissle 1917 was used to out compete pathogenic bacteria causing intestinal infections (Hancock et. al., 2010).

The commensal *E. coli* HS compete with pathogenic *E. coli* O157:H7 for the nutrients that are important for bacterial colonization (Maltby et. al., 2013), in addition to the production of bacteriocins (Dezfulian et.al., 2008; Kamada, et. al., 2013), and the expression of the adhesion factors such as type 1 fimbria and flagella that enable them to displace pathogenic bacteria (Dhakal et. al., 2008; Rendon et.al., 2007).

In the current chapter, the work is based on the anti-adhesion principles to inhibit bacterial infection via the interference with bacterial attachment to host cells without affecting their viability. Herein, adhesion inhibitors based on the MAM7 protein from the commensal *E. coli* strain HS (HSMAM7); semisynthetic adhesion inhibitor (beads coupled to purified GST- HSMAM7) and an engineered bacterium expressing HSMAM7 on the surface (BL21-HSMAM7) will be tested for their potential to outcompete with both Gram positive and Gram negative pathogenic bacteria causing wound infections. The aim of this study was to identify antibacterial agents to be used as wide spectrum inhibitors for the treatment of wound infections.

Wound infections are a major problem contributing to mortality and morbidity. This is due to the rise in the infections that are caused by multidrug resistant bacteria which makes the treatment more difficult and takes more time and increases the cost of medical care (Anderson et. al., 2007; Alebachew et. al., 2012). Factors which contribute to wound infections include obesity, diabetes, age, anaemia, malnutrition and unhygienic conditions (Bandaru et. al., 2012).

A broad range of aerobic and anaerobic pathogenic bacteria can cause wound infections. Common bacteria isolated from wound infections comprise *P. aeruginosa, S. aureus*, coagulase-negative staphylococci, Enterococci and Enterobacteriaceae sp. (Cantlon et.

al., 2006). Among the isolated pathogens from infected wounds, *S. aureus* is the most frequent pathogen (25.8%), followed by enterococci species (7.9%), and *P. aeruginosa* (6.7%), (Cantlon et. al., 2006).

In the current study, *S. aureus, E. faecalis* and *P. aeruginosa* were used due to their clinical importance and their role in wound and tissue infections. Gram positive cocci such as *S. aureus* and *Enterococcus spp* constitute the main burden of wound infections (Weiss et.al., 1999; Cantlon et. al., 2006). Strains that are broadly resistant to antibiotics, such as vancomycin resistant enterococci and methicillin resistant *S. aureus* (MRSA) make the treatment of infected patients more difficult and are associated with longer stays in hospitals and higher healthcare costs (Rajkumari et.al.,2014; Cantlon et. al.,2006). In addition to wound infections, *S. aureus* can result in a wide range of infections such as skin infections including atopic dermatitis (AD), boils and impetigo, and systemic infections such as osteomyelitis, urinary tract infections, endocarditis, meningitis, food poisoning and pneumonia (Ogston and Witte, 1984; Tong et.al., 2015).

*E. faecalis* can cause several diseases in addition to wound infections such as deep tissue infections, bacteraemia and urinary tract infections (Huycke et.al., 1998; Agudelo Higuita and Huycke, 2014).

*P. aeruginosa* is the most common Gram negative bacillus isolated from wound and burn infections. *P. aeroginosa* is able to cause chronic wound infections as a result of its capability to form biofilms that make the treatment with antibiotics less effective (Rasamiravaka et. al., 2015). Several studies have revealed that multi-drug resistant strains of *P. aeruginosa* were isolated from wound infections (Alharbi and Zayed, 2014;

Godebo et. al., 2013). In addition, *P. aeruginosa* can cause cystic fibrosis and septicaemia (Sousa, and Pereira, 2014; Vitkauskiene, et. al., 2010).

Due to the increasing resistance of these bacteria to available antibiotics, there is an urgent need to find potential compounds that prevent bacterial infections without affecting bacterial viability.

Targeting bacterial virulence factors is a promising strategy to treat bacterial infections. Bacterial adhesins are important factors for virulence, that enable tight binding with host cells towards infection. Therefore, using inhibitors based on bacterial adhesins is a potential way to compete with the attachment of pathogens and inhibit bacterial infection (Huebinger et. al., 2016).

The well characterized MAM7 from the pathogenic bacteria *V. parahaemolyticus* has been used as an adhesion inhibitor to inhibit bacterial infections both *in vivo* and *in vitro*. Recently, using a rat model, polymer beads coupled to MAM7 from *V. parahaemolyticus* were used to treat burn wounds infected with *P. aeruginosa* (Huebinger et. al., 2016). In addition, MAM7 has been used to displace a variety of pathogenic bacteria from host cells and inhibit their attachment and cytotoxic effects. MAM7 has been used against variety of pathogenic bacteria such as *Y. pseudotuberculosis*, *V. cholerae*, *V. parahaemolyticus* or enteropathogenic *E. coli* (EPEC) (Krachler et. al., 2011) and *P. aeruginosa* (Krachler et. al., 2012). Furthermore, it has been used to compete with the Gram positive bacterium *S. aureus* (Hawley et. al., 2013). However, *V. parahaemolyticus* MAM7 was recently shown to interfere with cellular signaling in intestinal epithelial cells and to increase the permeability of intestinal cell junctions *in vitro*, on the basis of its ability to bind to and cluster the signaling lipid phosphatidic acid in host cells (Lim et. al., 2014). Since the

commensal *E. coli* strain HS colonizes the human gut and has the ability to compete pathogenic bacteria (Maltby et. al., 2013), MAM7 ortholog from the commensal *E. coli* HS is chosen in this study to investigate its functionality as an adhesion inhibitor.

This chapter shows the potential role of adhesion inhibitors based on the MAM7 protein from the commensal *E. coli* strain HS; a semisynthetic adhesion inhibitor (beads coupled to GST-HSMAM7) and an engineered bacterium expressing HSMAM7 (BL21-HSMAM7), in combatting infections of *Staphylococcus aureus, Enterococcus faecalis* and *Pseudomonas aeruginosa.*

## 4.2 Anti- adhesion activity of HSMAM7 based inhibitors

In the previous chapter, the capability of HSMAM7 to adhere to host cells has been shown, using both beads coupled to GST-HSMAM7 and an engineered bacterium expressing HSMAM7 on the surface (BL21-HSMAM7). Herein, the ability of beads coupled to GST-HSMAM7 and BL21-HSMAM7 to out compete pathogenic bacteria was investigated, aiming to identify anti–adhesion agents involved in wound infection treatment. The anti–adhesion activity of HSMAM7 based inhibitors was done *in vitro* against *S. aureus, E. faecalis* and *P. aeruginosa*.

### 4.2.1 The attachment of pathogenic bacteria to host cells

The ability of the pathogenic bacteria *S. aureus, E. faecalis* and *P. aeruginosa* to adhere to host cells and their effect on host cell morphology was investigated prior to testing the effect of HSMAM7 based inhibitors on bacterial attachment and cytotoxic effects. Hela cells were infected with *S. aureus, P. aeruginosa* and *E. faecalis* followed by an attachment assay and immunostaining assay. The results revealed the ability of those bacteria to bind to Hela cells. In addition the incubation of Hela cells with those pathogens for 4 hours resulted in changes in cell morphology. Most Hela cells were rounded and others were lysed (Figure 4.1).

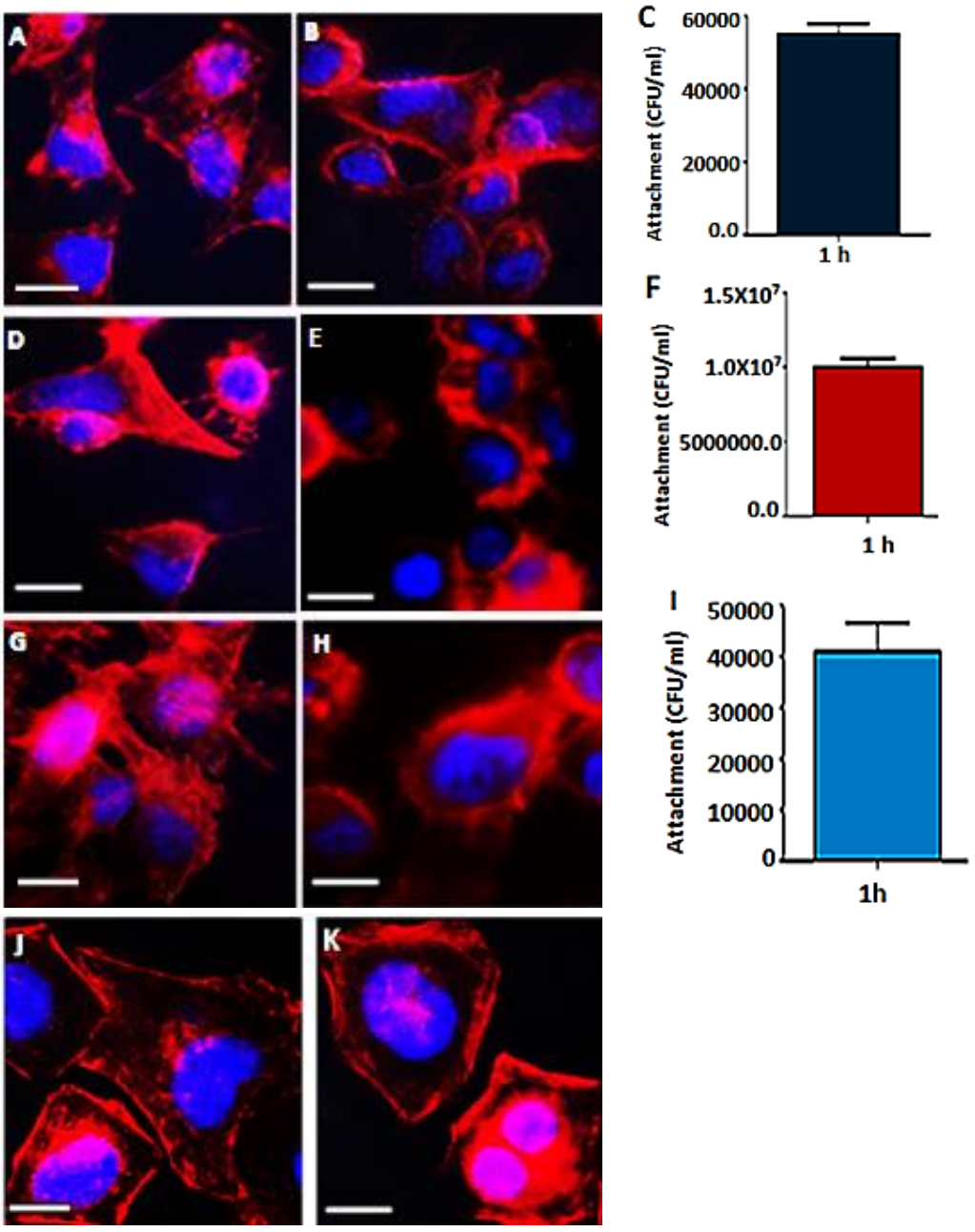



**Figure 4.1The attachment of pathogenic bacteria to Hela cells.** The figure shows the attachment of *S. aureus* (A, B, C), *P. aeruginosa* (D, E, F) and *E. faecalis* (G, H, I) to Hela cells. Hela cells were seeded on coverslips and incubated with pathogenic bacteria at an MOI of 10 for 1 hour (A, D, G) and 4 hours (B, E, H) or uninfected cells (control)(J,K). Cells were fixed and stained with Hoechst for DNA (blue) and rhodamine-phalloidin for actin (red). Scale bar 20 μm. The attachment of bacteria to Hela cells was also quantified by dilution plating (C, F, I). The values shown are means ± SEM.

### 4.2.2 Beads coupled to HSMAM7 compete with pathogens for attachment sites

Due to the ability of beads coupled to the recombinant protein GST-HSMAM7 to adhere to host cells (chapter 3, figure 3.9), they were used as adhesion inhibitors to inhibit bacterial attachment and cytotoxic effects. Herein, Hela cells were infected with the infection medium containing *S. aureus*, *P. aeruginosa* or *E. faecalis* at an MOI of 10 with10% v/v GST-HSMAM7 coupled beads and incubated for 4 hours, followed by immunostaining, an attachment assay and LDH release assay. The results revealed a significant decrease in bacterial adherence and cytotoxicity towards Hela cells in the presence of beads coupled to GST-HSMAM7 compared to the control GST-beads. The infection in the presence of beads coupled to GST-HSMAM7 resulted in decreased attachment of *S. aureus* (8-fold reduction), *E. faecalis* (2-fold reduction) and *P. aeruginosa* (2-fold reduction) to host cells. In addition, the inhibitor also resulted in the inhibition of the cytotoxic effects of *S. aureus*, *E. faecalis* and *P. aeruginosa* (7-fold reduction, 2-fold reduction and 4-fold reduction, respectively). In contrast, no marked effect on bacterial binding and cytotoxic effects was detected in the presence of the control beads (Figure 4.2, Figure 4.3 and Figure 4.4). These results were confirmed using an immunostaining assay to visualize the treatment. Microscopic analysis of multiple images suggests that the infection of Hela cells in the presence of beads coupled to GST-HSMAM7 result in the protection of Hela cells from the cytotoxic effects of *S. aureus, E. faecalis* and *P. aeruginosa*. In contrast, the morphology of Hela cells was affected in the presence of these pathogens or the pathogens with beads coupled to GST alone (Figure 4.2, Figure 4.3 and Figure 4.4).

In combination, these results indicate the ability of beads coupled to HSMAM7 to protect host cells from bacterial infections due to their ability to bind to host cells and compete with pathogens for the attachment sites.

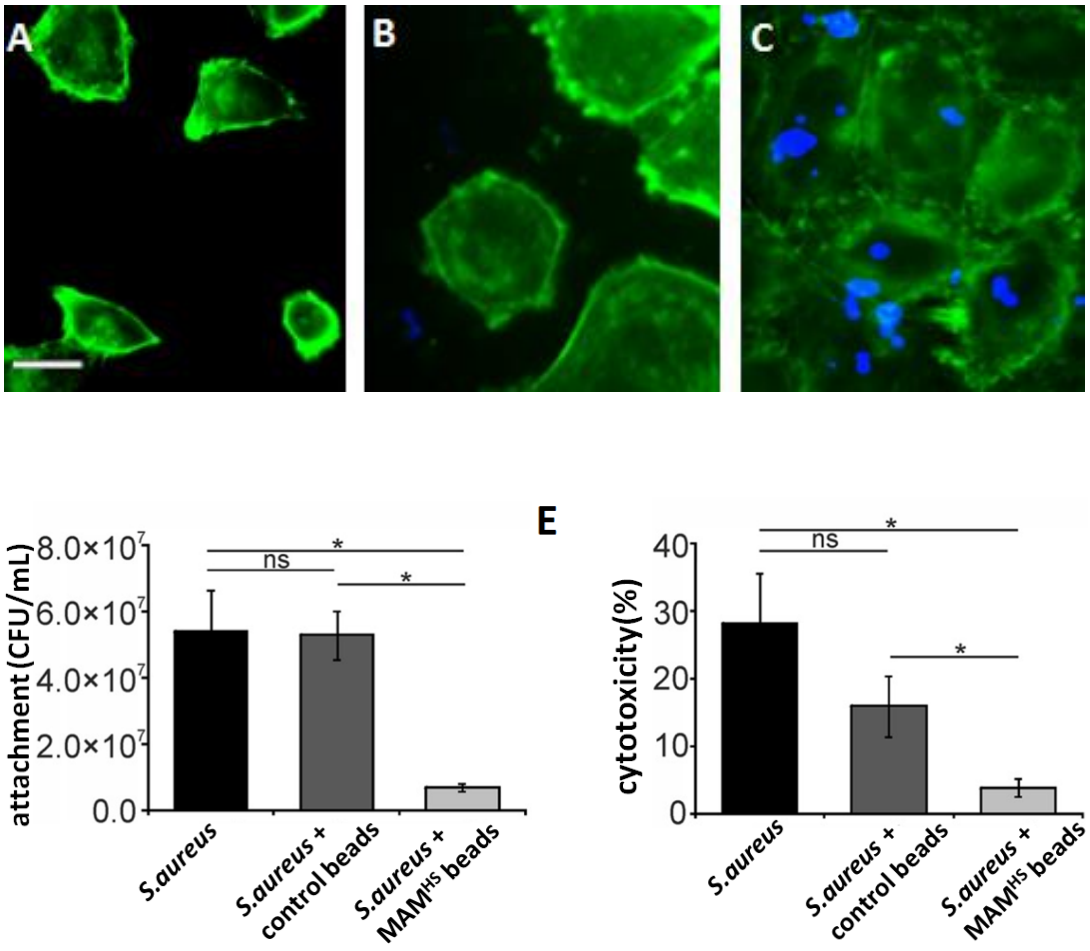



**Figure 4.2 Beads coupled to GST- HSMAM7 compete with *S. aureus*.** Hela cells were infected with *S. aureus* at an MOI of 10 (A). Attachment was visualized after the addition of 10% v/v GST coupled beads (control), (B) or beads coupled to GST-HSMAM7 (C). After incubation for 4 hours, cells were stained with phalloidin (green). Scale bar 20 µm. For the bacterial attachment assay, DMEM medium was removed and Hela cells were washed three times with PBS. 0.5% Triton X-100 was added to lyse Hela cells, and then serial dilutions of attached bacteria were cultured on LB agar. The number of colonies was counted after incubation at 37 °C for 24 hours (D). Cytotoxicity was determined using LDH release assays (E). The values shown are means ±SEM. Significance was determined using two tailed student's t test (* $p \leq 0.05$).

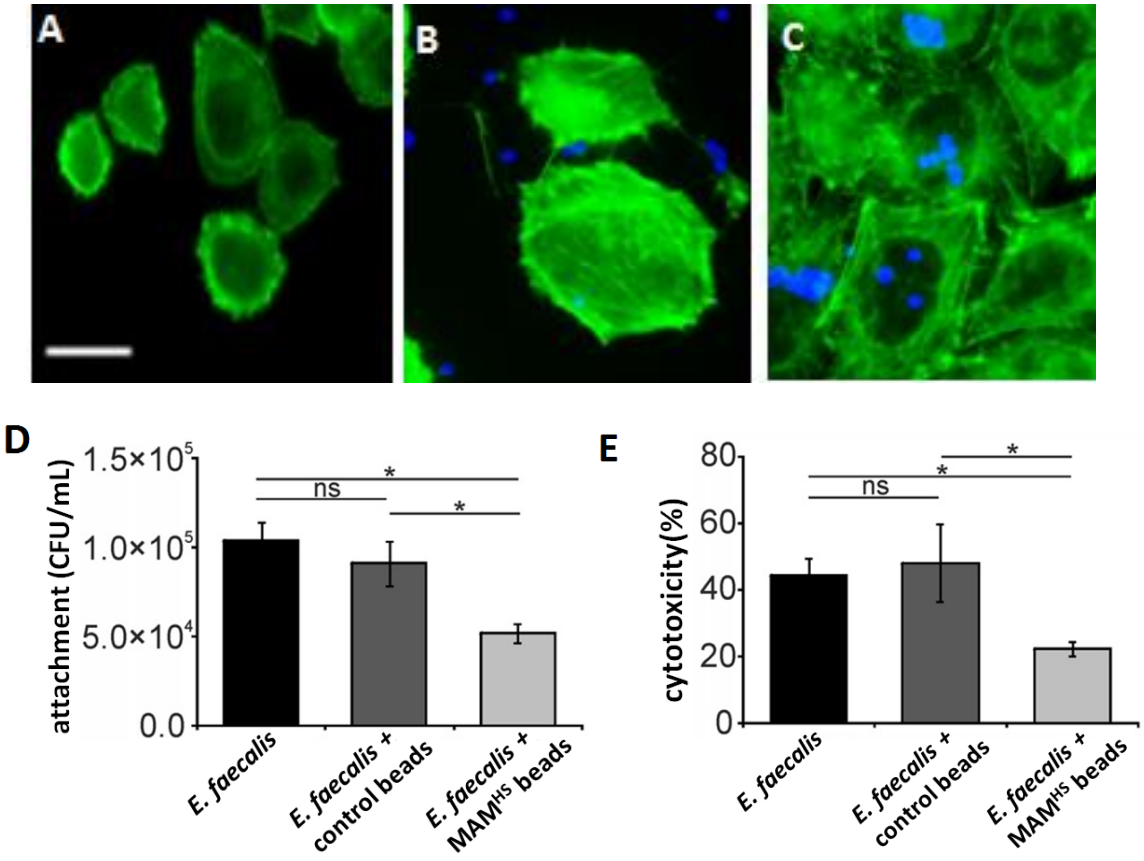



**Figure 4.3 Beads coupled to GST-HSMAM7 compete with *E. faecalis*.** Hela cells were infected with *E. faecalis* at an MOI of 10 (A). Attachment was imaged following the addition of 10% v/v GST coupled beads (control), (B) or beads coupled to GST- HSMAM7 (C). After incubation for 4 hours, cells were stained with phalloidin (green). Scale bar 20 µm. For the bacterial attachment assay, DMEM medium was removed and Hela cells were washed three times with PBS. 0.5% Triton X-100 was added to lyse Hela cells, and then serial dilutions of attached bacteria were cultured on LB agar. The number of colonies was counted after incubation at 37 °C for 24 hours (D). Cytotoxicity was determined using LDH release assays (E). The values shown are means ± SEM. Significance was determined using two tailed student's t test (* $p \leq 0.05$).

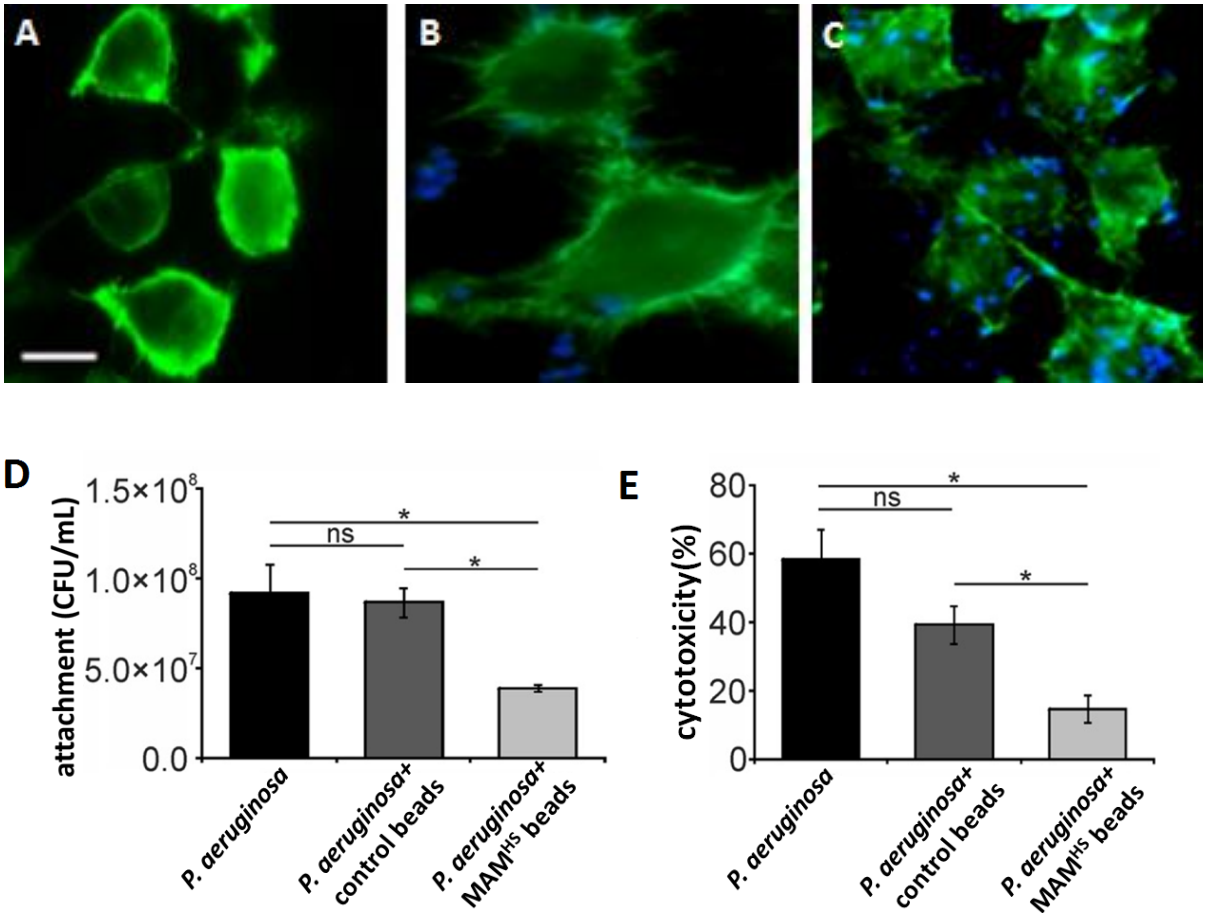



**Figure 4.4 Beads coupled to GST-HSMAM7 compete with *P. aeruginosa*.** Hela cells were infected with *P. aeruginosa* at an MOI of10 (A). Attachment was imaged after the addition of 10% v/v GST coupled beads (control), (B) or beads coupled to GST-HSMAM7 (C). After incubation for 4 hours, cells were stained with phalloidin (green). Scale bar 20 µm. For the bacterial attachment assay, DMEM medium was removed and Hela cells were washed three times with PBS. 0.5% Triton X-100 was added to lyse Hela cells, and then serial dilutions of attached bacteria were cultured on LB agar. The number of colonies was counted after incubation at 37 °C for 24 hours (D). Cytotoxicity was determined using LDH release assays (E). The values shown are means ±SEM (n=3). Significance was determined using two tailed student's t test (* $p \leq 0.05$).

### 4.2.3 Anti adhesion activity of *E. coli* BL21-HSMAM7

#### 4.2.3.1 *E. coli* BL21-HSMAM7 is not cytotoxic to host cells

Prior to investigation of the ability of the engineered bacterium expressing HSMAM7 (BL21-HSMAM7) to displace pathogens and inhibit their infections, it was necessary to determine whether BL21-HSMAM7 can cause cytotoxic effects to host cells. Hela cells were infected with BL21-HSMAM7 at an MOI of 100 and incubated at 37 ℃ for 1 hour, followed by an LDH release assay. The results show there was no cytotoxic effects due to incubation of Hela cells with *E. coli* BL21-HSMAM7 or BL21 (control) for 1 hour compared to the complete lysis result by the positive control (1% Triton- X100), (Figure 4.5).

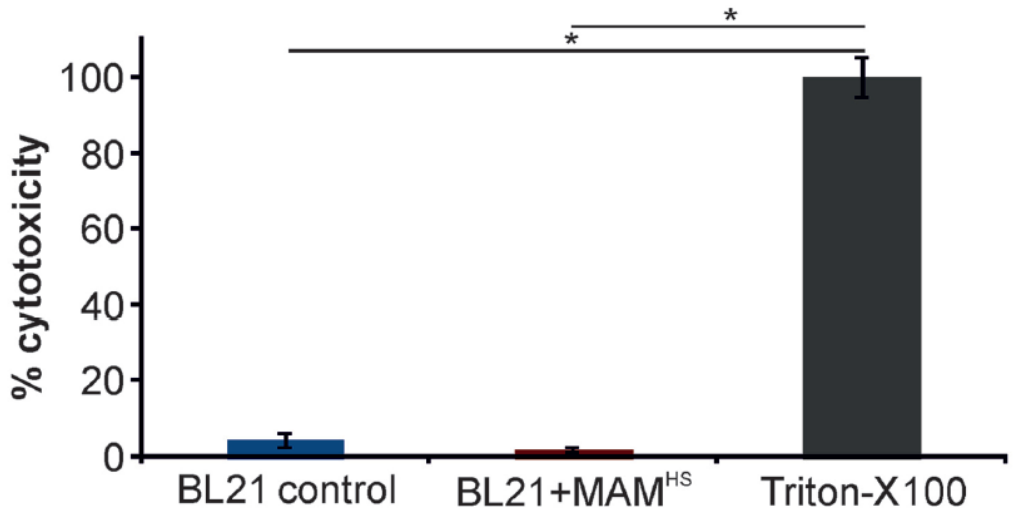



**Figure 4.5 *E. coli* BL21 HSMAM7 was not cytotoxic on Hela cells.** Hela cells were infected with BL21-HSMAM7 or BL21 (control) at an MOI of 100 for 1 hour. Cytotoxicity was determined using LDH release assays. The values shown are means ± SEM (n=3). Significance was determined using two tailed student's t test (* $p \leq 0.05$).

### 4.2.3.2 Displacement of pathogens by BL21-HSMAM7 depends on attachment competition and interspecies interactions

#### 4.2.3.2.1 The competition between BL21-HSMAM7 and *S. aureus* is based on competition for attachment sites and interspecies antagonism

The ability of BL21-HSMAM7 to inhibit *S. aureus* infection was investigated. Herein, Hela cells were incubated with *E. coli* BL21-HSMAM7 or BL21 (control) at an MOI of 100 for 1 hour prior to infection with *S. aureus* at an MOI of 10 for 4 hours, followed by immunostaining assay, attachment assays and LDH release assays.

Microscopic analysis of multiple images suggest that the pre- incubation of Hela cells with BL21-HSMAM7 prior to infection with *S. aureus* result in the protection of Hela cells compared to the BL21(control) (Figure 4.6 A, B, C). The results of the competition experiments revealed that both attachment and cytotoxic effects of *S. aureus* were reduced 6-fold in the presence of BL21-HSMAM7 compared to a 2-fold reduction using the BL21 control (Figure 4.6 D, E). Further investigation was done to study the interactions between BL21 and *S. aureus*. In LB broth, BL21 at an MOI of 100 was mixed with *S. aureus* at an MOI of 10 and incubated for 4 hours. Serial dilutions from the mixed culture were then plated on selective media to count the number of bacterial colonies of each species. The results revealed that BL21 does not affect *S. aureus* growth when *S. aureus* and *E. coli* BL21 were grown together in LB broth (Figure 4.6 F). However, BL21 growth was significantly decreased in the presence of *S. aureus* (Figure 4.10). The inhibition in BL21 growth may be due to secreted agents produced by *S. aureus*.

The results indicate that the inhibitory effect of BL21-HSMAM7 on *S. aureus* is as a result of both attachment competition and interspecies antagonism.

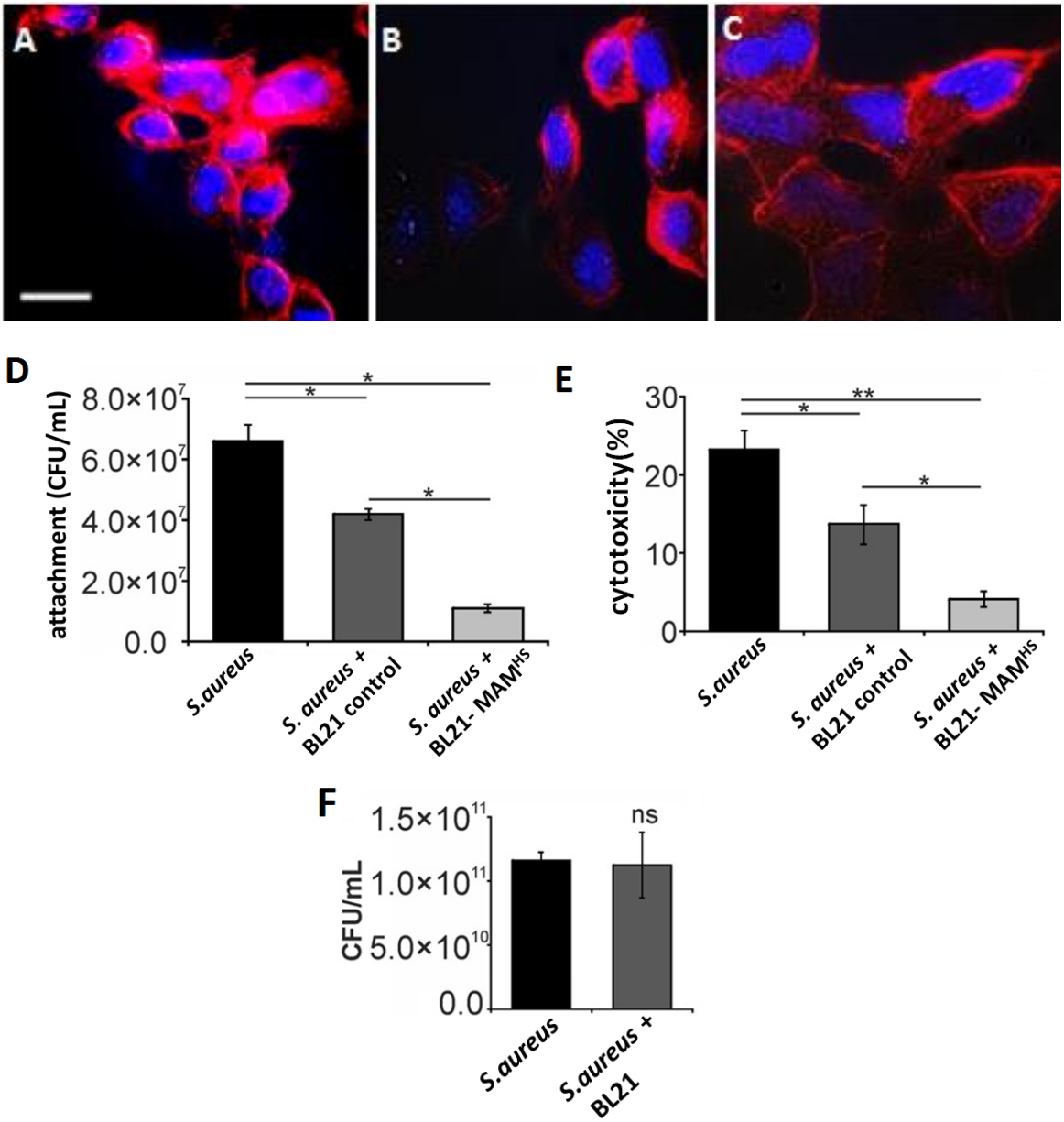



**Figure 4.6 Inhibitory effect of BL21- HSMAM7 on *S. aureus*.** Hela cells were infected with *S. aureus* only (A), *S. aureus* with BL21 control (B) or *S.aureus* with BL21-HSMAM7(C) for 4 hours. Cells were fixed and stained with Hoechst for DNA (blue) and phalloidin for actin (red). Scale bar 20 μm. For the bacterial attachment competition, 0.5% Triton X-100 was added and then serial dilutions were cultured on mannitol salt agar medium, and the number of colonies was counted (D). The cytotoxicity was determined using LDH release assays (E). *S. aureus* and BL21 were mixed and grown in liquid medium for 4 hours, followed by plating *S. aureus* on mannitol salt agar medium for counting bacterial colonies (F).The values shown are means ± SEM, (n=3). Significance was determined using two tailed student's t test (* $p \leq 0.05$, ** $p \leq 0.01$).

#### 4.2.3.2.2 The competition between BL21-HSMAM7 and *E. faecalis* is a combination of competition for attachment sites and nutrients.

The inhibitory role of BL21-HSMAM7 on *E. faecalis* was investigated. Hela cells were incubated with *E. coli* BL21- HSMAM7 or BL21 (control) at an MOI of 100 for 1 hour prior to infection with *E. faecalis* at an MOI of 10 for 4 hours. Immunostaining assays, attachment assays and LDH release assays were then performed.

Microscopic analysis of multiple images suggests the protective role of BL21-HSMAM7 compared to the BL21 (control) (Figure 4.8 A, B, C). The results of the competition experiments revealed that *E. faecalis* attachment was reduced 2-fold in the presence of BL21–HSMAM7 or BL21 (control). In addition, inhibition of cytotoxicity to host cells 10-fold reduction in the presence of BL21-HSMAM7 and 3-fold reduction in the presence of BL21 was observed (Figure 4.8 D, E). The interaction between *E. coli* and *E. faecalis* was further investigated via co incubation in LB broth. The results revealed that the co-incubation of both *E. coli* BL21 and *E. faecalis* in LB broth showed that the growth of both species was significantly decreased and this decrease might be due to a competition for nutrients (Figure 4.8 F and Figure 4.10). These results indicated that the competition between BL21-HSMAM7 and *E. faecalis* is both for attachment sites and for nutrients.

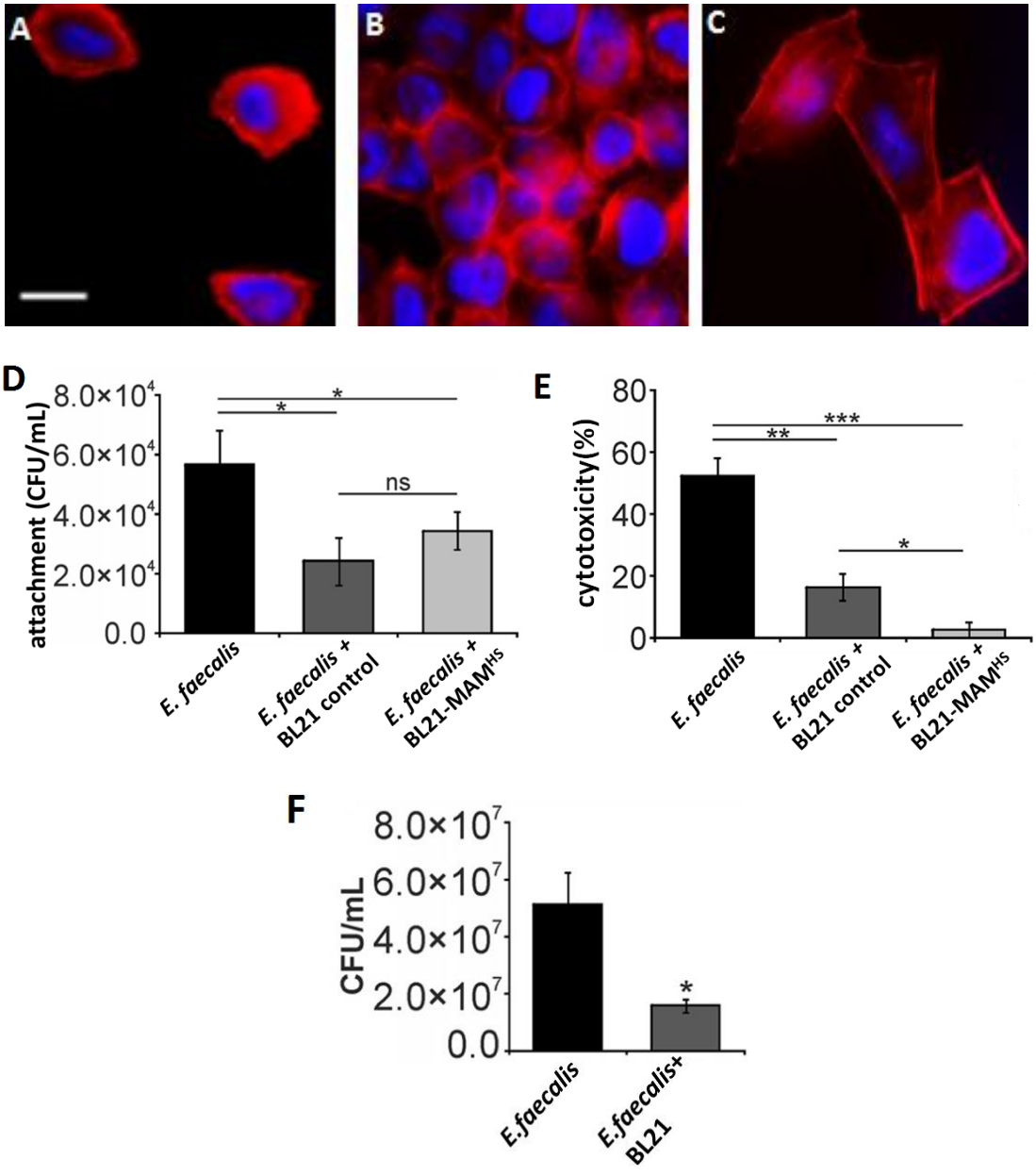



**Figure 4.8 Inhibitory effect of BL21- HSMAM7 on *E. faecalis.*** Hela cells were infected with *E. faecalis* alone (A), *E. faecalis* with BL21 control (B) or *E. faecalis* with BL21-HSMAM7(C) for 4 hours. Cells were fixed and stained with Hoechst for DNA (blue) and phalloidin for actin (red). Scale bar 20 μm. For bacterial attachment competition, 0.5% Triton X-100 was added and then serial dilutions were cultured on enterococcosel agar medium, and the number of colonies was counted (D). The cytotoxicity was determined using LDH release assay (E). *E. faecalis* and BL21 were mixed and grown in liquid medium for 4 hours, followed by plating *E. faecalis* on enterococcosel agar medium for counting bacterial colonies (F). The values shown are means ± SEM, (n=3). Significance was determined using two tailed student's t test (* p ≤0.05, ***p≤0.001).

#### 4.2.3.2.3 The competition between BL21-HSMAM7 and *P. aeruginosa* is only for attachment sites

Hela cells were incubated with *E. coli* BL21- HSMAM7 or BL21 (control) at an MOI of 100 for 1 hour prior to infection with *P. aeruginosa* at an MOI of 10 for 4 hours. Immunostaining assays, attachment assays and LDH release assays were then performed.

Microscopic analysis of multiple images suggest that the pre- incubation of Hela cells with BL21-HSMAM7 prior to infection with *P. aeruginosa* result in the protection of Hela cells compared to the BL21(control) (Figure 4.9 A, B, C). The results of the competition experiments revealed a significant decrease in both attachment (3-fold reduction) and cytotoxic effects (18-fold reduction) of *P. aeruginosa* to the host cells in the presence of BL21-HSMAM. No significant changes in bacterial attachment and the cytotoxic effects were observed in the presence of the BL21 control (Figure 4.9 D, E). The interactions between *P. aeruginosa* and BL21 in liquid medium was investigated to test if another factor was involved in this competition in addition to the competition for the binding sites. Co incubation of *E. coli* BL21 with *P. aeruginosa* in LB broth revealed no changes in the growth of both species (Figure 4.9 F and Figure 4.10). These results indicate the inhibitory effect of BL21-HSMAM7 on *P. aeruginosa* is via the competition for the binding sites alone.

Considering all competition results, this study demonstrates that the competition between BL21 and pathogens does not solely depend on binding sites, but other factors can also affect this competition depending on the bacteria involved.

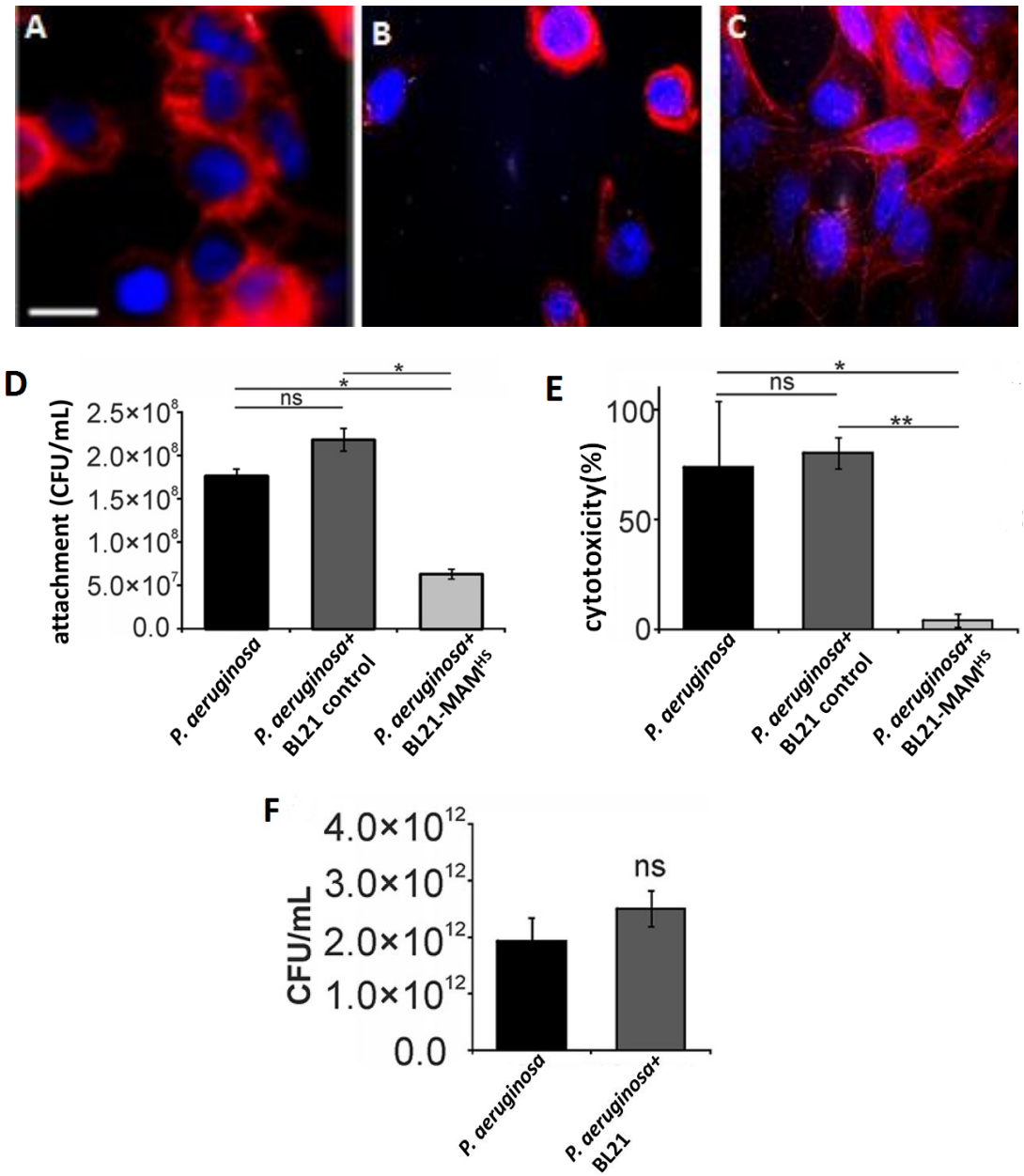



**Figure 4.9 Inhibitory effect of BL21- HSMAM7 on *P. aeruginosa.*** Hela cells were infected with *P. aeruginosa* alone (A), *P. aeruginosa* with BL21 control (B) or *P. aeruginosa* with BL21-HSMAM7(C) for 4 hours. Cells were fixed and stained with Hoechst for DNA (blue) and phalloidin for actin (red). Scale bar 20 μm. For the bacterial attachment competition, 0.5% Triton X-100 was added and then serial dilutions were cultured on cetrimide agar medium, and the number of colonies was counted (D). The cytotoxicity was determined using LDH release assays (E). *P. aeruginosa* and BL21 were mixed and grown in liquid medium for 4 hours, followed by plating *P. aeruginosa* on cetrimide agar for counting bacterial colonies (F). The values shown are means ± SEM, (n=3). Significance was determined using two tailed student's t test (* $p \leq 0.05$, ** $p \leq 0.01$).

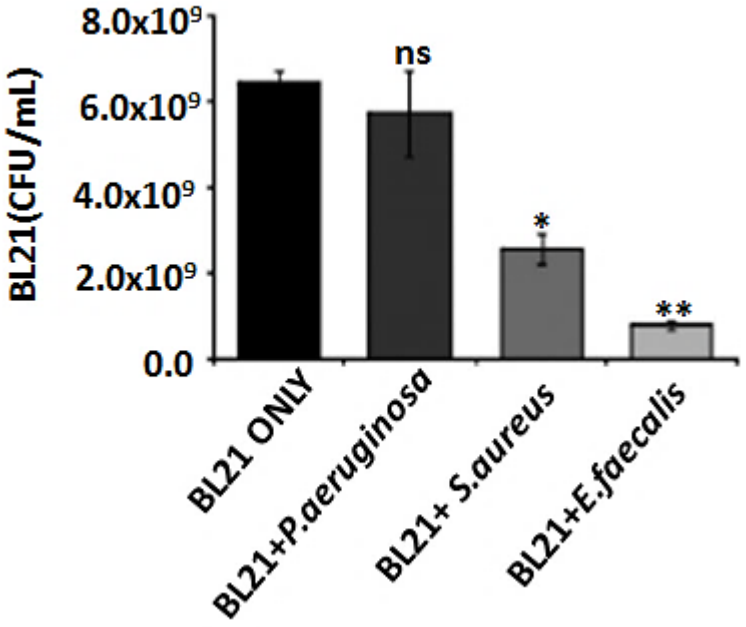



**Figure 4.10 The interactions between *E. coli* BL21 and the pathogens.** BL21 at an MOI of 100 was mixed with *P. aeruginosa, S. aureus* or *E. faecalis* at an MOI of 10 in LB broth, and incubated at 37 ℃ for 4 hours. Serial dilutions from the mixed cultures were prepared and cultured. BL21 colonies were counted after incubation at 37 ℃ for 24 hours. The values shown are means ± SEM, (n=3). Significance was determined using two tailed student's t test (* $p \leq 0.05$, ** $p \leq 0.01$).

## 4.3 Discussion

The current chapter describes targeting bacterial adherence to host cells as a potential method of treating bacterial infections. It is well known that commensal *E. coli* strains have the ability to bind to epithelial cells via the expression of a range of adhesins, such as fimbriae and flagella (Nowrouzian et. al., 2001; Dhakal et. al., 2008; Haiko and Westerlund-Wikstrom, 2013). The adherence ability of the commensals leads to the protection of host cells due to the competition with pathogenic bacteria. It has been revealed that commensal strains of *E. coli* have the capability to displace pathogenic *E. coli* strains during intestinal infections (Hancock et. al., 2010). In this study, adhesion inhibitors based on the MAM7 adhesin homolog from the commensal *E. coli* strain HS were designed, using beads coupled to GST-HSMAM7 and an engineered BL21-HSMAM7 to adhere to host cells. Herein, the inhibitory effects of beads coupled to GST-HSMAM7 and BL21-HSMAM7 on *S. aureus, P. aeruginosa* and *E. faecalis* was investigated. The findings revealed that both HSMAM7 based inhibitors have the ability to decrease bacterial attachment and cytotoxic effects.

Beads coupled to GST-HSMAM and BL21-HSMAM7 inhibited *P. aeruginosa* infection. However, no effects were observed using the controls. The inhibition was due to the competition for the attachment sites. Similar results were obtained when *V. parahaemlyticus* MAM7 based inhibitors were used against *P. aeruginosa* (Krachler et. al., 2012).

Beads coupled to HSMAM7 inhibited *S. aureus* attachment and cytotoxic effects. These results agree with the previous results obtained using beads coupled to *V. parahaemolyticus* MAM7 against *S. aureus* (Hawley et. al., 2013).

BL21 expressing MAM7 from *V. parahaemolyticus* was used to inhibit the infections caused by Gram negative bacteria such as *V. cholerae*, *V. parahaemolyticus*, *Y. pseudotuberculosis*, enteropathogenic *E. coli* (Krachler et.al., 2011), *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Acinetobacter* spp (Krachler et.al., 2012). However, no studies were done using BL21 expressing MAM against Gram positive bacteria. Herein, BL21 expressing HSMAM was used against Gram positive bacteria; *S. aureus* and *E. faecalis*. Using BL21 -HSMAM7 as an adhesion inhibitor against *S. aureus* showed significant effects on both bacterial attachment and cytotoxicity (6-fold reduction). However, the incubation with the BL21 control results in a slight inhibition (2 fold) in both adherence and cytotoxicity. Co-incubation of BL21 with *S. aureus* in liquid medium resulted in the decrease of BL21 growth but no effects on *S. aureus* growth (Figures 4.6 and Figure 4.10). This inhibition could be because of the production of secreted inhibitory agents by *S. aureus*. It has been shown that *S. aureus* produces antimicrobial agents such as bacteriocin (staphylococcins) (Al-Gosha'ah et. al., 2014). Bacteriocins from Gram positive bacteria have inhibitory effects against Gram positive in addition to some of Gram negative bacteria (Hsu and Wiseman, 1971; Ahmad et.al., 2003). Nevertheless, the secretion of antibacterial factors by *S. aureus* does not affect BL21-HSMAM7 inhibitory activity. However, its production of antibacterial factors does impact its potential to attach and cause cytotoxicity. The inhibitory role of BL21-HSMAM7 was also confirmed via visualization of the treatment.

Beads coupled to GST-HSMAM7 reduced *E. faecalis* cytotoxicity (2 fold reduction), while BL21–HSMAM7 reduced the cytotoxicity (10-fold reduction) and the BL21 control cause a 3-fold reduction. Further investigating showed that the growth of both BL21 and *E. faecalis*

was inhibited when both bacteria were co incubated in liquid medium. This inhibition could be because of the competition for nutrients. The limitation of *E. coli* growth does not affect its ability to outcompete *E. faecalis*. This could be because of the initial excess of *E. coli* over *E. faecalis* in the experiment.

This study demonstrates that HSMAM7 based inhibitors have the ability to displace the tested pathogenic bacteria from host cells. In addition, using an engineered live bacterium (BL21-HSMAM7) may increase this displacement effect over that of semi-synthetic materials, depending on the competing pathogen. Besides the competition for the attachment sites, the competition between live bacteria with pathogens may involve other factors such as competition for nutrients or the production of antimicrobial agents that impact the production of virulence factors. Therefore, these factors need to be considered when designing adhesion inhibitors.

It seems that the anti-adhesion property is a conserved feature among MAMs. In the case of *Vibrio* MAM7-based inhibitors, it has been shown that MAM7 has the ability to displace pathogens from the host surface and it utilizes phosphatidic acids as a receptor to bind to host cells. In this study, the next steps would involve identification of host surface receptors that interact with HSMAM7.

# Chapter 5. The *E. coli* HSMAM7 adhesin binds to host receptors through a 3-O-sulfogalactosyl group

## 5.1 Introduction

An important step in bacterial colonization is the adherence to the host cells. Most bacteria that interact with host cells express adhesins on their surface that aids their attachment. Bacterial attachment occurs via recognizing and adhering to specific structures on the host cells such as the extracellular matrix (ECM) proteins and/or host lipids. The successful interaction depends on the affinity and the concentration of adhesins and receptors, in addition to the presence of inhibitory and nutritional materials (Khan et.al., 1996). Multivalent Adhesion Molecule7 (MAM7) is expressed on the cell surface of Gram negative bacteria and mediates the interactions with the host receptors. It has been shown that MAM7 from the pathogenic bacterium *V. parahaemolyticus* adhere to a wide range of host cells such as macrophages, epithelial cells and fibroblasts. *V. parahaemolyticus* MAM7 recognizes phosphatidic acid and fibronectin to achieve a tight binding to the host (Krachler et. al, 2011; Krachler and Orth, 2011). These interactions stimulate host signalling that aid pathogenic invasions (Lim et. al., 2014).

Based on the fact that intestinal commensals colonize the human gastrointestinal tract and confer a protective barrier to inhibit infections by pathogens, the MAM7 ortholog of the commensal *Escherichia coli* strain HS was chosen for further analysis.

In this chapter, the results revealed that sulfates are important for the binding of MAM7 from the commensal *Escherichia coli* HS to the host ligands. The interactions between HSMAM7 with sulfated receptors and mucin have not been reported previously.

Mucosal surfaces have carbohydrate moieties as binding sites for pathogens and carbohydrate moieties exist either in the form of glycolipids or glycoproteins. Sulfatide (3-O-sulfogalactosylceramide) is present in the membranes of many cell types (Takahashi and Suzuki, 2012) and is mainly found in myelin and accounts for 4% of total membrane lipids (Ishizuka, 1997). Sulfatide is known to mediate the binding of variety of bacteria to the host cell surface and is recognized by a variety of bacteria such as the respiratory pathogen, *Bordetella pertussis*. It has been shown that filamentous hemagglutinin (FHA) adhesin in *B. pertussis* binds to sulfated glycolipids to initiate infection (Julie et. al., 2007). In addition, the binding of *Mycoplasma pneumoniae* to sulfated glycolipids was investigated. It has been found that *M. pneumoniae* bound to sulfatide and the binding to purified sulfatides and human colon adenocarcinoma cells WiDr was inhibited using Dextran sulfate (Krivan et. al., 1989). *H. pylori* bind to sulfatide via the heat-shock protein Hsp 70 (Huesca et.al., 1998; Kobayashi et.al., 2009).

Mucus covers epithelial surfaces and acts as lubricant and a protective barrier to protect the underlying epithelial cells from damage that could be caused by drugs, toxins, acids and microbial infections. Goblet cells secret the mucus layer that cover both of the small and large intestine. In the small intestine, the mucus layer is a discontinuous loosely-adherent layer (Johansson et. al., 2011; Atuma et. al., 2001). Whereas, the colon is covered with a thick continuous layer composed of two layers; an outer loosely-attached

layer and an inner firmly-attached layer. The outer layer provides nutrients for the commensals that contribute to the protection against pathogens.

Most of the commensal bacteria that inhabit the colon are obligate anaerobic bacteria. The majority of bacteria from the phyla Firmicutes and Bacteroidetes (Mariat et al. 2009), in addition to Actinobacteria, Verrucomicrobia, Fusobacteria, Proteobacteria and Spirochaetes (Rajilic-Stojanovi et. al., 2007; Zoetendal et. al., 2008). Facultative anaerobes such as *Lactobacillus* spp, *Escherichia coli* and *Enterobacter* spp are present as well. It is well known that commensals do not cause infection in the healthy host and the interactions between commensal bacteria and the host is useful. Therefore, commensals contribute in limiting pathogenic colonization and the invasion of epithelial cells due to the competition for nutrients and binding sites. Moreover, commensals release antimicrobial peptides known as bacteriocins that inhibit bacterial growth (Cunliffe and Mahida, 2001). The mucus inner layer does not contain bacteria due to the high content of digestive enzymes, immunoglobulins and antimicrobial peptides (Phillipson et. al., 2008). The major constituents of the mucus are mucins that contribute in the interactions between epithelial cells surfaces and microorganisms (Linden et. al., 2008; Johansson et. al., 2011). Bacterial binding to the mucin oligosaccharides facilitates removal of bacteria and inhibits their colonization of epithelial cells (Thornton and Sheehan, 2004). Although many pathogens can be trapped when they bind to the secreted mucin within the mucus, some pathogens go through this barrier to infect the cell surface. Therefore, cell surface mucins act as hindrance to release these pathogens from the cell surface (McGuckin et. al., 2011).

Depending on the composition of sugars on the mucins, they can be classified into two groups; acidic and neutral mucins. Acidic mucins comprise sialic acid and thus they are known as sialo mucins and/or sulfate groups, thus they are known as sulfo mucins (Derrien et. al., 2010; Brockhausen, 2003). In the sulfo mucins, the sulphate group is linked to specific sites on the sugar chain. Sulphate is linked to the C-6 of N-acetylglucosamine (GlcNAc), C-6 N-acetylgalactoseamine or the C-3 of terminal galactose groups (Robbe et. al., 2004). Mucin is a main factor for bacterial attachment and colonization because of its carbohydrate content (Shuter et. al., 1996; Trivier et.al., 1997).

Although mucin carbohydrates being considered as the binding sites for bacterial attachment (Roos and Jonsson, 2002), bacterial adhesins bind to a specific residue on carbohydrates. Some bacterial adhesins recognize and bind to sialic acid groups while others recognize sulfate residues in mucin as well as in cells receptors. For example, sialic acid recognition has been reported for *Streptococcus pyogenes.* It has been found that the M protein in *Streptococcus pyogenes* binds to pharyngeal epithelial cells and mucins through recognizing sialic acid residues (Ryan et. al., 2001). However, *Lactobacillus reuteri* JCM1081 binds to sulfated carbohydrate moieties of mucins and glycolipids (Nishiyama et. al., 2013). *H. pylori* recognize and bind to sulphated oligosaccharides on salivary mucins but a very weak binding was also observed to sialic acids (Veerman et.al., 1997).

In a healthy colon, mucus contains heavily sulphated mucins that protect the mucus from bacterial degradation (Nieuw Amerongen et.al., 1998). The sulfation process is important to protect mucins from degradation by bacterial enzymes. Under some conditions the interactions between commensals and host harm the host which results in an

opportunistic bacterial infection. *B. thetaiotaomicron* is a prominent bacterium in the gastrointestinal tract (Qin et. al., 2010). The interactions between *B. thetaiotaomicron* and the host are beneficial for both. Although *B. thetaiotaomicron* is a commensal bacteria, it can cause opportunistic infections such as meningitis (Feuillet et. al., 2005), bacteraemia and intraabdominal sepsis (Aldridge and O'Brien, 2002; Goldstein, 1996; Redondo et.al., 1995).

The ability to forage both dietary polysaccharides and the mucosal glycans is a strategy by which *B. thetaiotamicron* is able to adapt and compete with other microorganisms in the gut. *B. thetaiotaomicron* has carbohydrate active enzymes (CAZymes) that contributes to the metabolism of carbohydrates (Hehemann et.al., 2012). In addition, the *B. thetaiotaomicron* genome contains 28 putative sulfatase genes and an anaerobic sulfatase-maturating enzyme (anSME) (BT0238) which is essential for the activity of all sulfatases (Benjdia et. al., 2011). It was reported that the deletion of the gene that encodes an SME resulted in the loss of the ability to desulphate the sulfated polysaccharides as a result of loss of sulfatase activity (Benjdia et. al., 2011). *B. thetaiotaomicron* uses mucosal glycan for degradation when the dietary sources of glycans are unavailable (Benjdia et. al., 2011). It has been shown that a diet high in fiber and thus complex glycans promotes both the growth of the gut microbiota and the protection from disease (Sonnenburg and Sonnenburg, 2014). The protection may occur via the degradation of the dietary carbohydrates instead of mucus glycans. A recent study has shown that feeding mice colonized with synthetic microbiota with fibre rich dietary sources resulted in a thicker mucus layer compared with those that were fed with fibre free sources (Desai et. al., 2016).

Degradation of mucus by the microbiota may affect human health and cause disease. Inflammatory bowel disease (IBD), a chronic inflammation in the gut, could result from both genetic problems and alteration in the microbiota composition (Khor et. al., 2011; Andrew et. al., 2015). The major categories of inflammatory bowel disease are Crohn's disease and ulcerative colitis. *B. thetaiotaomicron* may have a role in causing inflammatory bowel disease. It has been shown that *B. thetaiotaomicron* produces sialidase to release the sialic acid from the host glycans and this process results in supplying sugars to pathogens such as *Salmonella enterica* and *Clostridium difficile* (Katharine et. al., 2013). In another study, it has been reported that the inflammation results from the contact between the outer membrane vesicles secreted from *B. thetaiotaomicron* containing sulfatase, and host immune cells (Hickey et. al., 2015).

This chapter describes the interactions between MAM7 from the commensal *E.coli* HS with sulfated ligands such as sulfatide, several components of extra cellular matrix and mucin and how the desulfation affects this binding. In addition to describe the interactions between the gut microbiota and the host via investigating the ability of *B. thetaiotaomicron* to desulfate and degrade mucosal glycans that might provide a new aspect of how this bacterium contributes to human health and disease.

## 5.2 HSMAM7 binds to phosphatidylinositol -3- phosphate and sulfatide

The purpose of the current study is to investigate the interactions between HSMAM7 protein and host lipids. The binding of *V. parahaemolyticus* MAM7 to host lipids has been investigated and it has been revealed that MAM7 binds to phosphatidic acids (Krachler and Orth, 2011).

To test the binding of MAM7 from the commensal *E. coli* HS to host lipids, recombinant purified GST- HSMAM7 protein was used in this study. Lipid overlay and indirect ELISA assays have been done for this purpose. For lipid overlay assay, PIP-Strips ™ and Sphingo-Strips were used. These membranes contain different immobilized lipids on the surface. The assay involves incubation of the purified recombinant protein GST-HSMAM7 or GST protein (control) with these lipids. Then anti GST–mouse antibody and anti- mouse IgG-HRP antibodies were used to probe for bound GST- HSMAM7, followed by ECL detection. The results revealed that GST-HSMAM7 protein binds to phosphatidylinositol -3- phosphate (PI (3) P) on the PIP- Strip and sulfatide on the Sphingo-Strip. Whereas, no binding was detected with the control GST protein (Figure 5.1).

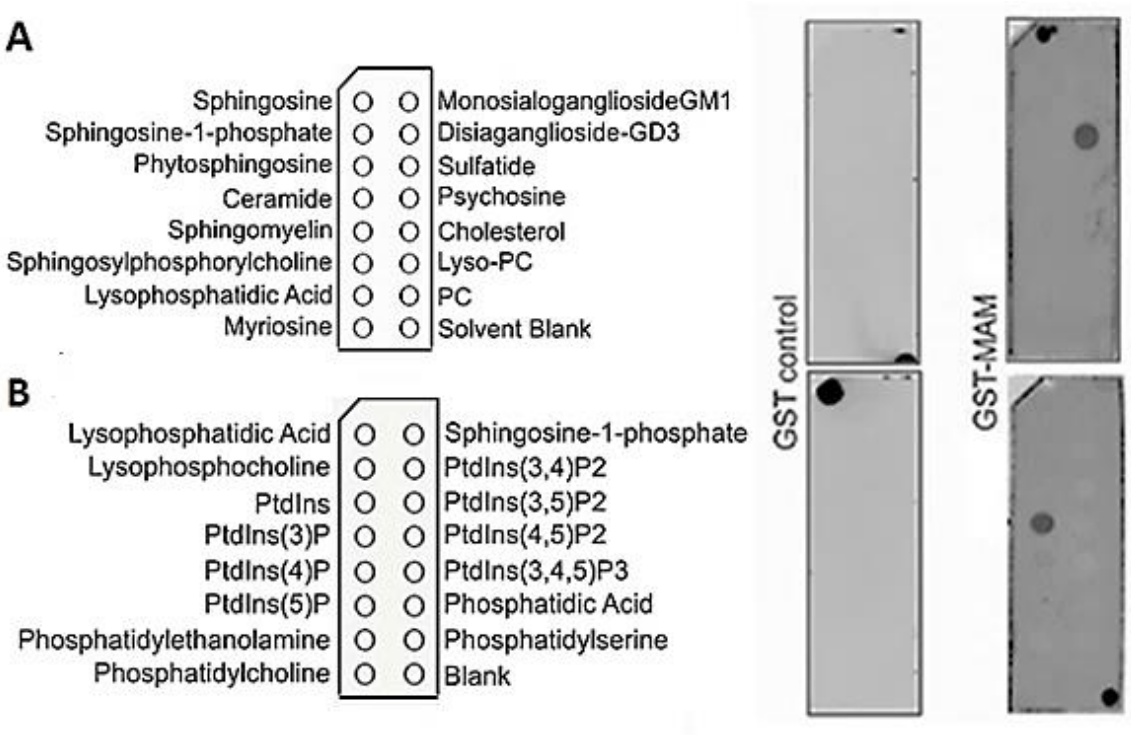
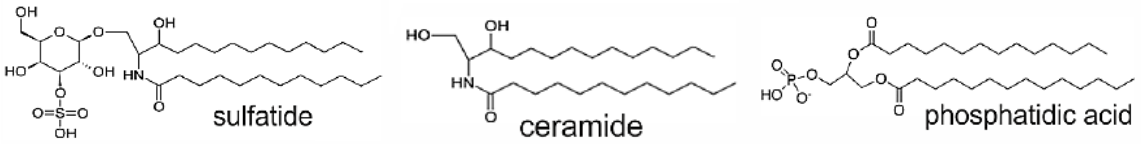



**Figure 5.1 Lipid overlay assay of GST- HSMAM7 protein**. (A) Layout and lipid contents of Sphingo-Strip. (B) Layout and lipid contents of PIP –Strip. GST-HSMAM7 protein and GST(control) at concentration of 10 μM was incubated with membranes which contain 100 pmol of spotted lipids, and bound proteins detected by probing membranes with anti GST–mouse and anti-mouse IgG-HRP antibodies, and developing with enhanced chemiluminescence (ECL) detection reagent.

Because phosphatidylinositol -3- phosphate is intracellular and does not have a role in bacterial adhesion, the interaction between *E. coli* HSMAM7 and phosphatidylinositol -3-phosphate has not been investigated further. However, a large variety of bacteria recognizes and binds to sulfatide, so this interaction was investigated in more detail. An ELISA assay was used to quantitate the binding of HSMAM7 to the host lipids sulfatide, ceramide, which is the same scaffold as sulfatide but lacks the sulfate group, and phosphatidic acid, which binds *V. parahaemolyticus* MAM7. Wells of a high-bind microtiter plate were coated with 50 µl of 200 µg/ml sulfatide, ceramide, phosphatidic acid or were left uncoated. GST-HSMAM7 was added at indicated concentrations and binding quantified by incubation of plates with anti GST–mouse followed by incubation with anti-mouse IgG-HRP antibodies and ECL detection. The results demonstrate that GST-HSMAM7 bound to sulfatide with high affinity, but displayed much lower binding to phosphatidic acid and ceramide, and no significant binding to uncoated wells (Figure 5.2).

A

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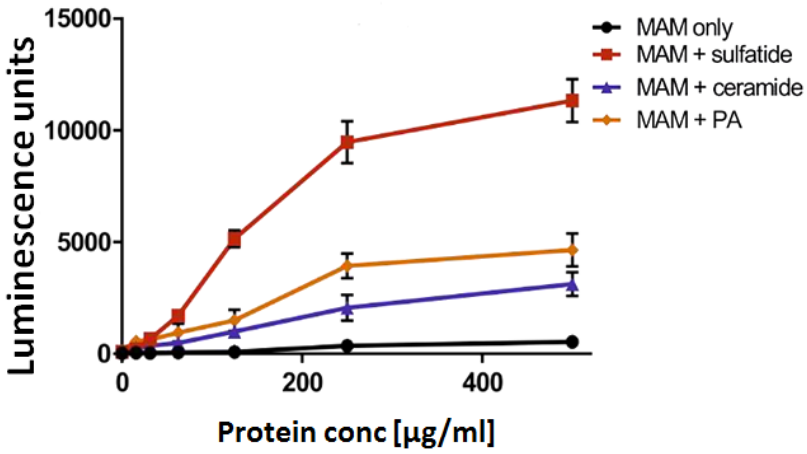
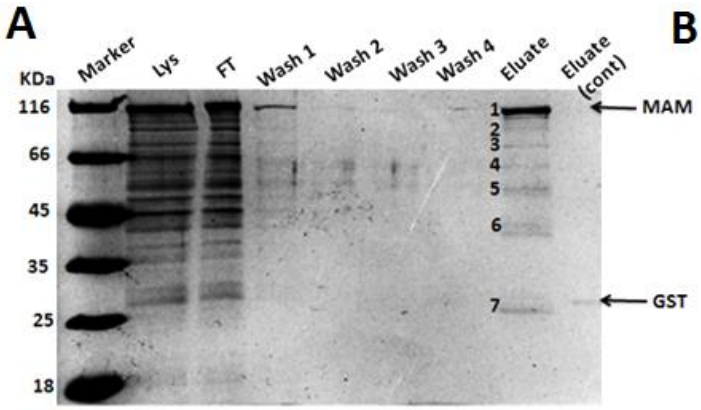



**Figure 5.2 ELISA to quantitate binding of GST-HSMAM7 to host lipids.** (A) Diagram of the structures of sulfatide, ceramide and phosphatidic acid (PA). (B) The luminescence output is plotted against GST- HSMAM7 protein concentration. Results shown are means ± SEM from three independent experiments.

## 5.3 HSMAM7 binds to host sulfated proteins

This study was aiming to investigate the interactions between HSMAM7 with the proteins of the host cells using a pull down assay. The principle of this assay is that GST fusion protein binds to the glutathione beads and pulls down specific proteins in the cell lysate. These proteins are collected by centrifugation of the beads and boiling them in the SDS buffer. Then they are subjected to SDS–PAGE and Coomassie staining to separate and visualize protein bands. In this study, Hela cells lysate was prepared as mentioned in section 2.12.5. The purified recombinant proteins (GST-HSMAM7) or GST protein (control) were incubated with Hela cells lysate. Then the mixture was added to the glutathione agarose beads. The reaction mixture was centrifuged and the pellet was washed with washing buffer to remove unbound proteins, followed by separating bound proteins using SDS- PAGE. Commassie stain was used to visualize the bands. The result of the SDS–PAGE revealed the presence of seven individual protein bands that were pulled down by the recombinant protein GST- HSMAM7, and only one band for GST control. To analyze and identify the bands, they were cut and sent for mass spectrometry. The results revealed identification of the following proteins specifically bound to GST-HSMAM7; perlecan, mucin, fibronectin, collagen IV and laminin (Figure 5.3).




B

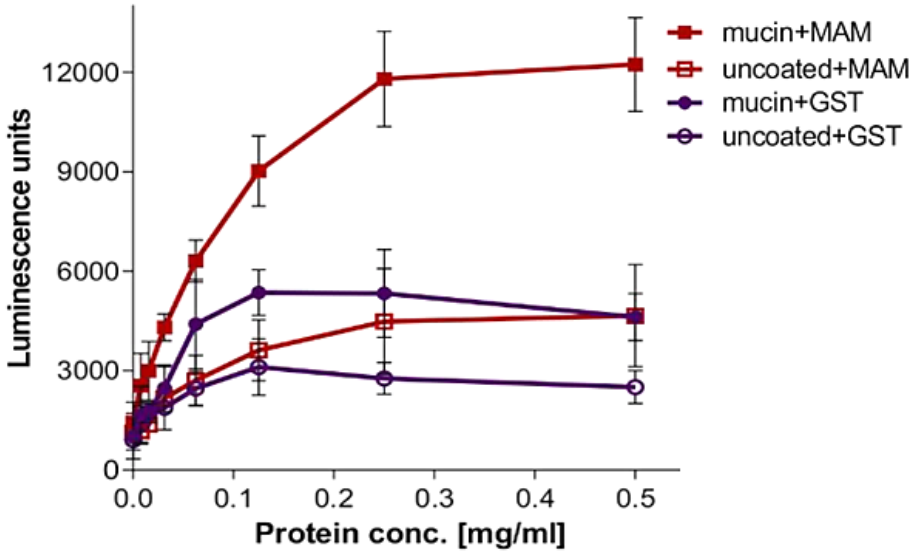
| Band | Description | Score | Coverage | # Unique peptides | Mw [kDa] |
|---|---|---|---|---|---|
| 1 | MAM | NA | NA | NA | NA |
| 2 | perlecan | 14.6 | 5.3 | 4 | 463 |
| 3 | mucin | 9.3 | 6.8 | 4 | 356 |
| 4 | fibronectin | 6.8 | 1.8 | 3 | 221 |
| 5 | collagen IV | 16.0 | 5.6 | 5 | 161 |
| 6 | laminin | 24.5 | 17.3 | 9 | 174 |
| 7 | GST | 180.9 | 83.6 | 34 | 27 |

**Figure 5.3 *E. coli* HSMAM7 binds to host sulfated proteins.** (A) SDS-PAGE of the recombinant HSMAM7 protein using a pull down assay. The purified recombinant protein HSMAM7 at a concentration of 200 µg/ml was incubated with Hela lysate. Followed by adding DSP at a final concentration of 100 µg/ml and incubated at room temperature for 30 minutes. GSH beads were added and incubated overnight at 4 °C. The reaction mixture was centrifuged and the pellet was washed 4 times with washing buffer. Proteins were eluted into SDS sample buffer. SDS -PAGE was run followed by Commassie stain to detect the protein bands. Lysate (Lys), Flow through (Flt), pellet washes (W1-W4), eluate fractions (Eluate) and the control (Eluate cont). Bands 1-7 were cut out and subjected to tryptic digest and protein ID by LC-MS/MS. (B) Top hits for protein bands 1-7 depicted in (A).

## 5.4 HSMAM7 binds to gastric mucin but does not bind to bovine submaxillary mucin

Based on the mass spectrometry analysis, mucin was one of the glycoproteins that were pulled down by HSMAM7. Since mucin is one of the first host receptors intestinal bacteria will encounter, this interaction was investigated further. An indirect ELISA assay was performed to quantify the interaction of the GST- HSMAM7 protein with mucin. Different types of gastric mucins and salivary mucin were used in this study to test if MAM7 binding was limited to a specific type of mucin. Mucin from porcine stomach type II, mucin from porcine stomach type III and mucin type I-S from bovine submaxillary glands (Sigma) were used. Different concentrations of GST-HSMAM7 protein were added to the immobilized mucin in 96 well microtiter plates for 2 hours. Anti GST–mouse antibody (1:1000), then secondary anti mouse IgG–HRP antibody (1:5000) in blocking buffer was added, followed by ECL detection. The results revealed that GST-HSMAM7 strongly bound both mucin type II and III from porcine stomach (Figure 5.4 and Figure 5.5), while no binding was detected to mucin type I-S from bovine submaxillary glands (Figure 5.6). All three mucins contain the same Muc2 core structure, but differ in their level and quality of glycosylation, with mucin type I-S carrying shorter O-linked glycans and being most heavily sialylated. It has been shown that the amount of sialic acid in porcine gastric mucin (0.5-1.5%) is less than mucin type I-S bovine submaxillary glands (9-17%) of the total mass (Madsen et. al., 2016). These data suggest that HSMAM7 specifically recognizes glycosylation patterns on mucin.

A

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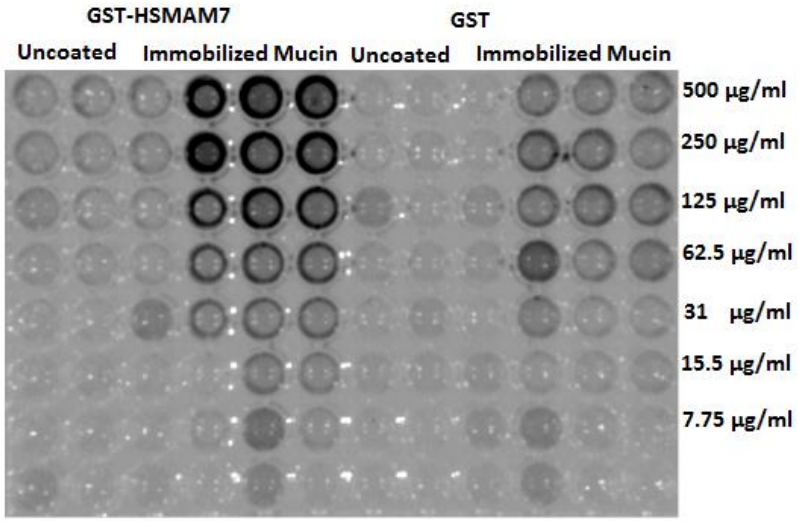
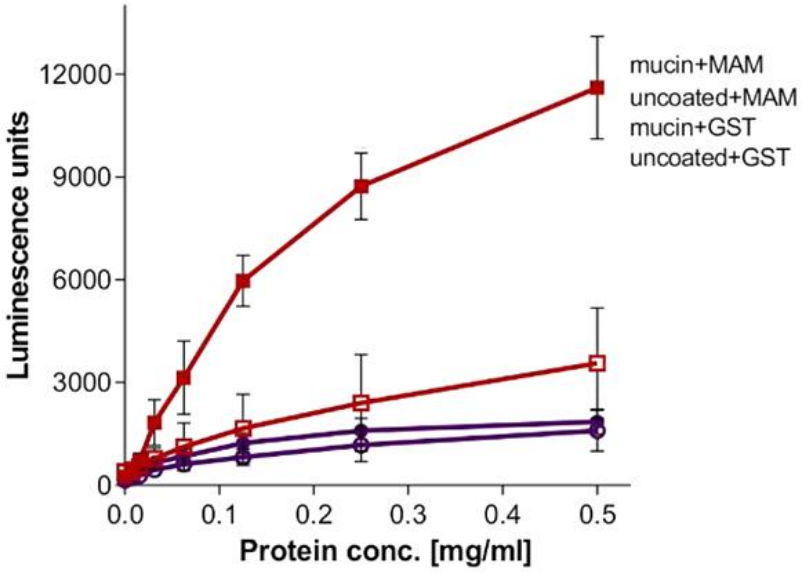



**Figure 5.4 ELISA to detect the binding of HSMAM7 protein to mucin from the porcine stomach type II.** (A) The luminescence output is plotted against protein concentrations. (B) Imaging the 96 well microtiter plate. Wells coated with either 50 µg/ml mucin from the porcine type II or were left uncoated. GST-HSMAM7 or GST protein (control) were added at concentrations between 0-500 µg/ml. Binding of GST-HSMAM7 or GST to mucin was quantified using anti GST-mouse and anti-mouse HRP antibodies coupled with chemiluminescence detection.

A

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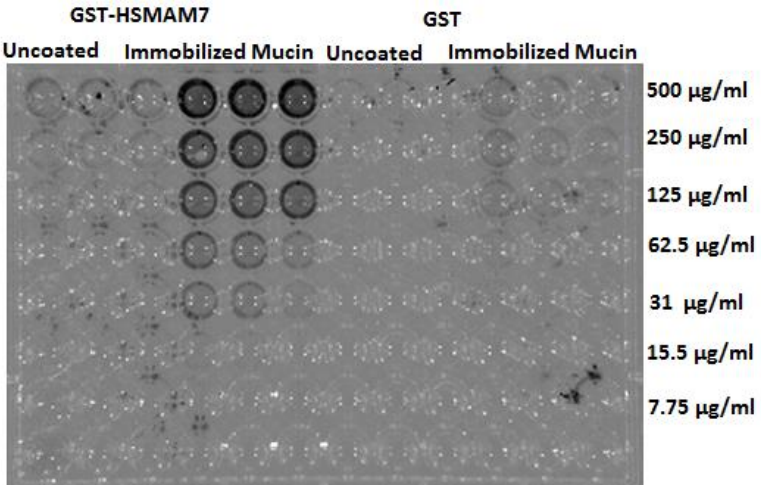



**Figure 5.5 ELISA to detect the binding of HSMAM7 protein to mucin from the porcine stomach type III.** (A) The luminescence output is plotted against proteins concentrations. (B) Imaging the 96 well microtiter plate. Wells coated with either 50 µg/ml mucin from the porcine type III or were left uncoated. GST-HSMAM7 or GST protein (control) were added at concentrations between 0-500 µg/ml. Binding of GST-HSMAM7 or GST to mucin was quantified using anti GST-mouse and anti-mouse HRP antibodies coupled with chemiluminescence detection.

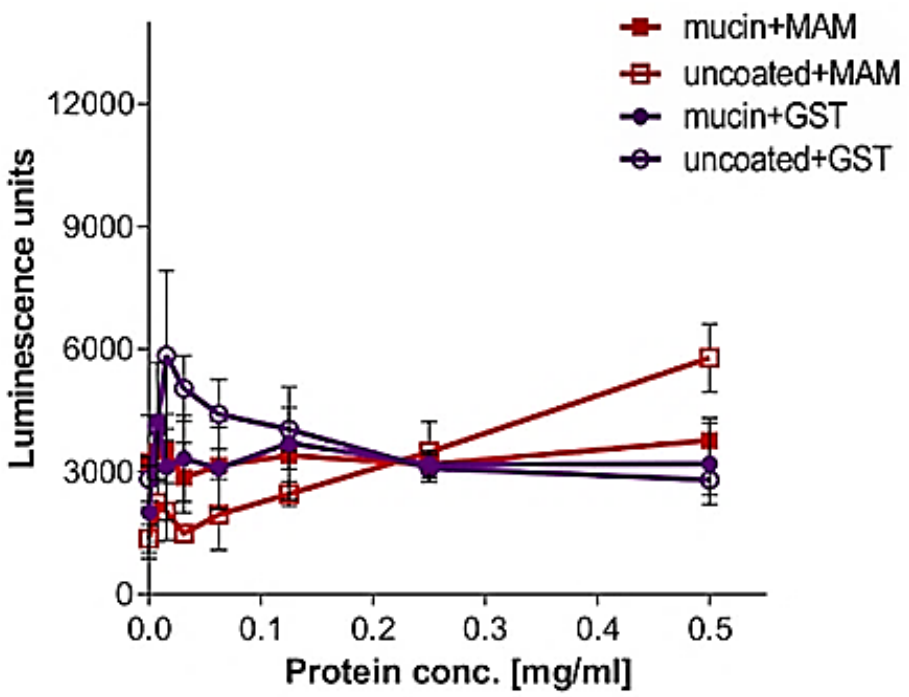



**Figure 5.6 ELISA to detect the binding of HSMAM7 protein to mucin type I-S from bovine submaxillary glands.** Wells contained either 50 µg/ml mucin type I-S from bovine submaxillary glands or were left uncoated. GST-HSMAM7 or GST protein (control) were added at concentrations between 0-500 µg/ml. Binding of purified GST-HSMAM7 or GST protein (control) to mucin was quantified using anti GST-mouse and anti-mouse HRP antibodies coupled with chemiluminescence detection. The luminescence output is plotted against protein concentrations.

## 5.5 Mucin inhibits bacterial binding to epithelial cells

Since mucus, in the form of the mucus layer is usually the first surface encountered by bacteria entering the intestinal lumen, and contributes in keeping the microbiota at a distance from the epithelial cells. Herein, the ability of mucin to compete with *E.coli* expressing HSMAM7 on their surfaces was investigated. *In vitro*, mucin from porcine stomach type II was used to compete with *E.coli* expressing HSMAM7 and inhibit their binding to Hela cells. In triplicate, Hela cells were infected by adding *E. coli* expressing HSMAM7 (BL21-HSMAM7) or *E. coli* (BL21 control) at an MOI of 100 with mucin at a final concentration of 50 µg/ml and incubated for 1 hour. The attachment was assessed via counting the colony forming units that adhered to Hela cells. In addition, the infected cells were fixed and stained with Hoechst and green phalloidin for imaging the bacterial attachment of mCherry expressing bacteria and counting bacterial cells that adhered to the Hela cells. The results revealed that pre-incubation of HSMAM7-expressing BL21 with mucin type II decreased bacterial attachment to Hela cells to background levels (BL21 control without HSMAM7), while the non-specific adherence of BL21 without HSMAM7 was not affected by incubation of bacteria with mucin (Figure 5.7 and Figure 5.8). These results demonstrate that interaction of HSMAM7 with mucin inhibits bacterial attachment to the epithelial surface.

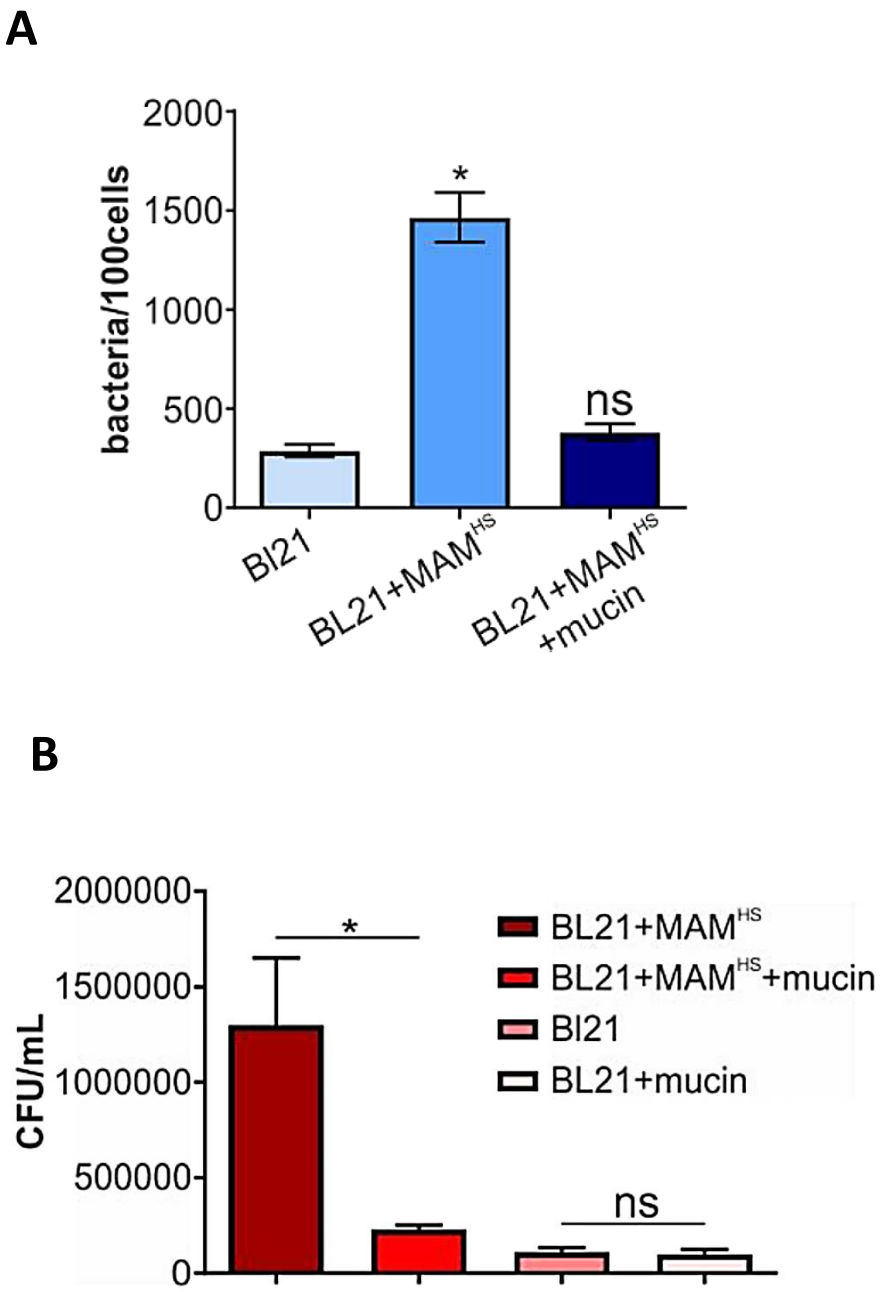



**Figure 5.7 Mucin inhibits the attachment of *E. coli* HSMAM7 to Hela cells.** Hela cells were incubated with *E. coli* BL21-HSMAM or *E. coli* BL21 at an MOI of 100 and mucin from porcine stomach type II at a final concentration of 50 µg/ml for 1 hour. (A) The adhered bacterial cells were counted using image analysis. (B) CFU counts of the attached bacterial cells to Hela. Values show means ± SEM. Significance was determined using two tailed student's t test (* p ≤0.05).

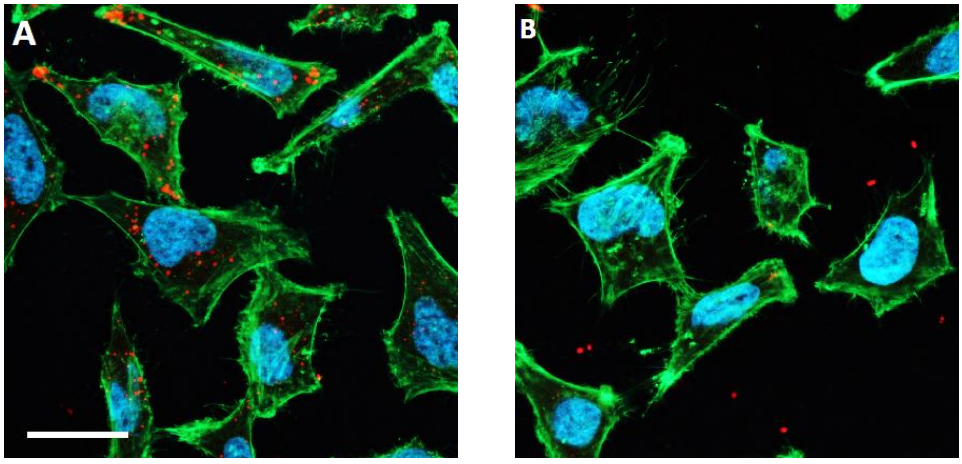



**Figure 5.8 Mucin inhibits the attachment of *E. coli* HSMAM7 to Hela cells**. (A) Adherence of *E. coli* (BL21-HSMAM7) expressing mCherry (red) to Hela cells. (B) Mucin from porcine stomach type II inhibits attachment of *E. coli* (BL21-HSMAM) to Hela cells. Cells were fixed and stained for actin (green) and DNA (blue) following a 1 hour infection. Scale bar 10 μm.

## 5.6 Sulfation is necessary for the binding of HSMAM7 to mucin

*E. coli* strain HS inhabits the human colon, which is covered by mucus containing heavily -sulphated mucins (Nieuw Amerongen et.al., 1998). Based on the results above showing that HSMAM7 binds to the gastric mucins, but does not bind to mucins type I-S from bovine submaxillary glands. The aim of this work was to investigate if mucin sulfation was required for the interaction between HSMAM7 and mucin. An indirct ELISA assay was used to study the interaction of HSMAM7 protein with desulfated mucin. To desulfate the mucin, a pure sulfatase from *Helix promatia* was used. Different concentrations of sulfatase were added to the immobolized mucin (mucin from porcine stomach type II) and incubated at room temperature for 24 hours, followed by blocking with the blocking buffer. To detect HSMAM7 binding, 125 µg/ml of the recombinant GST-HSMAM7 was added for 2 hours. For quantitation of binding, anti GST–mouse antibody, secondary anti mouse IgG –HRP antibody were used followed by the ECL detection. The results revealed that the binding of the recombinant GST-HSMAM7 protein to mucin was decreased when the protein was incubated with desulfated mucin. Desulfation decreased the binding of GST-HSMAM7 in a dose-dependent manner ( Figure 5.9).

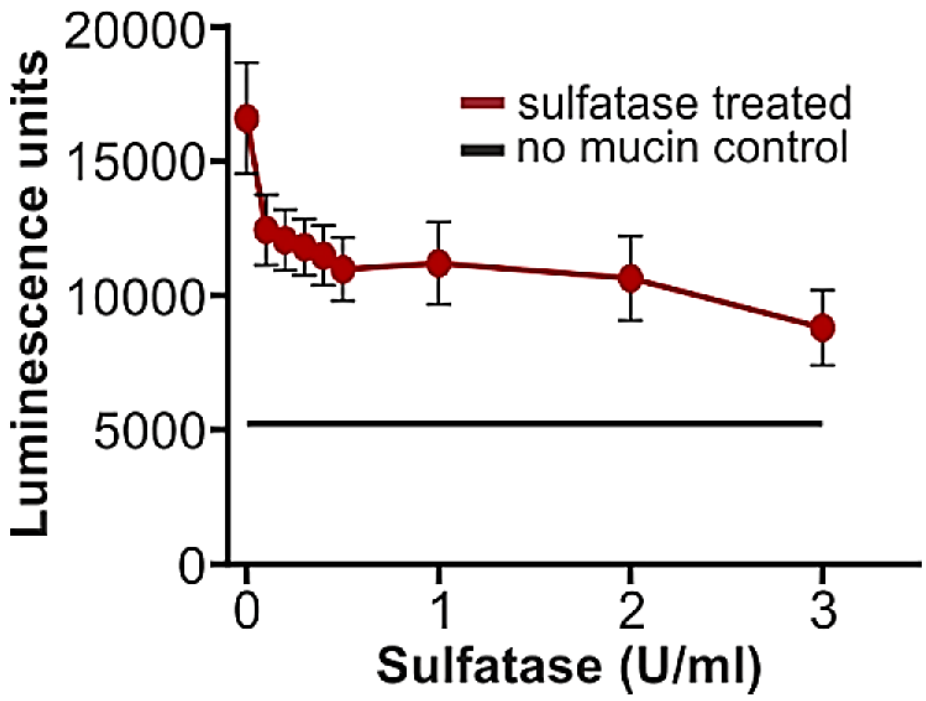



**Figure 5.9 ELISA to detect binding of HSMAM7 to the desulfated mucin.** 50 µg/ml mucin was treated with different concentrations of sulfatase and 125 µg/ml of the recombinant GST-HSMAM7 protein was added. Anti GST–mouse and anti- mouse IgG -HRP antibodies were used. Followed by ECL detection. The luminescence output is plotted against sulfatase concentrations. Results shown are means ± SEM from three independent experiments.

## 5.7 Barium chloride to quantify sulphate released from mucin

Barium chloride was used to quantify sulphate that was released from mucin upon sulfatase treatment. It is well known that adding barium chloride to sulphate ions result in forming a white precipitate of barium sulphate: $Ba^{2} + SO_4^{-2} = BaSO_4$ (Atkinson and Hibbert, 2000). This method was adapted to measure the released sulfate, so it could be used to measure the efficiency of desulfation. Herein, $Na_2SO_4$ at concentrations (3-200 µM) was prepared for calculating the calibration curve. In triplicates, 100 µl of 20% barium chloride was added to 96 -well plate containing 100 µl/well of each concentration of $Na_2SO_4$. The absorbance was read at OD 600 nm (Figure 5.10).

To quantify sulphate released from mucin upon sulfatase treatment, 50 µg/ml of mucin was immobilized in a 96 well plate at 4 °C overnight. To desulphate mucin, 100 µl of sulfatase at concentrations of 0.1- 3 U/ml was added and incubated at 37 ℃ overnight. To quantify the released sulphate, 100 µl of the mixture was removed into a new 96- well plate and 100 µl barium chloride 20% were added to each well. The absorbance was read at OD 600 nm. The results revealed the presence of sulphate ($SO4^{-2}$) and the concentration of the released sulphate increased with increasing concentration of sulfatase enzyme. The concentration of the released sulphate ($SO4^{-2}$) from the used mucin was ranging from 250 -350 µM and showed complete desulfation was achieved by adding 1 U/ml of sulfatase (Figure 5.11).

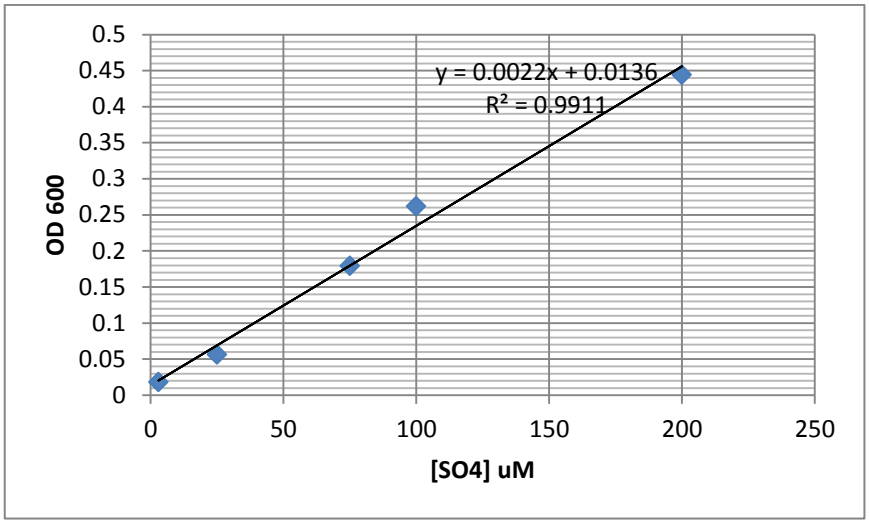



Figure 5.10 Standard curve to quantify sulphate.

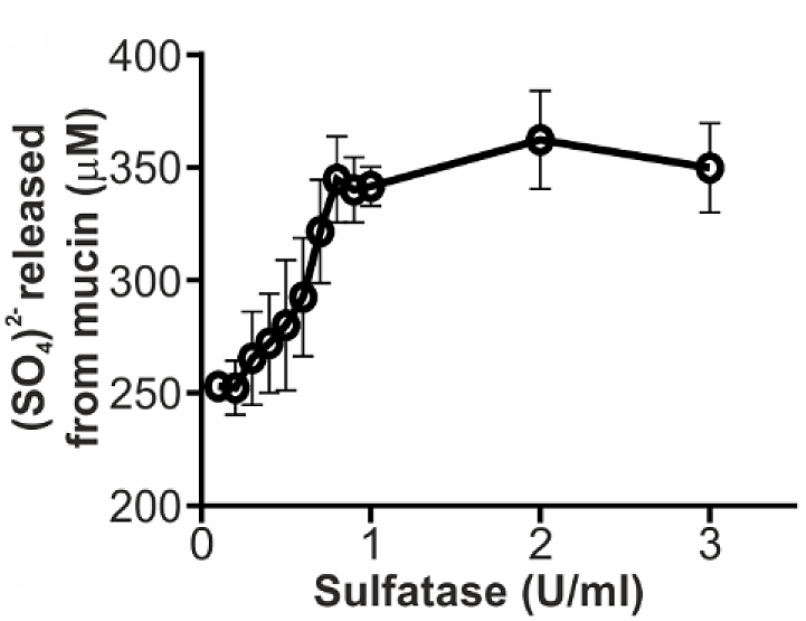



Figure 5.11 Sulfate quantification using 20% barium chloride.

## 5.8 The ability of mucin to block bacterial adhesion to epithelial cells is dependent on mucin sulfation

The aim of this work was to study the binding of *E. coli* expressing HSMAM7 to mucin and desulphated mucin to confirm the effect of sulphation in the interaction of HSMAM7 protein to sulphated receptors and mucin. Competition assays and immunostaining were used. Caco-2 cells were seeded on coverslips for two days before the experiments. For HSMAM7 protein expression, *E. coli* HSMAM7 was grown in LB broth containing arabinose to 0.05%. In triplicate, Caco-2 cells were infected with colourless DMEM containing *E. coli* expressing HSMAM7 at MOI of 100 with 50 µg/ml mucin, or de-sulphated mucin. The results indicate there was a significant difference in the number of colony forming units (CFU) of *E. coli* BL21HSMAM7 that attached to Caco-2 cells in the presence of mucin and the number of CFUs of *E. coli* BL21HSMAM7 that attached to Caco-2 cells (control). In contrast, no differences between the number of CFUs of *E. coli* BL21 HSMAM7 that attached to Caco-2 cells in the presence of desulphated mucin and the control were detected (Figure 5.12 E). Immunostaining results revealed that the attachment of *E. coli* BL21 HSMAM7 to Hela cells and Caco2 cells was inhibited in the presence of mucin (Figure 5.12 A,B,C,D). These data demonstrate that desulfated mucin lost the ability to inhibit *E. coli* expressing HSMAM7 from attaching to the epithelial surface.

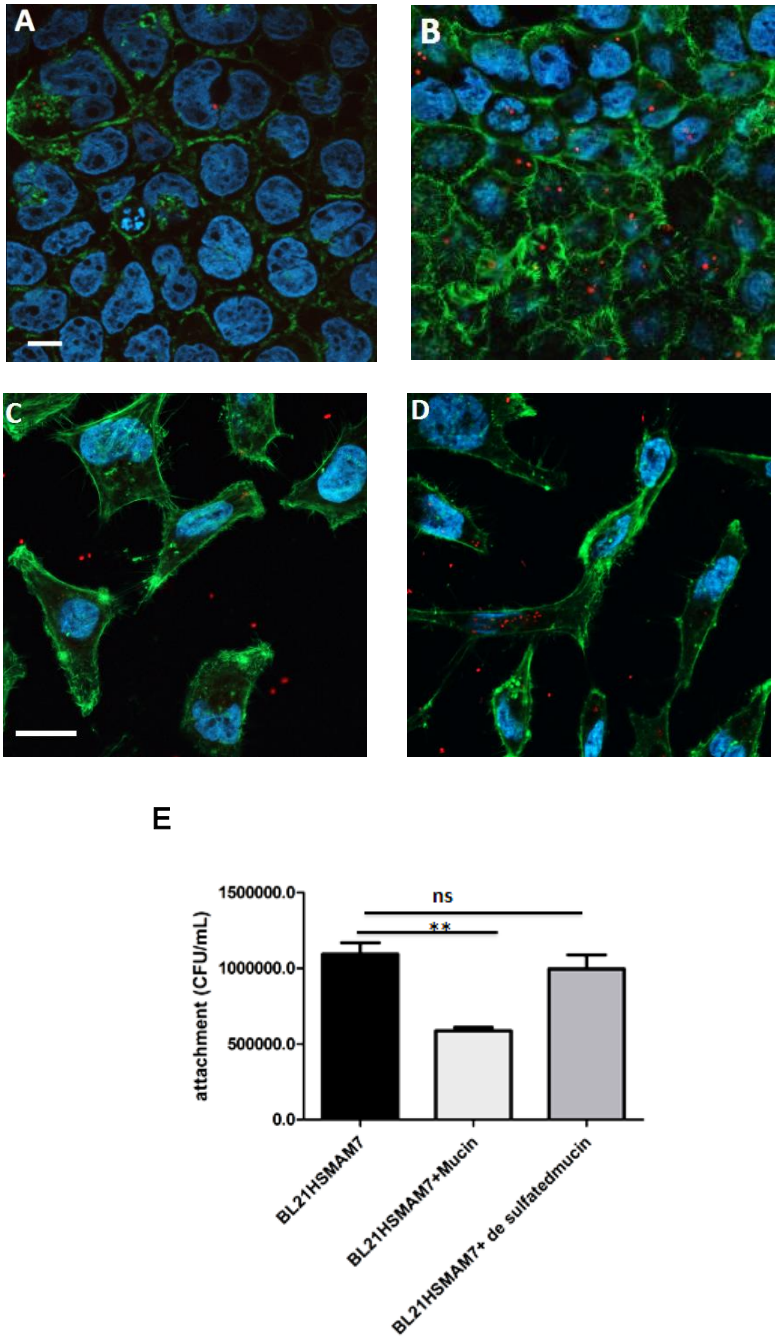
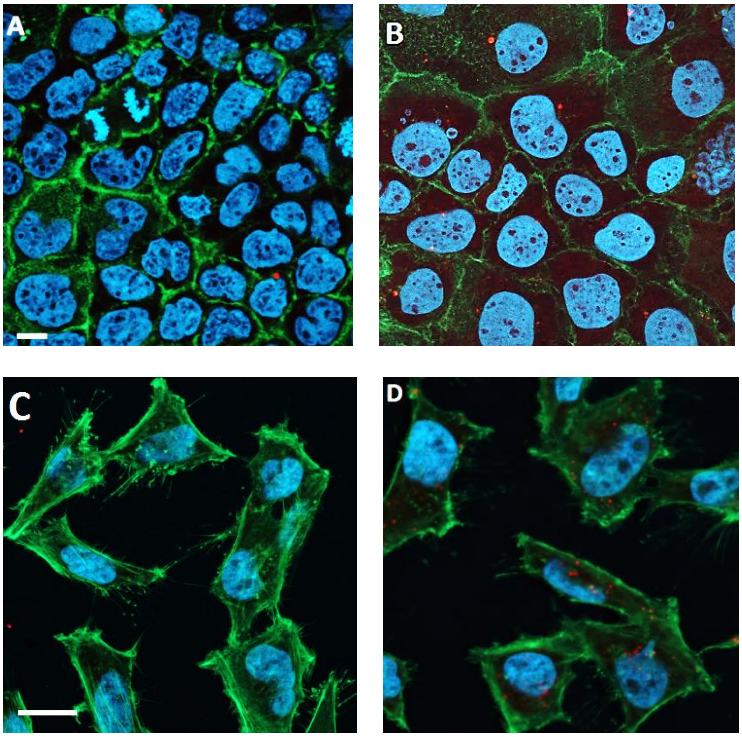



**Figure 5.12 *E. coli* BL21 expressing HSMAM7 attaches to sulfated mucin.** Caco-2 cells (A and B) or Hela cells (C and D) were infected with infection medium containing BL21HS MAM7 at an MOI of 100 with 50 µg/ml mucin (A, C), or de sulphated mucin (B, D) for 1 hour. Cells were fixed and stained with Hoechst for DNA (blue) and phalloidin for actin (green). Bar 10 µm. For attachment, 0.5% Triton X-100 was added to lyse cells, and serial dilutions were cultured on LB agar and incubated at 37 °C for 24 hours (E). Data represent the means ± SEM (n=3). Significance was determined using two tailed student's t test (**p ≤0.01).

## 5.9 HSMAM7 recognizes 3-o-sulfo-galactosyl moieties found in host receptors

In the sulfomucins, the sulphate group is linked to specific sites on the sugar chain. Sulphate is linked either to the C-6 of N-acetylglucosamine (GlcNAc), C-6 of N-acetylgalactoseamine or the C-3 of terminal galactose (Gal) residues (Robbe et. al., 2004). Herein, the role of sulfated sugars in inhibiting the binding of *E. coli* BL21HSMAM7 to host cells was investigated. Competitive attachment assays and immunostaining assays were used. Caco-2 cells were seeded on coverslips for two days before the experiments. Caco-2 cells were infected with colourless DMEM containing *E. coli* expressing HSMAM7 at MOI of 100 with 200 μM of lactose 3 –sulphate or lactose (control), D- galactose-6-sulphate or D-galactose (control), N-acetyl glucosamine-6-sulfate or N-acetyl glucosamine (control) and incubated for 1 hour. The results revealed that HSMAM7 -mediated adhesion was specifically inhibited by lactose-3-sulfate. However, the adhesion was unaffected by the presence of of lactose, galactose, galactose-6-sulfate, N-acetyl-glucosamine, or N-acetyl-glucosamine-6-sulfate (Figure 5.13, Figure 5.14 and Figure 5.15). Lactose-3-sulfate shares the 3-O-sulfo-galactosyl group found in sulfatide and mucin. Taken together, these data show that recognition is glyco-specific, but also specific to the position of the sulfoglycosylation, since galactose-6-sulfate did not inhibit binding.




E

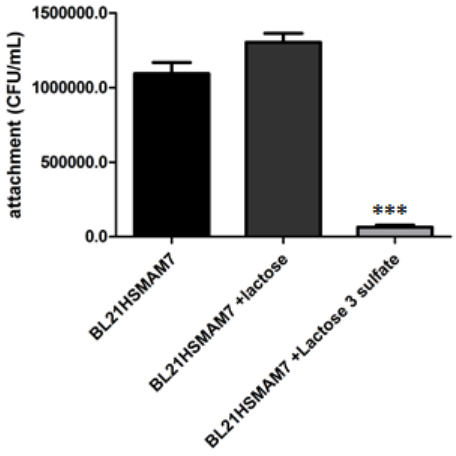



**Figure 5.13 *E. coli* BL21 expressing HSMAM7 binds to lactose 3- sulphate.** Caco-2 cells (A and B) or Hela cells (C and D) were infected with infection medium containing BL21 HSMAM7 at an MOI of 100 with 200 µM lactose 3 sulfate (A, C), or lactose (B, D) for 1 hour. Cells were fixed and stained with Hoechst for DNA (blue) and phalloidin for actin (green). Bar 10 µm. For attachment, 0.5% Triton X-100 was added to lyse cells, and serial dilutions were cultured on LB agar and incubated at 37 °C for 24 hours (E). Data represents the means ± SEM (n=3). Significance was determined using Student's two-tailed t test (*** p ≤0.001).

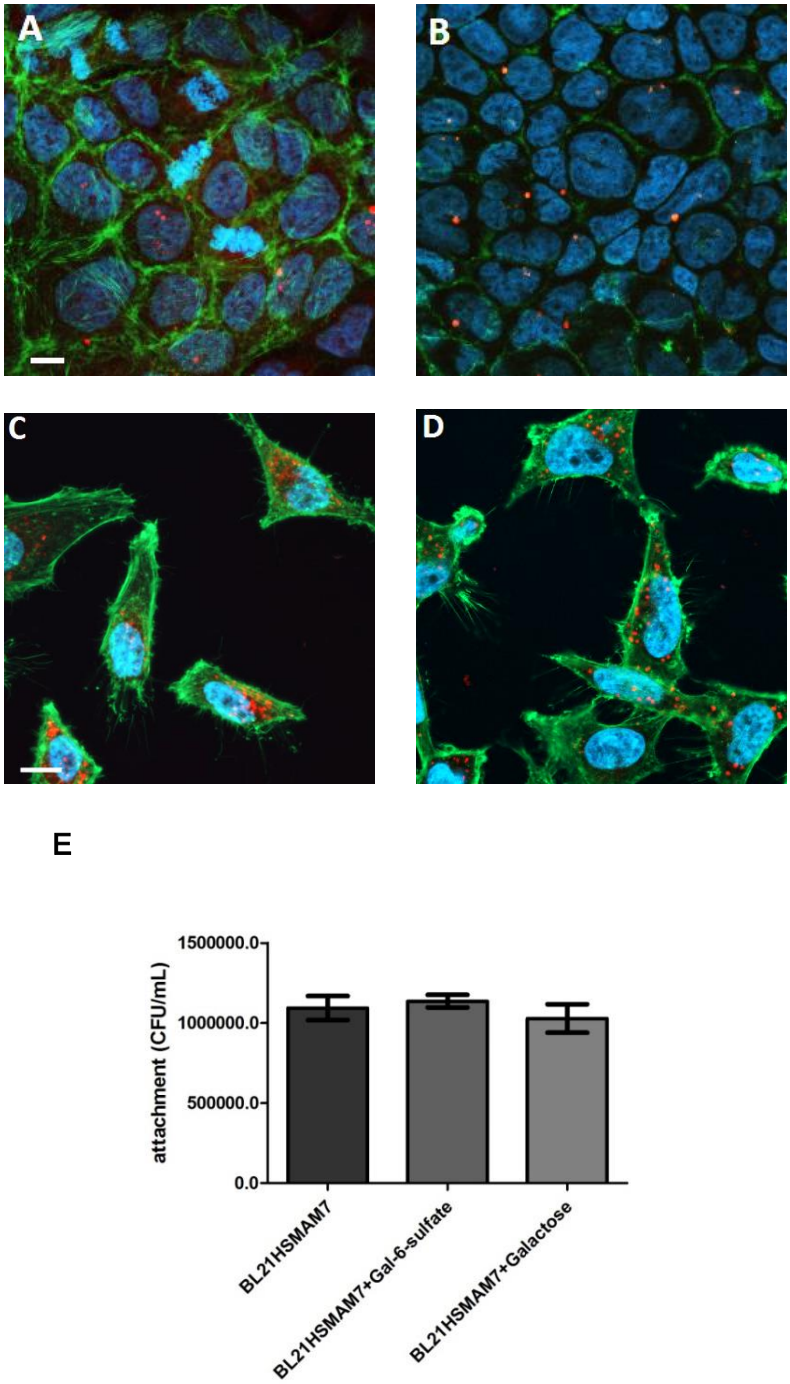
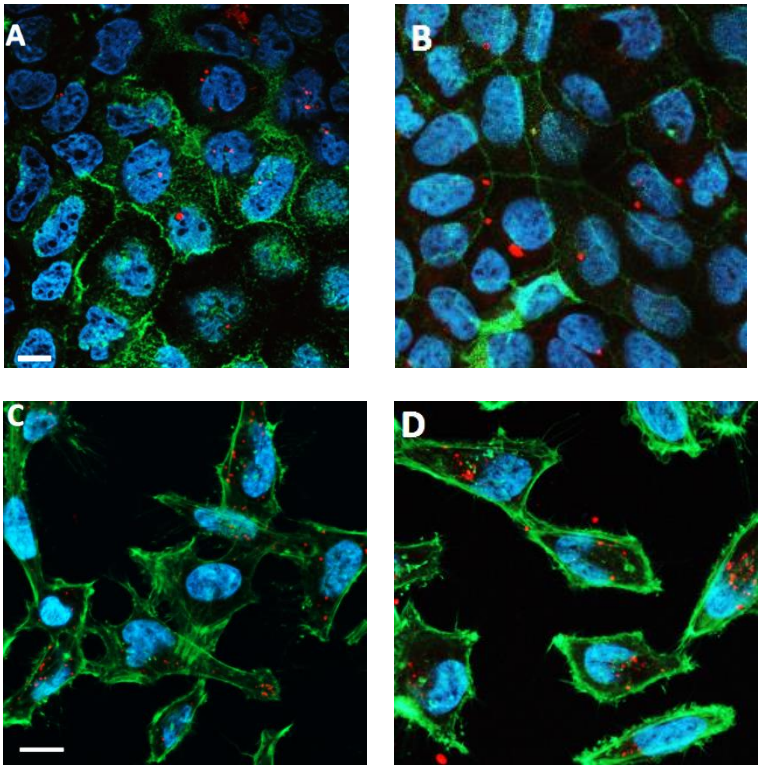



**Figure 5.14 *E. coli* BL21 expressing HSMAM7 does not bind to Gal-6-sulfate.** Caco-2 cells (A and B) or Hela cells (C and D) were infected with infection medium containing BL21 HSMAM7 at an MOI of with 200 µM D-Gal-6- sulfate (A, C), or D –Galactose (B, D) for 1 hour. Cells were fixed and stained with Hoechst for DNA (blue) and phalloidin for actin (green). Scale bar 10 µm. For attachment, 0.5% Triton X-100 was added to lyse cells, and serial dilutions were cultured on LB agar and incubated at 37 °C for 24 hours (E). Data represents the means ± SEM (n=3).




E

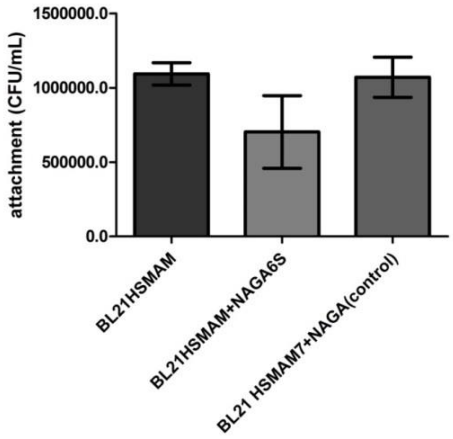



**Figure 5.15 *E. coli* BL21 expressing HSMAM7 does not bind to N-acetyl glucose amine 6 sulfate.** Caco-2 cells (A and B) and Hela cells (C and D) were infected with infection medium containing BL21HSMAM7 at an MOI of 100 with 200 μM NAGA6S (A, C), or NAGA (B, D). Cells were fixed and stained with Hoechst for DNA (blue) and phalloidin for actin (green). Scale bar 10 μm. For attachment, 0.5% Triton X-100 was added to lyse cells, and serial dilutions were cultured on LB agar and incubated at 37 °C for 24 hours (C). Data represents the means ± SEM (n=3).

## 5.10 The effect of *B. theta* on *E. coli* HS MAM7 binding to mucin

### 5.10.1 Quantification of *B. theta* sulfatase activity

Because of the highly sulphated mucins in the colon, mucins are resistant to bacterial degradation and thus protect the host from bacterial invasion. However, some bacteria use desulfation of mucins to increase their degradation by bacterial enzymes and infect host cells. The commensal *B. thetaiotaomicron* produces sulfatase and carbohydrate active enzymes which are involved in the carbohydrates metabolism. Bacterial sulfatase was quantified using enzymatic assay of sulfatase protocol from Sigma. The principle of this assay is that sulfatase enzymes catalyse the hydrolysis of sulfate ester of p - Nitrocatechol sulphate into 4-nitrocatechol which can be detected at OD 515 nm:

p - Nitrocatechol Sulphate + sulfatase **---------->** p - Nitrocatechol + sulphate

Brain heart infusion (BHI) broth containing 2.5 mg/ml mucin from porcine stomach type II was inoculated with *B. theta* wt and *B. theta* anSME mutant and incubated 48 hours at 37 ℃ under anaerobic conditions. Bacterial growth was centrifuged and serial dilutions from the supernatant were prepared using 0.2% NaCl. Then follow the protocol steps as mentioned in section (2.17). Sulfatase from *Helix promatia* (Sigma) at different concentrations was used to prepare the standard curve (Figure 5.16). The results revealed that the supernatant from *B. theta* wt contain sulfatase enzyme. Sulfatase concentration was reduced when the supernatant was diluted. No sulfatase was detected in the supernatant from *B. theta* anSME mutant (Figure 5.17).

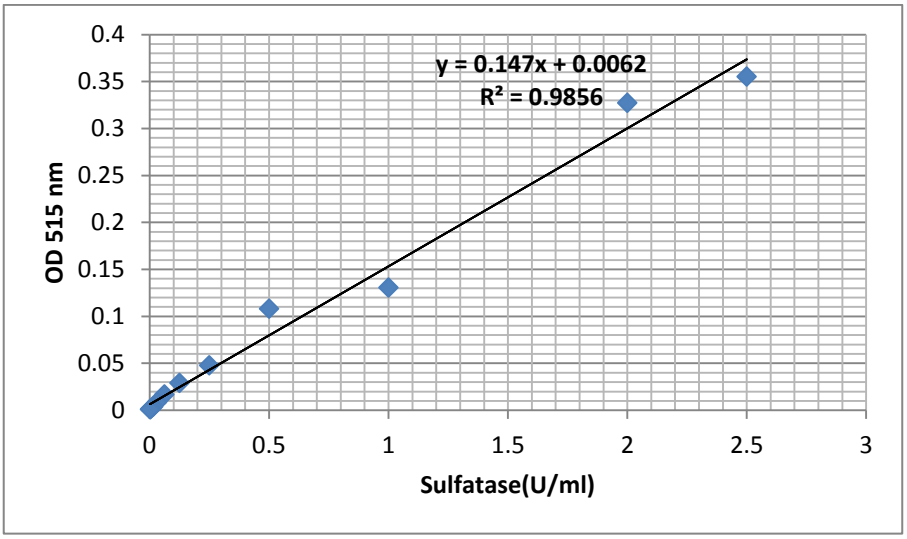



Figure 5.16 The standard curve to quantify sulfatase activity from *B. theta*.

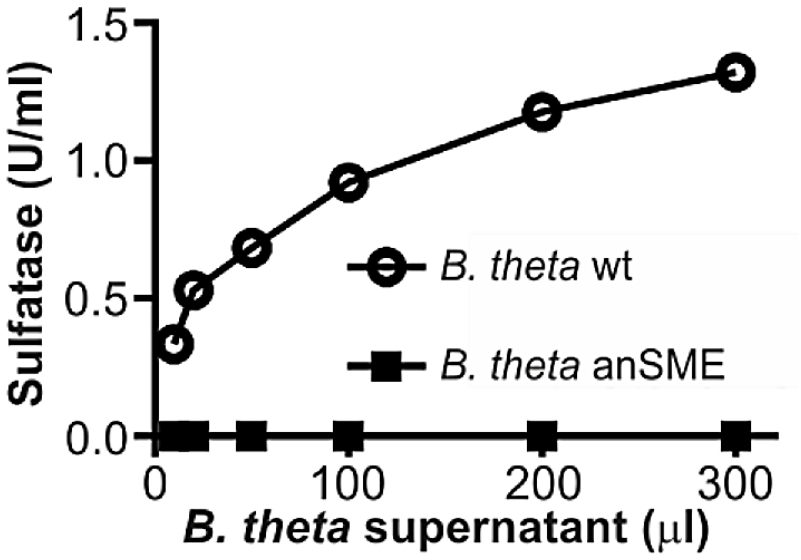



**Figure 5.17 *B. theta* wt sulfatase activity.** Sulfatase activity was quantified via hydrolysis of p - nitrocatechol sulphate into 4-nitrocatechol that was detected at OD 515 nm. Values are means ± SEM (n=3).

### 5.10.2 Bacterial attachment to HT-29MTX cells

#### 5.10.2.1 Immunocytochemistry (ICC) staining to visualize HT-29MTX mucin

Prior to investigating bacterial binding to mucin on HT-29MTX cells and investigating the effect of *B. theta* on this binding, immunocytochemistry (ICC) staining was used to visualize mucin on HT-29MTX cells. The results show the presence of mucus spots covering HT-29MTX cells and not continuous mucus layer. This indicates that the mucus layer does not cover the cells completely under the experimental conditions (Figure 5.18).

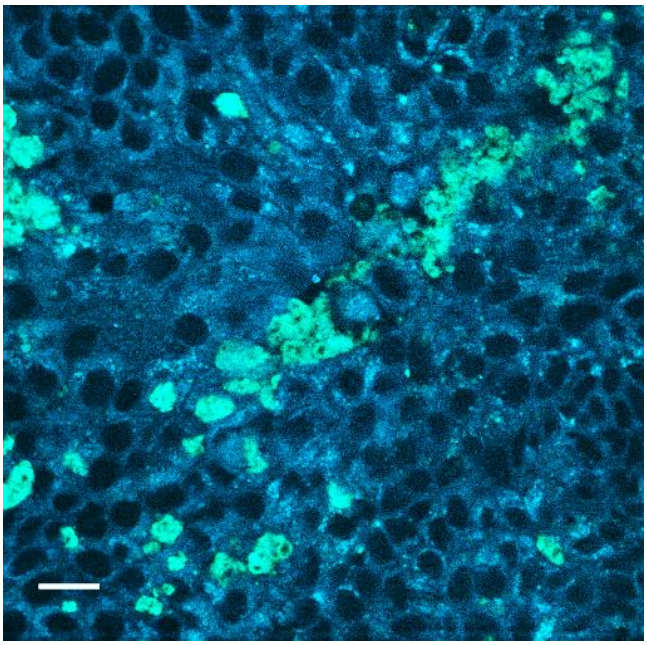


**Figure 5.18 Immunocytochemistry staining of HT-29MTX mucin.** The fixed cells were treated with 0.1% Triton X-100 and blocked with blocking buffer. Followed by adding Anti muc 2 antibody produced in mouse (1:400) and the the secondary antibody (anti mouse IgG FITC) (1:1000). ConA was added to stain cell membrane. The image shows cell membrane (blue colour) and mucin (green colour). Scale bar 20 μm.

### 5.10.2.2 Alcian Blue stain to visualize HT-29MTX sulfomucin

Alcian Blue stain was used in this study to stain mucins on HT-29MTX cells. Herein, Alcian Blue stain pH 1 was used to stain sulfomucins. Sulfomucins can be detected using Alcian blue at pH 1 or less because they are ionized at pH 1. The results show the presence of sulfomucin spots on HT-29MTX cells (Figure 5.19).

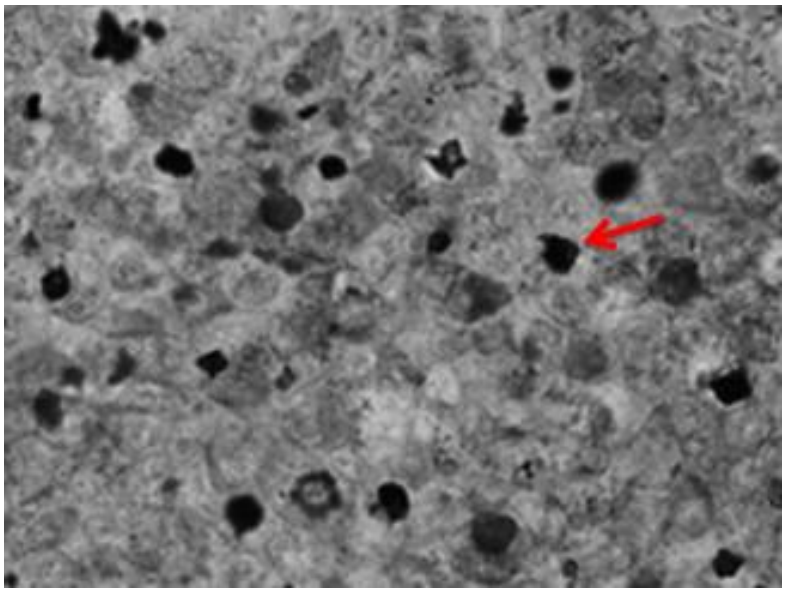


**Figure 5.19 Alcian Blue stain to visualize HT-29MTX sulfomucins.** The fixed cells were incubated with Alcian blue stain pH 1 for 30 minutes. Cells were washed and mounted using anti- fade reagent. The image shows sulfomucin spots (red arrow) on HT-29MTX cells.

### 5.10.2.3 Bacterial binding to mucin on HT-29MTX cells

In order to visualize bacterial adherence to HT-29MTX mucin, immunocytochemistry staining was used. HT-29MTX cells were seeded on coverslips in 24-well plate and incubated for three weeks. HT-29MTX cells were infected with BL21-HSMAM7 expressing mCherry and BL21 expressing mCherry at an MOI of 100 for 1 hour. After the infection, ICC staining was done. The images show that the tested bacteria adhere to the mucin spots on HT-29MTX cells (Figure 5.20 and Figure 5.21). The results indicate that the mucin is important for bacterial colonization due to mucin carbohydrates that might be used as a source of nutrient and/or as binding sites.

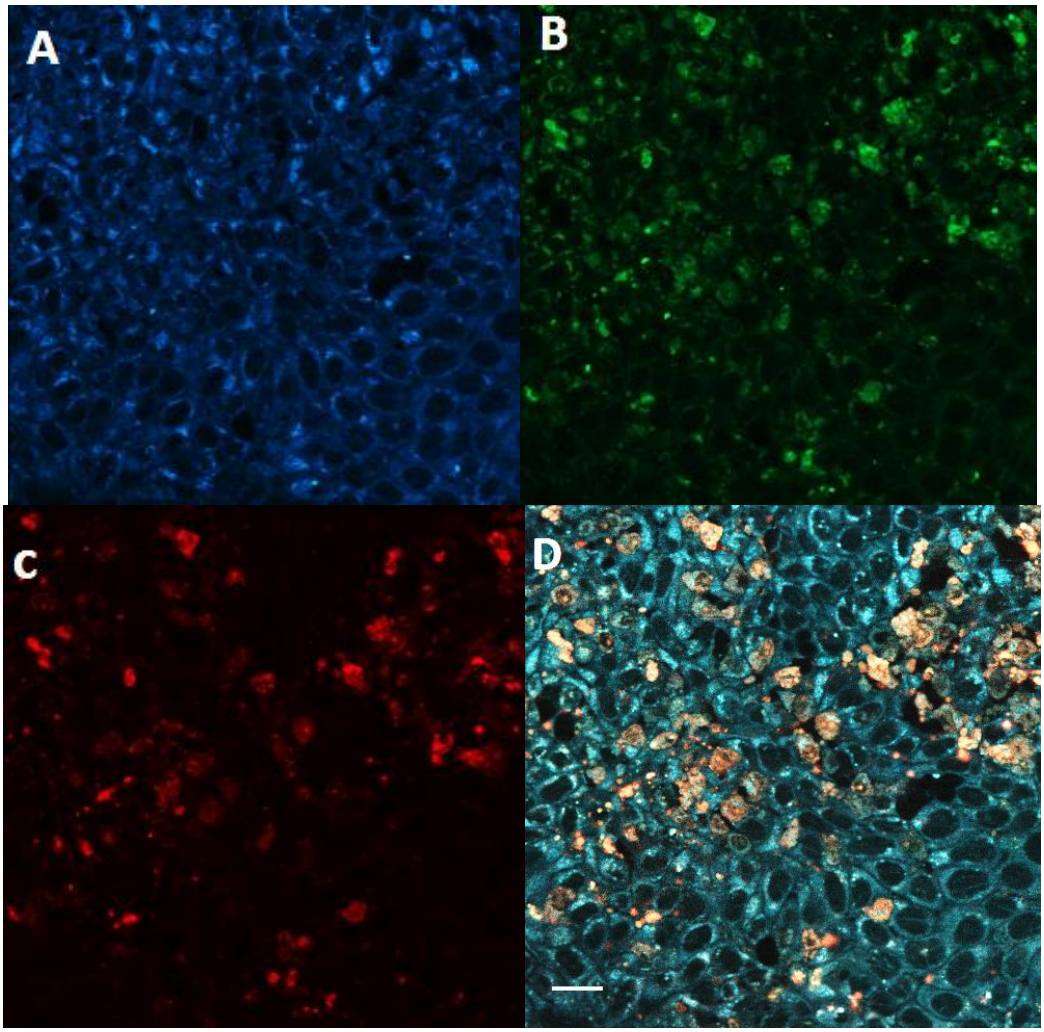



**Figure 5.20 Adherence of *E. coli* BL21 HSMAM7 to HT-29MTX mucin.** Cells were infected with *E. coli* HS (BL21-HSMAM7) expressing mCherry for 1 hour. Cells were fixed and stained for ICC. The image shows cell membrane (blue) (A), mucin (green) (B), *E. coli* BL21 expressing m Cherry (Red) (C) and merged image (D).Scale bar 20 μm.

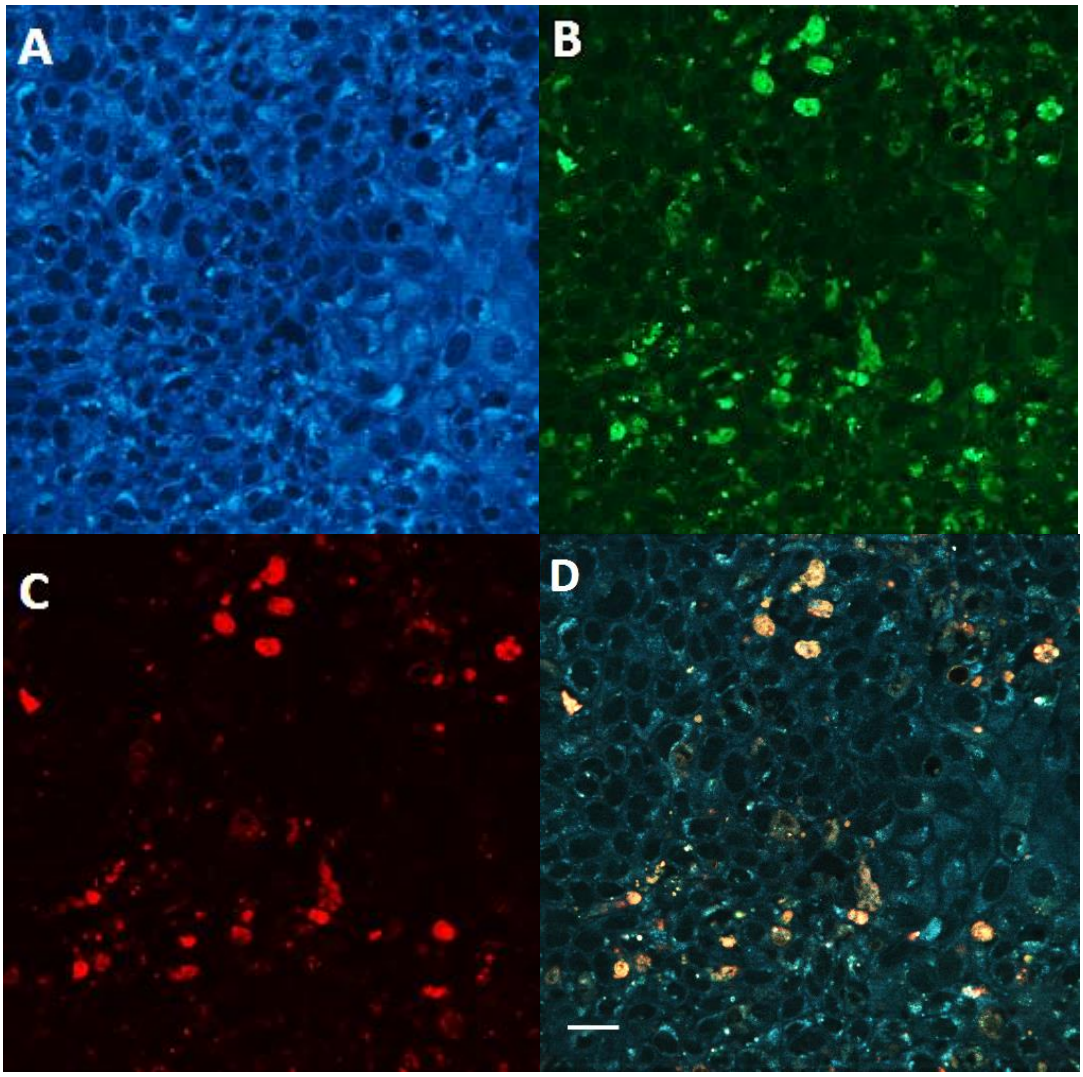



**Figure 5.21 Adherence of *E. coli* BL21 to HT-29MTX mucin.** Cells were infected with *E. coli* BL21 expressing mCherry for 1 hour. Cells were fixed and stained for ICC. The image shows cell membrane (blue) (A), mucin (green) (B), *E. coli* BL21 expressing m Cherry (Red) (C) and merged image (D). Scale bar 20 μm.

To study the effect of *B. theta* on the binding of *E. coli* HSMAM7 to mucin, indirect ELISA and transmigration assays were set up using a purified mucin instead of using HT-29MTX cells. The reason is because the mucus layer does not cover the cells completely under the experimental conditions. Attempts to optimize the protocol to obtain HT-29MTX cells with a more even mucin layer formation by mixing HT-29MTX cells in different ratios with Caco-2 cells, introducing movement, or by adding endogenous mucin for the last day of the cell growth all failed.

### 5.10.3 *B. theta* inhibits HSMAM7 binding to mucin

According to my previous results, sulfate is important for the binding of HSMAM7 to mucins. The aim of these next experiments was to investigate the role of sulfatase produced from *B. theta* in the inhibition of HSMAM7 binding to the mucin. An indirect ELISA assay was used and involved testing the interaction between GST-HSMAM7 and mucin in the presence of live *B. theta*. Different volumes of *B. theta* wt or *B. theta* anSEM mutant growth at OD 0.6 was added to the immobilized mucin in 96-well microtiter plate and incubated for different time points. Bacterial growth was discarded and the wells were washed once using washing buffer followed by blocking step. 125 µg/ml of HSMAM7 protein was added and incubated for 2 hours with gentle shaking. GST–mouse antibody, then secondary anti mouse IgG–HRP antibody were added, followed by ECL detection. The results revealed that incubation of immobilized mucin with the wild type *B. theta* resulted in inhibition of HSMAM7 binding to mucin and this inhibition was increased with increasing the incubation time of the wild type *B. theta* with mucin. However, incubation of

mucin with the sulfatase-deficient *B. theta* anSME mutant resulted in loss the binding of HSMAM7 to mucin after extended incubation time (Figure 5.22). These results indicate that binding of HSMAM7 to mucin depends on the presence of sulfoglycan on mucin. Thus, desulfation of mucin by sulfatase-secreting *B. theta* resulted in inhibition of the binding of HSMAM7 to mucin.

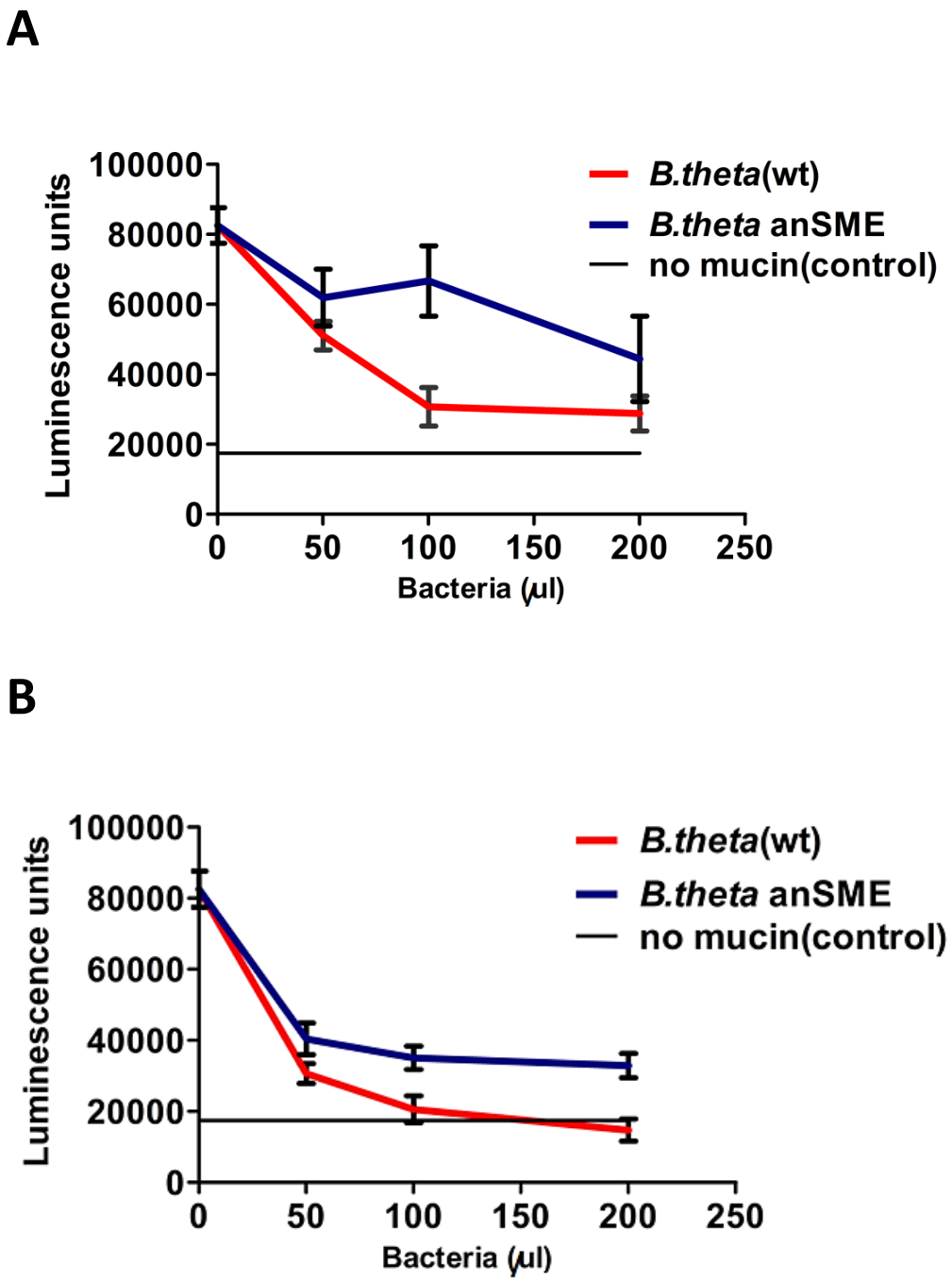



**Figure 5.22 ELISA to detect the effect of *B. theta* on the binding of HSMAM7 to mucin.** Different volumes of *B. theta* wt or *B. theta* anSME mutant growth was incubated an aerobically with the immobilized mucin for 48 hours (A) and 72 hours (B). GST-HSMAM7 was added at concentration of 125 µg/ml. Anti GST–mouse and anti- mouse IgG -HRP antibodies were used. Followed by ECL detection. The luminescence output is plotted against bacterial volumes. Results shown are means ± SEM from three independent experiments.

### 5.10.4 Desulfation of the mucin layer increases bacterial transmigration

In a healthy colon, mucus contains heavily sulphated mucins that protect them from bacterial degradation that result in the infection of the epithelial cells. Bacterial attachment to mucin facilitates their removal and inhibits their colonizing on epithelial cells. Herein, the effect of mucin sulfation on *E. coli* transmigration through an artificial mucus barrier was investigated. 100 µl of 10 mg/ml mucin was treated with 100 µl of 1U/ml *Helix promatia* sulfatase enzyme and incubated at room temperature for 24 hours with gentle shaking. Trans-well filters were either coated with 50 µl of 10 mg/ml mucin, desulfated mucin or un coated and incubated at 4 °C for overnight, then placed onto 24 well plate containing 600 µl colourless DMEM. Followed by adding 100 µl of $10^{6}$ *E. coli* BL21 expressing HSMAM7 or BL21 (control) growth to the trans-well filter and incubated for 2 hours. Serial dilutions were prepared from the filtrate and cultured on LB agar medium and incubated at 37 °C for 24 hours. Followed by counting bacterial colonies. The results revealed that *E. coli* rapidly migrated through uncoated transwells and motility was unaffected by the presence or absence of HSMAM7. Coating of transwells with a mucin gel inhibited the transmigration of *E. coli*, but retained *E. coli* expressing HSMAM7 more efficiently, suggesting the mucin layer constitutes a physical barrier but its effect is enhanced through specific interactions between mucin and the HSMAM7 adhesin. Treatment of mucin with *Helix pomatia* sulfatase increased the transmigration of *E. coli* expressing HSMAM7 to levels seen with BL21 control bacteria, while mucin desulfation had no significant effect on the transmigration of *E. coli* without HSMAM7 (Figure 5.23). These results suggest that

bacteria were trapped by mucin that acts as a physical barrier to inhibit bacterial invasion and desulfation of mucin makes it more susceptible and aids bacterial invasion.

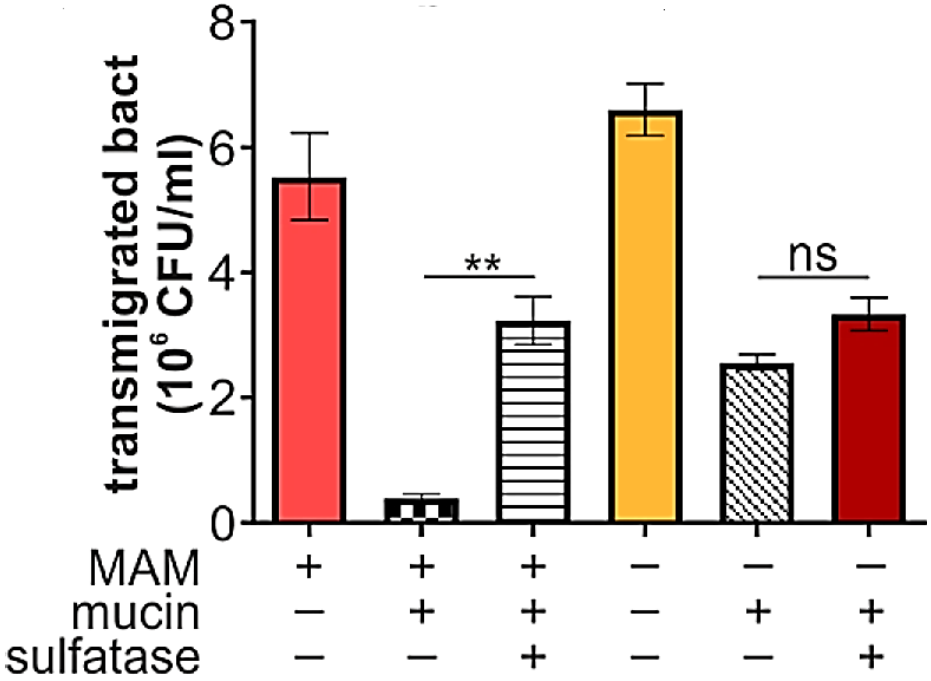



**Figure 5.23 Recovery of *E. coli* transmigrated through the mucin and desulfated mucin coated transwells.** *E. coli* BL21HSMAM7 or BL21 were added to the top compartment of a transwell either coated with type II mucin (mucin +) or left uncoated (mucin -). Mucin used for coating was either desulfated with sulfatase from *Helix pomatia* (sulfatase +) or left untreated (sulfatase -) prior to coating transwells. Bacterial transmigration (recovery from bottom well following 2 hours of incubation at 37 °C) was measured by serial dilution and plating. Values represent means ± SEM (n=3). Significance was determined using two tailed student's t test (** $p \leq 0.01$).

### 5.10.5 *B. theta* desulfates and degrades mucin and aids bacterial transmigration

In order to survive and compete with other microorganisms, *B. theta* has mucin degradation enzymes that are involved in the metabolic breakdown of carbohydrates either from dietary sources or glycoproteins in the mucus. *B. theta* uses mucus glycoproteins when dietary glycan sources are unavailable. Because mucins are highly sulphated in the colon, *B. theta* releases sulfatase enzymes to desulfate them prior to degradation. Herein, the effect of *B. theta* on bacterial transmigration using transmigration assay was investigated. 100 µl from *B. theta* wt or *B. theta* anSME mutant supernatant was incubated with mucin at room temperature for 24 hours. 50 µl of the treated mucin was added into trans-well filter and incubated at 4 ℃ overnight. Followed by adding 100 µl of $10^6$ *E. coli* BL21HSMAM7 growth to the coated trans-well filters and incubated at 37 ℃ for 2 hours. Serial dilutions were prepared from the filtrate and cultured on LB agar medium. The results indicate that the number of CFUs of *E. coli* BL21HSMAM7 that migrated through the treated mucin coated filters with *B. theta* wt or *B. theta* anSME mutant was significantly higher than the number of CFUs that passed through the mucin coated filters (Figure 5.24). The results indicate that desulfation and mucin degradation by *B. theta* can accelerate transmigration of *E. coli* expressing MAM7, which otherwise get stuck on the mucin.

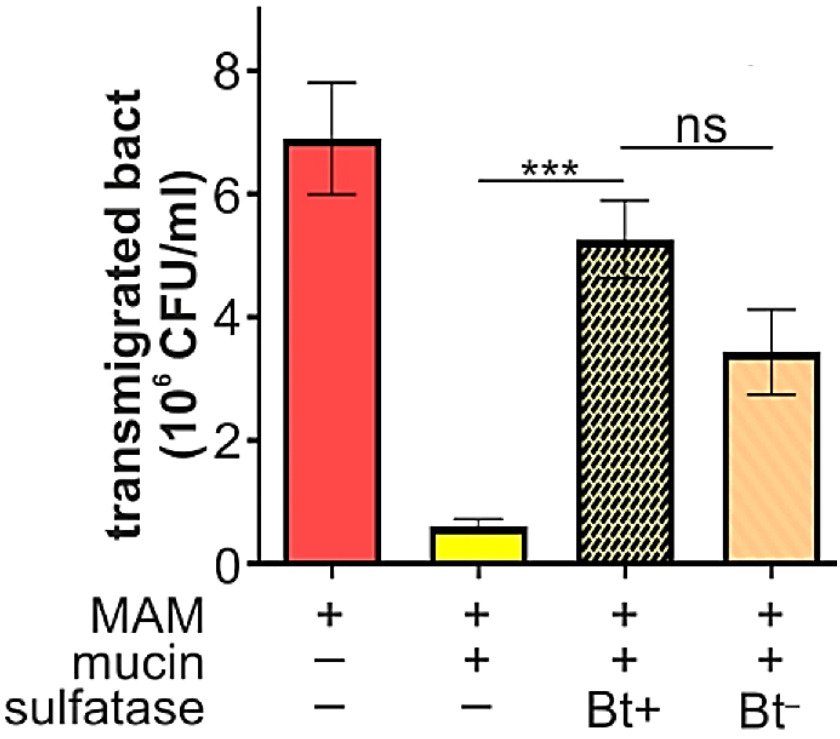



**Figure 5.24 Recovery of *E. coli* BL21-HSMAM7 transmigrated through the desulfated and degraded mucin coated transwells.** *E. coli* BL21HSMAM7 was added to the top compartment of a transwell either coated with type II mucin (mucin +) or left uncoated (mucin -). Mucin used for coating was either treated with supernatant from wild type *B. theta* (Bt+) or from the sulfatase-deficient *B. theta* (anSME) mutant (Bt-) prior to coating transwells. Bacterial transmigration (recovery from bottom well following 2 hours of incubation at 37 °C) was measured by serial dilution and plating. Values represent means ± SEM (n=3). Significance was determined using two tailed student's t test (*** $p \leq 0.001$).

## 5.11 Discussion

The aim of this work was to study the interactions between HSMAM7 protein and host ligands in detail, and identify the biochemical basis for adhesin-ligand interactions. Previously, it has been reported that the well characterized MAM7 from *Vibrio parahaemolyticus* recognizes the host lipid phosphatidic acid and the extracellular matrix protein fibronectin as receptors (Krachler et. al., 2011; Krachler and Orth, 2011). Herein, the results revealed that MAM7 from the commensal bacteria *E. coli* strain HS (HSMAM7) uses the host lipid sulfatide for adherence. Sulfatide is an abundant host cell lipid, accounting for 4 % of total membrane lipid content (Ishizuka, 1997). Sulfatides participate in a wide range of cellular functions; including protein trafficking, cell adhesion and aggregation, as well as immune responses ((Xiao et. al., 2013; Takahashi and Suzuki, 2012; Takahashi and Suzuki, 2015). Additionally, both viruses and bacteria have been found to utilize sulfatides as host receptors for adherence. It has been revealed that sulfatides serve as a receptor for *P. aeruginosa* (Xia et. al., 2007), *Bordetella pertussis* (Julie et. al., 2007), *H. pylori* (Kamisago et. al., 1996; Kobayashi et.al., 2009), enterotoxigenic *Escherichia coli* (ETEC) (Janson et. al., 2009) and Influenza A virus (Takahashi and Suzuki, 2015). .

Pull-down experiments with purified recombinant GST-HSMAM7 protein as a bait identified five host epithelial proteins specifically interacting with the adhesin; perlecan, mucin, fibronectin, collagen IV and laminin. All identified binding partners are extracellular proteins, and have been reported to be heavily glycosylated and sulfoglycosylated (Hummel et. al., 2007; Colburn et.al., 1987), or to bind to sulfated ligands, such as in the case of laminin, which binds to sulfatides (Roberts et.al., 1985; Baron et.al., 2014). It has

been found that a variety of bacteria utilize these proteins as host receptors for adherence (Bucior, et.al., 2012; Oh et. al., 2016; Longhi et. al., 2009; Bober et. al., 2010; Tailford et. al., 2015).

The identified receptors are either localized at the epithelial surface or at the basement membrane. Therefore, further work was done on the interaction between HSMAM7 and mucin. Mucin is a major component of the mucosal layer of the gastrointestinal tract and is one of the earliest targets faced by bacteria entering the gastrointestinal tract. The interactions between microbes and mucin oligosaccharides have been shown to facilitate bacterial clearance and to inhibit colonization of epithelial cells (Martin Sosa et. al., 2002; Ruiz Palacios et. al., 2003). The deficiency in mucin results in a severe disease and intestinal inflammation (Bergstrom et. al., 2010; Van der Sluis et. al., 2006).

A variety of bacteria use mucin specifically as a binding site such as *P. aeruginosa* (Lillehoj et. al., 2002), *S. aureus* (Shuter et. al., 1996) and *H. pylori* (Linden et. al., 2004). The specificity in the binding could be because of the similarity in structure between secreted mucins and the glycoproteins on the cell surfaces (Ryan et. al., 2001). The current study involves characterization of the binding of HSMAM7 protein to mucin. Herein, the results revealed that HSMAM7 protein interacts with mucin in a sulfation-dependent manner. The interaction of HSMAM7 protein with sulfated mucin competitively inhibits attachment of *E. coli* expressing HSMAM7 to epithelial cells, and desulfation of mucin using purified sulfatase decreased the binding of HSMAM7 protein to mucin. In addition, desulfated mucin also lost the ability to inhibit *E. coli* expressing HSMAM7 from attaching to the epithelial surface.

Further analysis of competitive inhibition with sugars or sulfosaccharides showed that HSMAM7 protein recognize 3-O-sulfo-galactosyl moiety, found in both mucin and sulfatide. The identification of 3-O-sulfo-galactosyl as a common binding moiety for MAM7 binding to both host lipids and proteins has not been reported previously. Similar results have been shown when the 47-kDa adhesin from *Lactobacillus reuteri* JCM1081 binds to mucin and sulfatide via the 3-O-sulfo-galactosyl moiety (Nishiyama et. al., 2013). Sulfation of colonic mucins has been shown to play a protective role in animal models of colitis (Tobisawa et. al., 2010), and reduced sulfation has been associated with impaired barrier function and with infectious disease (Dawson et. al., 2009; Xia et. al., 2005).

It is known that gut microbiota contribute in the protection of the host epithelial cells by inhibition of pathogens colonization and activates the immune response. However, abnormalities in microbiota composition have been contributed in human diseases (Chow and Mazmanian, 2010). The competition for polysaccharides is considered to be an important factor that shape microbiota composition. Thus some bacterial species develop strategies to compete with others for the host glycans when the dietary sources are an available such as producing sulfatase that increases glycans susceptibility to be degraded by bacterial glycosidases (Tsai et.al., 1995). However, degradation of the mucus layer by the endogenous leads to damage of the mucus layer and helps pathogens to cause infections (Gazzaniga and Kasper, 2016; Sonnenburg and Sonnenburg, 2014; Katharine et. al., 2013). The commensal an aerobic bacterium *B. thetatiomicron* compete with others for the sulphated glycans by producing sulfatase enzyme. Sulfatases are essential for mucosal foraging, and have a direct impact on the chemical composition and architecture of the mucus layer (Benjdia et. al., 2011; Desai et. al., 2016).

Enhanced sulfatase activity by the intestinal microbiota has been detected in fecal samples from patients suffering ulcerative colitis (Corfield et.al., 1993). Herein, the findings suggest that the sulfation of mucin effects specific bacterial adherence to, and thus retention of bacteria by the mucin gel. However, desulfation and degradation of mucin by *B. thetatiomicron* can act to modulate the adherence of commensals to mucin, and may directly affect their retention by the mucus barrier. A limitation in predicting *in vivo* modulation of the mucus barrier effect from our *in vitro* assays is that the physiological mucus layer has components in addition to mucin, which contribute to the barrier's structure and function. Additionally, mucus viscosity is not uniform, since the barrier consists of two layers; a loose outer layer and a denser, cell-adherent layer that is largely free of microbes in the healthy intestine. This complexity cannot be fully reproduced by the assays mentioned this study. Because the work showed in this chapter focused on the biochemical determinants of HSMAM7-receptor interactions, further work is suggested to study the effect of *B. thetatiomicron* sulfatase on commensals binding to mucin in a physiologically relevant model. Such experiment will determine if the observed changes in adherence in response to desulfation described herein may have an impact on bacterial localization and mucosal inflammation *in vivo*.

# Chapter 6. General discussion and recommended future work

Antibiotic resistance is a serious problem in the treatment of infectious diseases. Antibiotics are highly effective against bacteria, as they kill or prevent bacterial growth by targeting processes which are critical for bacterial proliferation or physiology. However, bacteria have developed strategies to resist the threat of antibiotics. Therefore, looking for alternatives to antibiotics to treat bacterial infections is indispensable.

Since bacterial attachment to host cells is a critical step of bacterial infections, targeting bacterial binding to host cells is a potential strategy to treat bacterial infections without killing them.

Because the commensal *E. coli* strains have the ability to bind to host without harming the host, and they can provide a barrier against infections by pathogenic bacteria via the competition for nutrients, binding sites and secretion of antibacterial agents known as bacteriocins that kill pathogens, they have been used to compete with pathogens and inhibit their infections. Examples include using the commensal *E. coli* strain Nissle 1917 to out compete pathogenic bacteria causing intestinal infections (Hancock et.al, 2010), and using the commensal *E. coli* strain HS to compete with pathogenic *E. coli* O157:H7 for nutrients that are important for bacterial colonization and inhibit infections (Maltby et.al., 2013).

In this thesis, there were several investigations based on the interactions between the adhesin Multivalent Adhesion Molecule (MAM7) from the commensal *E. coli* strain HS with

the host cells. MAM7 is an outer membrane protein found in most Gram negative bacteria and contributes in bacterial attachment in the early stages of infections.

The first chapter of the results involves the identification and characterization of a MAM7 ortholog (accession number YP_001458619) from the commensal *E. coli* strain HS. I demonstrated that *E. coli* HS contain a MAM7 ortholog consisting of 879 amino acids with an identity of 35.78% to the well characterized MAM7 from *V. parahaemolyticus*. Previously, it had been shown that several Gram negative pathogens such as *Y. pseudotubercolusis*, *V. cholera*, enteropathogenic *E. coli*, and *Shigella* sp harbour MAM7 (Krachler et. al., 2011; Mahmoud et. al., 2016), but orthologs from commensal bacteria had not been studied.

The potential role of MAM7 from the commensal *E. coli* HS in bacterial attachment to host cells was investigated. An engineered bacterium expressing HSMAM (BL21-HSMAM7) and polymer beads coupled to the purified recombinant HSMAM were designed and their attachment ability was investigated. I demonstrated that both BL21-HSMAM7 and beads coupled to HSMAM7 were able to bind to host cells. HSMAM7 confers adhesive properties to these agents to bind to host cells. Previously, it had been shown that BL21 expressing MAM7 from the pathogenic bacteria *V. parahamolyticus* and beads coupled to MAM7 are able to bind to host cells (Krachler et. al., 2012; Lim et. al., 2014). MAM7 contributes to bacterial attachment and deleting MAM7 from bacteria affects bacterial binding. It has been shown that the attachment of a variety of pathogenic bacteria such as *V. parahaemolyticus, Y. pseudotuberculosis*, *V. cholera*, or enteropathogenic *E. coli* to host cells was inhibited by disruption of their MAM7 gene (Krachler et. al., 2011).

The second results chapter involves investigating the ability of the MAM7 from *E. coli* HS to compete with pathogenic bacteria and inhibit infection. Herein, I used BL21-HSMAM7 and beads coupled to HSMAM7 depend on their ability to attach to host cells. In this study, *S. aureus, E. faecalis* and *P. aeruginosa* were used due to their clinical importance and their role in wound and tissue infections. I demonstrated that beads coupled to HSMAM were able to protect host cells from bacterial infections due to their ability to bind to host cells and compete with pathogens for the attachment sites. However, the protection of host cells using BL21-HSMAM7 does not solely depend on binding sites, but other factors also affected the competition between BL21-HSMAM7 and pathogens, and these factors depended on the bacteria involved. I found that the competition between *S. aureus* and BL21-HSMAM7 is caused by both attachment competition and interspecies antagonism. It is well known that *S. aureus* produces antimicrobial agents such as bacteriocin (Al-Gosha'ah et. al., 2014). It has been shown that bacteriocins from Gram positive bacteria have activity against Gram positive as well as some of Gram negative bacteria (Hsu and Wiseman, 1971; Ahmad et.al., 2003). I found that the co incubation of *S. aureus* with the BL21 in liquid medium resulted in inhibition of BL21 growth. This inhibition could be resulted by the production of bacteriolytic enzymes or bacteriocins by *S. aureus*, as the supernatant of an *S. aureus* growth caused inhibition zone on *E. coli* BL21 growth. The results showed that the secretion of inhibitory agents by *S. aureus* affects it's potential to adhere and cause cytotoxic effects, but does not affect BL21-HSMAM7 inhibitory activity, which was confirmed by visualization of the treatment.

The competition between BL21-HSMAM7 and *E. faecalis* is both for attachment sites and for nutrients. The competition between BL21-HSMAM7 and *P. aeruginosa* is due to the competition for the binding sites alone. Previously, beads-coupled MAM7 and live bacteria expressing MAM7 inhibited the adherence and infections of *P. aeruginosa* (Krachler et. al., 2012). Beads-coupled MAM7 was used to inhibit *S. aureus* (MRSA) that cause wound infections without affect the host cellular functions (Hawley et. al., 2013). *S. aureus* bind with high affinity to host fibronectin via the surface adhesin fibronectin binding protein A (FnBPA) (O'Neill et. al., 2008). MAM7 interact with the host cell lipid, phosphatidic acid in high affinity and use fibronectin as a co-receptor to facilitate this binding (Krachler and Orth, 2011). This means that the stable interaction of beads-coupled MAM7 with host cells can impede the attachment of *S. aureus* that use fibronectin as a binding site without affecting fibronectin related cellular functions (Hawley et. al., 2013). BL21 expressing MAM7 from *V. parahaemolyticus* inhibited the infections of a broad range of pathogenic bacteria (Krachler et. al., 2011).

Targeting MAM7 and using MAM7 based inhibitors seems to be a potential way to inhibit bacterial infections. In case of using an engineered live bacterium *E. coli* (BL21-HSMAM7) to displace pathogenic bacteria *in vitro,* the competition depends on the bacteria involved, and includes different factors such as competition for nutrients, the competition for the binding sites or the production of antimicrobial agents. However, the competition between beads coupled to HSMAM7 with pathogens depends on the binding sites only. Thus, using BL21-HSMAM may increase the displacement effect over that of beads coupled to HSMA7. In contrast, BL21-HSMAM7 could be used *in vivo* to compete with enteric pathogenic bacteria but not for treating wound infections. Since

using them for treating wounds might result in inflammatory responses and thus, delay the treatment. Therefore, using beads coupled to MAM7 to deliver MAM7 to the site of infection could be a potential approach to treat burn wound infections (Krachler et. al., 2012; Huebinger et. al., 2016).

Herein, the recommended future work is using MAM7 from the commensal *E. coli* HS *in vivo*. For example it would be interesting to treat skin infections with beads coupled to HSMAM7, in addition to inhibit the infections caused by food- borne pathogens using BL21 expressing HSMAM7. Previously, MAM7 from *V. parahaemolyticus* has been used to treat bacterial infections *in vivo*. It has been reported that beads coupled MAM7 has the potential to treat burn wounds infected with multi drug resistant *P. aeruginosa* due to the competition with the pathogen for receptors on the host. The success of this treatment based on that MAM7 does not affect wound healing and does not enhance inflammatory response (Huebinger et. al., 2016). However, using *V. parahaemolyticus* MAM7 to compete pathogens *in vitro* has revealed to induce cellular signaling in intestinal epithelial cells and break of intestinal cell junctions due to the interactions with host cells phospatidic acid (Lim et. al., 2014). Since commensal *E. coli* are capable of binding to epithelial cells without any illness and HSMAM7 binds to sulfatid on cell membrane with no affinity to phosphatidic acids, this means that the host cell signalling will be different from those induced by *V. parahaemolyticus* MAM7. Furthermore, HSMAM7 has the ability to adhere to host, compete with pathogens and inhibit their infections *in vitro*. Thus, using MAM7 protein from a commensal bacterium may have a good efficacy as antibacterial agent than *V. parahaemolyticus* MAM7 to treat bacterial infections *in vivo*.

The third results chapter involves investigating of interactions between MAM7 from the commensal *E. coli* HS with host receptors. I demonstrated that HSMAM7 was able to bind to the host lipid sulfatide. Sulfatide is a glycolipid that has been used by a variety of bacteria to bind to host cells such as *Helicobacter pylori* (Kamisago et. al., 1996 Kobayashi et. al., 2009), *P. aeruginosa* (Xia et. al., 2007) and enterotoxigenic *Escherichia coli* (ETEC) (Jansson et. al., 2009). In addition to the binding to sulfatide, HSMAM7 was able to bind to the extracellular matrix constituents such as, collagen IV, fibronectin, perlecan and laminin. It is well known that extracellular matrix components are attractive receptors exploited by bacteria for the binding to host cells (Vazques et. al., 2011; Singh et. al., 2012). Previous work on MAM7 from *V. parahaemolyticus* revealed that MAM7 binds to the host cell lipid phosphatidic acid and fibronectin (Krachler and Orth, 2011).

The binding of *V. parahaemolyticus* MAM7 with phosphatidic acids on the host membrane results in the induction of host cellular signalling that involve actin rearrangements, the activation of the GTPase RhoA and disruption of the epithelial barrier which aid bacterial invasion of the underlying tissue (Lim et. al., 2014). Because HSMAM7 does not bind to the host cell phosphatidic acids, thus host cell signalling will be different from those induced by *V. parahaemolyticus* MAM7. Therefore, more work is recommended to study how host signalling is affected by the binding of HSMAM7 to the host cell membrane lipid, sulfatide.

In the same chapter, the binding ability of HSMAM7 to mucins was investigated in more depth. I demonstrated that HSMAM7 binds to mucins via a 3-O-sulfo-galactosyl moiety. It is well known that the sulfation of mucin occurs on the 3-position of Gal and the 6-position of GlcNAc of carbohydrates. Interestingly, sulfate position in the sulfatide (3-O-

sulfogalactosylceramide) also occurs at the 3-position of galactose. Thus, I demonstrated that HSMAM7 binds to mucin and the host lipid sulfatides via a common 3-O-sulfo-galactosyl moiety. Furthermore, the investigation was expanded to test whether mucin sulfation was required for the interaction between HSMAM and mucin. I demonstrated that mucin desulfation using sulfatase enzyme decreased the binding of HSMAM to mucin.

In a healthy colon, mucus contains heavily sulphated mucins that protect the mucus from bacterial degradation. Under some conditions, such as when *B. thetaiotaomicron* desulfate and degrade mucosal glycans when the dietary sources of glycans are unavailable (Benjdia et. al., 2011), mucus becomes more susceptible to degradation and the host more susceptible to infection. In the current study, I demonstrated that desulfation of mucin by the sulfatase producer *B. thetaiotaomicron* that inhabit the same niche as the commensal *E. coli,* resulted in inhibition of the binding of HSMAM7 to mucin. This means that mucin's role is to trap but also bind to the commensal *E. coli* HS, and inhibition of this binding by desulfation increases bacterial attachment to the epithelial surface which has been linked to increase inflammation and disease (Bergstrom and Xia, 2013). Further work is recommended to evaluate the effect of sulfatase-produced by *B. thetaiotaomicron* on commensal adherence in a physiologically relevant *in vivo* model.

In conclusion, since the inhibitors based on MAM7 from the commensal *E. coli* HS have the ability to bind to host cells, compete pathogenic bacteria and inhibit infections, HSMAM7 based inhibitors could be considered as a possible opportunity to treat bacterial infections; especially, in case of antibiotics resistant bacteria. In addition, the binding of HSMAM7 to sulfated mucin and the importance of sulfation for this binding may provide preliminary evidence of how inflammatory gut disease could happen and targeting

sulfatase producing bacteria, i.e. *B. thetaiotaomicron* may provide a possible approach for understanding and treating gut infectious diseases.

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