School of Pharmacy

Role of Active Efflux Proteins in the Defence of the Gastrointestinal Tract from Excessive Colonisation of Pathogenic Microflora

Aparna Warrier

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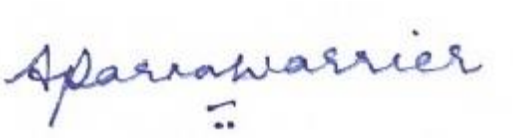
Declaration

To the best of my knowledge and belief this thesis contains no material previously published by any other person except where due acknowledgment has been made.

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university.

Signature: 

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List of Abbreviations

Abbreviation	Full Form
µM	Micro molar
ºC	Degrees Celsius
A-beta	Amyloid-beta
AMP	Antimicrobial peptide
AUC	Area under the curve
B.megaterium	*Bacillus megaterium*
BBB	Blood brain barrier
BCRP	Breast cancer resistance protein
BSA	Bovine Serum albumin
C.albicans	*Candida albicans*
CCL20	Chemokine Ligand-20
CCR6	Chemokine receptor type-6
cDNA	Complementary DNA
CEA	Carcinogenic embryo antigen
CFTR	Cystic fibrosis transmembrane conductance regulator
CFU	Colony forming unit
CMV	Cytomegalovirus
CNS	Central nervous system
CYP	Cytochrome P

Abbreviation	**Full Form**
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco minimal essential medium
dNTP	Deoxynucleotide
E.coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic reticulum
FCS	Foetal Bovine Serum
FXR	Farnesoid X receptor
GPCR	G-protein coupled receptor
H.pylori	*Helicobacter pylori*
hAPP	Human amyloid precursor protein
HBD-1	Human beta defensin-1
HBD-2	Human beta defensin-2
HBD-3	Human beta defensin-3
HBD-4	Human beta defensin-4
hCAP-18	Human cationic antimicrobial peptide
HD-5(α-defensin)	Human defensin
HNP-1	Human neutrophil protein-1
HNP-2	Human neutrophil protein-2
HNP-3	Human neutrophil protein-3
IC-50	Inhibitory concentration-50
IL-1β	Interleukin-1β
IL-6	Interleukin-6

Abbreviation	Full Form
IL-8	Interleukin-8
ITS-X	Insulin-transferrin-Selenium-ethanolamine
kDa	Kilodalton
L.monocytogenes	Listeria monocytogenes
LC/MS/MS	Liquid chromatography-Mass spectrometry
LPS	Lipopolysaccharide
LRP-1	Low-density lipoprotein receptor related protein-1
MDR1	Multidrug resistance-1
$MgCl_2$	Magnesium chloride
mM	Milli molar
MMP-7	Matrix Metallo Protease-7
MRP2	Multi resistant protein-2
NBD	Non-binding domain
NEAA	Non-essential amino acid
Nf-κB	Nuclear factor-κB
NK cells	Natural killer cells
nM	Nano molar
NP-40	Nonidet P-40
OAT	Organic anion-transporter
OATP	Organic anion-transporter peptide
P	Passage
PBS	Phosphate buffer saline
PEN/STREP	Penicillin/Streptomycin

Abbreviation	Full Form
PFIC	Progressive familial intrahepatic cholestasis
P-gp	Permeability-glycoprotein
PMSF	Phenylmethylsulfonyl fluoride
PPI	Proton pump inhibitor
PVDF	Polyvinylidene difluoride
PXR	Pregnane-X-receptor
Rho-123	Rhodamine-123
RNA	Ribonucleic acid
RT Buffer	Reverse transcriptase buffer
RXR	Retinoid-X-receptor
S.aureus	*Staphylococcus aureus*
S.epididermis	*Streptococcus epidermidis*
S.pneumoniae	*Streptococcus pneumonia*
S.typhimurium	*Salmonella typhimurium*
SDS	Sodium dodecyl sulphate
SEC	Size exclusion chromatography
SPLA2	Secreted phospholipase A2
SXR	Xenobiotic receptor
TAE	Tris-acetate-EDTA
TBS	Tris buffered saline
TBST	Tris buffered saline with Tween-20
TEER	Trans epithelial electrical resistance

List of publications arising from the thesis

- **The influence of defined medium on the epithelial properties of gastrointestinal cells** (manuscript in preparation). Aparna Warrier, Andrew Crowe.

- **Expression and regulation of antimicrobial peptides in P-gp sufficient cells** (manuscript in preparation). Aparna Warrier, Andrew Crowe.

- **Evaluation of putative antimicrobial factors in gastrointestinal cells** (manuscript in preparation). Aparna Warrier, Hendra Gunosewoyo, Andrew Crowe.

- **Role of P-gp in clearance of Amyloid beta from Caco-2 cells** (manuscript in preparation)**.** Aparna Warrier, Hendra Gunosewoyo, Andrew Crowe.

- **Assessment of neural peptides and its link to antimicrobial activity** (manuscript in preparation). Aparna Warrier, Hendra Gunosewoyo, Andrew Crowe.

List of conferences attended during thesis

- Aparna Warrier, Hendra Gunosewoyo, Andrew Crowe "Presence of known and unknown antimicrobial peptides and associated antimicrobial activity" at 7th International meeting on antimicrobial peptides in 2017 (August) to be held in Copenhagen-Abstract accepted for poster presentation (to be presented).

- Aparna Warrier, Hendra Gunosewoyo, Andrew Crowe "Characterisation of gastrointestinal cell lines in chemically defined medium and associated antibacterial activity" at ASCEPT-MPGPCR Joint Scientific Meeting in 2016 (November) held in Melbourne-Poster presentation
- Aparna Warrier, Hendra Gunosewoyo, Andrew Crowe "Evaluation of Active efflux proteins in gastrointestinal cell lines of chemically defined media" at Science on the Swan conference in 2016 (May) held in Perth-Poster presentation
- Aparna Warrier, Hendra Gunosewoyo, Andrew Crowe "Effect on P-gp on various passages in Caco-2 and LS174T cell lines in serum-free medium" at Mark Liveris Health Sciences Research Student Seminar in 2015 (September) held at Curtin University-Poster presentation
- Aparna Warrier, Andrew Crowe "Preliminary antimicrobial activity in low molecular weight fraction in gastrointestinal cells" at Mark Liveris Health Sciences Research Student Seminar in 2014 (October) held at Curtin University -Poster presentation

Abstract

This work aimed to isolate low molecular weight peptides with antimicrobial activity that used P-glycoprotein (P-gp) as a conduit to enter the exterior environment of cells where bacteria may be present. P-gp is an active efflux transporter in the ABC superfamily. Its main action is to secrete low molecular weight (LMW) substances out of the sensitive cellular environments and ultimately out of the body. Substrates of P-gp include many anti-cancer drugs. Earlier studies from the lab had demonstrated antimicrobial activity in gastrointestinal cells such as LS174T and Caco-2. A causal link between increased antibacterial activity and P-gp stimulation was observed. This suggested that unknown or putative P-gp substrates could be the cause for the observed activity, as direct P-gp was not known to yet show any identified antimicrobial resistance properties. To further probe the P-gp link, this current study used conditioned medium (CM) from these cells to observe effluxed putative antimicrobial factors that may be possible substrates for P-gp. The largest known substrate of P-gp is gramicidin, which is only 1.9 kDa. Hence, only the low molecular weight fraction was detected for antimicrobial activity.

To isolate excreted proteins/peptides, it was considered optimal to remove as much undefined protein and albumin from the growth medium as possible without adversely affecting the growth characteristics of the cells in order to easily extract the factor we were looking for in secretions from the cell monolayers. The expression of transporters in both cell lines and tight junction protein expression in Caco-2 cells were observed during the growth medium optimisation phase of the project. Using 2% FCS with defined concentrations of insulin, transferrin, selenium and ethanolamine instead of 10% FCS was acceptable for observation of the transporters, but tight junctions always needed 10% FCS for them to be high enough for bidirectional transport studies. Thus 2% FCS with the defined components was used to examine antimicrobial activity of any secreted material.

The assessment of antimicrobial activity was done in the low molecular weight fraction of CM which was enriched with MW cut off filters and an AKTA purifier with size exclusion columns. The sample was test against *E.coli, S.typhimurium and*

S.aureus with fractions collected from AKTA (based on size exclusion chromatography). Increased antimicrobial activity was detected, however it was not consistent across all bacterial species tested against.

The samples were also screened for putative antimicrobial peptide sequences using LC/MS/MS. Although histones are primarily a DNA binding protein, they have also antimicrobial activity and were detected in the samples of the current study. For histones to show antimicrobial activity it is expected to be present at very high concentrations of atleast 3.8 µM, which is not believed to be present in the CM of this project. Hence the antimicrobial activity observed in this study cannot be fully explained by the presence of histones. Based on some studies that suggest that amino acids such as valine, proline, cysteine, arginine and glycine are present in increasing concentrations in the known AMPs, it maybe speculated that some of the unknown peptide sequences detected in the samples may show some amount of antimicrobial activity.

It was also important to observe previously defined antimicrobial peptides to see if they were present in our cells and whether exposure to bacterial products such as lipopolysaccharide (LPS) could increase exposure of any known AMPs detected in the gastrointestinal cells. Amongst the many peptides observed, MMP-7 and Human Beta defensin 2 (HBD 2) showed higher expression in the presence of LPS, while HBD 1 was detected even without LPS stimulation. A decrease in HBD 1 related mRNA band intensity was observed when the cells were co cultured with LPS and a P-gp inhibitor (PSC-833). This suggests that at least when primed for expression of HBD 1, P-gp inhibition may block access to this antimicrobial peptide, which is in keeping with the previous results showing increased bacterial adherence to these cells when P-gp blockage has occurred. Other antimicrobial peptides were below levels of detection in the gastrointestinal cell lines, although mRNA was present in many cases.

Recent reports suggested beta amyloid may have antimicrobial properties and other reports had suggested that Caco-2 cells were able to express this small protein. The presence of amyloid-β-42 (Aβ-42) was analysed in this project. Although the 4 kDa peptide was not observed, the Amyloid precursor protein (110 kDa) was observed in the Caco-2 and LS174T cell lines leading to some potential PXR regulated outcome.

These results along with published reports from other groups led to the belief that Aβ may also be present in the fractions and can cause the antimicrobial effect that was observed.

Report from many other labs had indirectly suggested that Aβs are possible substrates of P-gp. Although beta-amyloid was unable to be detected in the secreted conditioned medium, the possibility of its presence in Caco-2 cells from the literature and its possible antimicrobial properties led to the examination of purified Aβ-42 through confluent suspended Caco-2 monolayers with high level of tight junctions to directly measure P-gp mediated efflux. From the study, it was observed that there was no net flux between the apical and basolateral chambers, hence Aβ-42 may not be a prospective P-gp substrate.

The current study thus developed a method for culturing cells in reduced serum and have investigated both known and unknown antimicrobial peptides that may have been involved in the observed antimicrobial activity. The study also experimentally evaluates the mechanism of substrate efflux of Aβ-42 by P-gp in Caco-2 cells.

Chapter 1 : Introduction and Review of Literature

1.1 ABC Transporters

The ABC super family of active transporters are the largest group of transporters present in the body and they play a pivotal role in our physiology (1). ABC transporters were characterized in 1992 by Chris Higgins because of the presence of highly conserved ATP binding cassette region in the representative proteins. These transporters are versatile and transport a plethora of compounds. They mainly assist in the transport of specific substances like amino acids, sugars, inorganic ions or complex polysaccharides across the extracellular and intracellular membranes (2). They also transport drugs (xenotoxins) and drug conjugates across the cells usually in an efflux direction. ABC transporters are known to have two units of a single polypeptide chain. Drug transport sites are formed in the lipid bilayer by the transmembrane domains.

ABC transporters are highly variable and may be altered by the presence of inhibitors or inducers. Other endogenous factors like genetic influence, sex hormone and cholesterol also affect their transport mechanism (3). These transporters are under tight transcriptional regulation by nuclear receptors. A number of human inborn metabolic diseases are reported to occur due to defects in one or more of the ABC transporter genes (4).

The transporters prevent the uptake of toxic compounds and that includes many drugs and food components thereby protecting the pivotal organs that include the brain, the testis, gastrointestinal tract and placenta, against toxins entering these organs or even the body generally when prevented from crossing the intestinal tract. Apart from serving to protect the body, their functions also include peptide transport (5). Mammalian epithelia use ABC transporters to excrete endogenous metabolites.

Some of the major ABC transporters observed in this study are:

- Multidrug resistance protein-1 (MDR1/P-gp)
- Breast cancer resistance protein (BCRP)
- Multidrug resistance-associated protein-2 (MRP2)

There are 48 identified human ABC genes to date. ABCB1 (MDR1/P-gp), ABCG2 (BCRP) and ABCC2 (MRP2) are some of the important efflux transporters

expressed primarily in the liver, the blood brain barrier, the gut and in mammary glands. Some other very important ABC transporters is ABCB4 that is important for secretion of phosphatidyl choline and other bile salts that regulates the normal bile function (5). Mutations in them are usually the cause of an autosomal recessive liver disorder called progressive familial intrahepatic cholestasis (PFIC) that is indicated by an early onset of cholestasis (6).

Some of the ABC transporters are called half transporters as they form heterodimers to transport short peptides into Endoplasmic Reticulum (ER). This process is important for antigen recognition by class I histocompatibility genes. Some families that may be faulty do not use this antigen recognition as they have defects in these genes. The half transporters present in the ER are ABCB2 and ABCB3 genes, while other half transporters present in mitochondria and lysosome are ABCB6-10, with the exception of ABCB9 which is present in the lysosome (5).

One main problem that is prevalent amongst the Australian community is cystic fibrosis which is caused by the malfunction of the CFTR protein that normally exchanges chloride and sodium ions across the cell membrane. CFTR protein is also an ABC transporter, but remains unique amongst them as it is a cAMP mediated chloride ion channel (7). P-gp, MRP2 and BCRP are some of the clinically relevant and the most important efflux transporters. They have also shown a trend towards an increased biliary or renal clearance, although currently observed studies do not suggest adequate levels of significance to confirm these trends (8).

1.1.1 Structure

The characteristic feature of an ABC transporter is a nucleotide binding domain (NBD) and at least one trans-membrane domain that contains six helices. A functional transporter consists of two units as a single polypeptide chain or composed of either a homo or heterodimer. Each NBD consists of highly conserved Walker A and Walker B motifs, Q, D and H loops and the signature domain of ABC proteins which is the LSGGQ motif, the linker peptide or C motif. The nucleotide binding sequence is composed of 25 aromatic amino acids that are upstream of Walker A. Amino acids present in the nucleotide binding sites prove to play a major

role by stacking of the adenosine ring of ATP, coordinating with the divalent cation and interacting with the gamma-phosphate of ATP through a water molecule. Crystal studies have shown that a functional ATP site is formed by the interaction of residues from both halves of the protein (9). Dimerisation of the ATP sites appears to be a prerequisite to ATP hydrolysis and the functional ATP sites are formed by the Walker A and Walker B motifs of one of the NBD against the signature sequence and the D loop of another. Inactivation of one of the two NBDs of the active efflux transporter by amino acid substitution or by nucleoside trapping, blocks drug transport and ATP hydrolysis by the unaffected NBD (9).

1.1.2 Mechanism of ABC transporters

All ABC transporters share a common mechanism wherein there is coordination of nucleotide and substrate-dependent conformational change. This results in substrate translocation across the membrane. The “alternating access” model, where there is an alternation of the substrate binding sites between outward and inward facing conformations, is an example of this characteristic mechanism (10). The conducive environment includes leakage of accumulated substrate across the membrane or the futile cycling of ATP hydrolysis (11).

One group had suggested that ATP is bound to the outward facing conformations of the transporter while ADP is bound to the inward conformation. ATP hydrolysis is responsible for the conversion from outward to inward facing states. The net rate of substrate translocation is given by the difference between the rates of import and export (11). For effective functioning of an ABC transporter or a pump that imports substances across the membrane, the rate of the forward reaction need to be maximised while efflux is minimised. An amplified rate of nucleotide exchange of ATP in the unliganded state of the transporter concludes the process. ATP hydrolysis stimulates substrate translocation resulting in hydrolysis of nucleotides, as hydrolysis of ATP remains the characteristic feature of ABC transporters (11).

1.1.3 Regulation of ABC transporter activity

The action of domains fused to the NBDs may determine the activity of ABC transporters. It should also be considered that non-covalently associating subunits could also be involved. The regulatory process which includes inhibition of uptake of an external ligand by intracellular concentrations of the same ligand is called trans inhibition (12). Kadner.,(13), observed this sequence in the uptake of methionine. ABC transporters may use particular subunits for binding of allosteric ligands to several other proteins, even though they appear to be very different from each other. The ABC subunits must associate to form the ATP bound state, hence keeping them separated will reduce the rate of ATP binding, thereby inhibiting the overall transport cycle.

ABC subunits do not only function as transporters. They have also been observed to be involved in DNA repair and chromosome maintenance. It has been proposed that the ATP binding and hydrolysis is responsible for reversible association and dissociation of the subunits to hinge the substrates together such as DNA or chromosomes (11, 14).

1.2 P-GLYCOPROTEIN

1.2.1 Introduction

MDR1 or P-glycoprotein (P-gp), was discovered by Rudy Juliano and Victor Ling in 1976 (15). It was observed as an anti-cancer agent in resistant chinese hamster ovary cells and seemed to alter the permeability of chemotherapeutics. Several years later it was found in the human blood brain barrier (BBB) and in the apical membrane of most secretory tissues. It is increased in brain tumours and is now one of the primary reasons for resistance to therapeutics. This is attributed to easy induction of P-gp in most cell tumour types. Resistance is a result of the ability of these transporters to efflux anticancer drugs from the cell, thus lowering the concentration at the site of its action (16). P-gp has also been gaining attention for central nervous system (CNS) drug targeting system owing to its significant endogenous presence on the blood brain barrier (17).

1.2.2 Structure

The basic structure of P-gp consists of 12 transmembrane segments and two ATP binding sites in a protein of about 1280 amino acids with 4.5 kilo base mRNA. This structure may be assembled as two equal or unequal halves (18). The amplified gene in tumour cells was termed MDR1 originally but is now known as ABCB1 and is located on the chromosomal locus 7q21 (18). The structural conformation is exhibited as a nucleotide-free inward facing structure arranged symmetrically with a twofold molecular symmetry of 136 Å which is off side to the plane of the bilayer. This inward facing conformation forms two bundles of six transmembrane helices which results in a cavernous hole capable of opening both to the cytoplasm and the inner leaflet. A negatively charged residue along with a hydrophobic surrounding and a drug binding pocket provided with many drug binding sites that may also show overlapping tendency is also seen (19). It is considered to be very important amongst ATP-dependant efflux transporters that confer multidrug resistance to chemotherapy in tumour cells. The expression of P-gp is inversely proportional to presence of primary tumours, although other transporters may also have additional predictive value clinically (17, 20).

1.2.3 Action

The action of efflux transporters is based largely on the transport of drugs (xenotoxins) and drug conjugates. The overlapping drug binding sites of P-gp give the basis for its promiscuity and transports a diverse group of molecules (21).

Although, P-gp has a broad range of substrates, its preference is for large amphipathic molecules that are neutral or weakly basic. The disadvantage of the substrate efficacy of P-gp is that it also interferes with the delivery of drugs to target tissues thereby limiting some medical treatment. P-gp in humans has only one gene product (ABCB1) as opposed to some other animals like rodents that have two, although the homology seen between all mammalian P-gps including human P- gp is high at about 90 % (22, 23).

1.2.4 Distribution

Some of the important barriers where P-gp is present are:

1.2.4.1 The Blood Brain Barrier

The blood brain barrier (BBB) is very important for protection against xenotoxins in the environment. A characteristic reason for its activity includes the presence of the active efflux transporters that help and regulate the uptake of drugs and other toxins into the most sensitive organ of the body, the brain. The BBB consists of endothelial cells of the brain capillaries that are connected to each other by tight junctions that restrict transport through the transcellular route unlike other capillaries elsewhere in the body where some paracellular transport can still occur. In addition, there are other ways to limit transcellular transport in the brain including additional efflux transporters in the capillary endothelial cells. P-gp is considered to be the most important efflux system in the BBB (24, 25).

P-gp was seen at the blood brain barrier as early as 8 weeks of gestation (26). Immunostaining analysis of P-gp also shows that they are present in the endothelial cells lining the primary cortex microvessels at 12 weeks of gestation and identified on the luminal and abluminal endothelial membranes at approximately 22 weeks. Therefore it could be suggested that the early presence of P-gp at the BBB is important in protecting the brain against teratogenic xenobiotics (27).

The action of efflux transporters was first noticed when Mdr1a (-/-) mice were analysed. They were increasingly more sensitive to ivermectin, which is a good P-gp substrate (28). These reports confirm the fact that drugs that may have significant therapeutic results and are substrates of P-gp could be co-administered with P-gp inhibitors, thereby leading to an effective penetration of the drug (29). Also in the absence of P-gp a sharp increase in the uptake of a large range of drugs was seen including anti-cancer drugs, cardiac drugs, antiemetic drugs, anti-diarrheal drugs and the immunosuppressant cyclosporine A. P-gp also limits the penetration of HIV protease inhibitors, like indinavir, nelfinavir and saquinavir in the brain.

P-gp is also responsible for affecting the treatment of primary brain tumours with intact brain vasculature. P-gp inhibitors may not completely inhibit the high concentrations of the transporter in the blood brain barrier thus making treatment of brain tumours, difficult (29). It is also seen at a very high level in patients diagnosed with multidrug resistant epilepsy and is seen as a stress response effect (30).

1.2.4.2 The Maternal-Foetal barrier

P-gp in the placenta was first discovered 1988 by Sugawara *et al*.,(31) This finding was confirmed in 1990 by Vahakangas and Myllynen (32). P-gp is present in the apical membrane of the placental synciotrophoblast that faces the maternal circulation. The location is such that it protects the foetal circulation from the amphipathic toxins that may pass from the maternal circulation. The protection is gained through transport back into maternal blood and that actively proceeds from the first trimester to term in mice (33). In human placenta, the expression of P-gp decreases with advancing gestation (34, 35).

In contrast to humans, rat placenta manifests the expression of both Mdr1a and Mdr1b genes (36). In comparison to their presence in the liver, the placental Mdr1a and Mdr1b mRNA show an increase by 4 times and 15 times than in the liver. The expression in kidney was also less than it in the placenta.

Various experiments have indicated that the presence of P-gp has inhibited movement of drugs such as saquinar, digoxin and paclitaxel in the materno-foetal direction. Administration of rhodamine-123 to rat foetus also suggested that P-gp may be responsible for removal of foetally derived substrates as well back to the maternal blood (35, 37). Blocking P-gp pharmacologically, leads to an exponential increase in the transfer of its substrates like saquinavir. Thus, clinical evidence of teratogenicity in humans might be exacerbated by the maternal use of P-gp inhibiting drugs (38).

1.2.4.3 The Liver

Hepatic expression of P-gp is important for the absolute clearance of P-gp substrates from the body. Its activity is compromised in the presence of inflammation and

endogenous mediators released during inflammation that may suppress P-gp function, while an overexpression leads to increased drug resistance (39). In mice, in addition to Mdr1a, liver also expresses Mdr1b that contributes to the efflux of xenotoxins. Schinkel had reported that Mdr1b (-/-) mice are twofold more sensitive to daunorubicin, which is an anti-cancer drug and is responsible for toxic effects in these animals (40).

It has been observed that in rats, the expression of the Mdr1b gene is up-regulated in stressful conditions and has therefore been called "the stress-responsive" gene. *In-vitro* studies have stated that there is an increase in its expression in the presence of cytotoxic drugs, insulin or other carcinogens (41-43). *In-vivo* studies have noted that the expression of Mdr1b gene shows an increase in hepatocarcinogenesis, liver regeneration and during cholestasis that may be induced by endotoxins. In addition, it has also been understood that tumour necrosis factor-α (TNF-α) may be responsible for the upregulation of Mdr1b (44-46).

In humans, the canalicular membrane of hepatocytes expresses P-gp (47). The expression levels are sevenfold lower than the intestine with high inter-individual differences in expression levels (42).

1.2.4.4 The Gut mucosa

The importance of the gastrointestinal tract cannot be underestimated as it is exposed to harmful exogenous substances and hence functions as a host defence and detoxification barrier equipped with many metabolic enzymes and efflux transporters. The intestinal epithelia is a semipermeable membrane that allows transport of nutrients and other molecules through passive diffusion as well as carrier-mediated transport facilitated by other trans-membrane transport proteins. In addition to this, efflux transporters are also present. These include P-gp, MRP 2, and BCRP on the apical membrane of the intestinal epithelia. P-gp is the main focus as it recognizes a variety of structurally and pharmacologically unrelated neutral and positively charged hydrophobic compounds. Intestinal P-gp is similar to the P-gp seen in other epithelial cells and in cancer cells (48). P-gp on enterocytes limits the influx and facilitates efflux of its substrates (17). Hence, P-gp serves as a determinant of oral bioavailability and intestinal efflux clearance for an array of

drugs. Drug-drug interactions are also important due to possible affinity concerns altering transport rates when co-administered (49).

P-gp is present in high levels at the apical surface of the superficial columnar epithelial cells of the ileum and colon, and their expression tapers proximally into the jejunum, duodenum and stomach (50). A distinct interindividual variation of expression has also been observed for intestinal P-gp. The target site of P-gp is present in the inner leaflet of the apical membrane, or the inner leaflet-cytosolic interface. According to Andre *et al.,* (51), there may be a variation in the presence of P-gp or an altered expression of P-gp in inflamed and malignant tissues. Under normal circumstances, P-gp may be the only efflux protein that confers apical directed polarity (52). During absorptive transport any substrate for P-gp may have to cross the apical interface first, while the substrate crosses the basolateral interface during basolateral transport fully and then enters the apical interface. This suggests an asymmetric pattern in the transport of P-gp substrates (52).

Presence of the transporter expression of P-gp and MRP2 was high in the jejunum, while its presence in the human adenocarcinoma cell line Caco-2 cells is reduced by 7 fold or more (53). The gene expression increased with cultivation time. In contrast a current study by Vaessen *et al.*,(54) suggests that the three transporters (P-gp, BCRP and MRP2) are much more significant in Caco-2 cells in comparison to human jejunum. The passage number could be a huge influence in this case.

P-gp plays a pivotal role in the pharmacokinetics of its substrates. The efflux ratio of the transporter drug assists in the determination of P-gp efflux activities and attenuation of oral absorption. However, although *in-vitro* efflux ratio is useful to provide relative transport roles of P-gp *in-vivo* absorption studies is necessary for quantification of drug absorption in patients. Hence *in-vitro* assays with P-gp expressing cells may not be an accurate method to gauge its clinical activity (52).

Apart from being present in the most important barriers, P-gp is also present in the blood-ocular barrier that includes retinal capillary endothelial cells, retinal pigmented epithelial cells, ciliary non-pigmented epithelium, conjunctival epithelial cells, iris and the ciliary muscle cells (55).

1.2.5 Transport mechanism of P-gp in the intestine

The passage of molecules through the intestinal epithelium consists of two routes, paracellular and transcellular. Small hydrophilic and ionized drugs are inclined to use the paracellular route. This route is not the optimal choice for transport of drugs, due to the presence of intercellular tight junctions that constrains the transepithelial movement and slows the flow of transport.

Tight junctions are present between adjacent cells close to the apical surface and are important as a barrier (56). They regulate the movement of ions, water and various other molecules through the paracellular spaces and are also responsible for maintenance of cell polarity. ATP is required for the regulation of the tight junctions (57). They are made of a complex association of many different proteins such as the transmembrane proteins that includes occludin, claudin and other junctional adhesion molecules, scaffolding proteins such as zona occludens and signalling proteins (58). Tight junction proteins like occludins are observed in well-differentiated adenocarcinoma cell lines. A weak or leaky tight junction results in transepithelial permeability. Occludin is one of the first integral proteins, a knockdown of which results in an increase in permeability to macromolecules. This specifies the role of occludin in maintaining tight junctions (59). The tight junctional pore size was first investigated in the adenocarcinoma cell line, Caco-2. The smaller pore size that is permeable to small molecules was 4.3-4.6 Å, while the larger pore size that is responsible for leaky junctions, being permeable to macromolecules was about 14.6 Å (60).

The transcellular route involves uptake from the lumen across the apical membrane, which then passes through the cytosol, to cross the basolateral membrane and reach its destination in the circulatory system. Transcellular transport is potentially used for substrates of active efflux transporters. They can be extruded back into the lumen via these active efflux transporters. Many of these drugs are also substrates for various intracellular metabolic systems such as the cytochrome P450 mediated metabolism (CYP) system. Conjugating enzymes produce metabolites that may also be substrates of the efflux transporters and in some cases the metabolites are more rapidly effluxed than the parent drug.

The excretion of xenobiotics from the intestinal epithelial cells cannot be attributed to the presence of P-gp alone. Although the substrates of P-gp can be broadly classified into amphipathic, cationic or neutral and have a wide molecular weight range for transport, there might be other transporters or proteins that may help in the excretion of anionic toxins (61). These include organic anion transporter (OAT) and organic anion transporting polypeptides (OATPs). The expression and metabolising properties of P-gp in the intestine *in-vitro* are best studied in Caco-2 cells. This is because they are the prime examples of enterocytic differentiation of human gastrointestinal cells and show a high endogenous expression of P-gp (62, 63). Spontaneous differentiation of Caco-2 cells into polarised apical brush border intestinal cells, presence of tight junctions and expression of hydrolases and microvillar transporters make them primary examples of enterocytic differentiation and paracellular and transcellular pathways (64). Moreover, an increased presence of P-gp on the cells in contrast to human colon also adds to the transport studies performed in Caco-2 cells (65).

There may be variations in the efflux of P-gp substrates due to differences in P-gp expression in the intestine. Hence competitive inhibition and interactions due to co-administration of P-gp substrates, inhibitors and inducers are also observed. There is greater than sixfold diference in P-gp efflux between the duodenum and the ileum (66). Inter individual variations are also reported (67). Variations may also occur due to overlapping substrate specificity between P-gp and CYP 450 3A. Since CYP3A occurs in close proximity to sites similar to P-gp, the possibility increases. The overlap of substrates would provide another avenue for research in the field of pharmacotherapeutics (68). Increased efflux of substrates by P-gp has been well demonstrated, *in-vivo*, using a known substrate of P-gp, Rhodamine-123 (rho-123). The transport of rho-123 is varied in the intestine with the rate being much higher in the ileum (68). This study is coincidental with earlier reports from the lab of Trezise *et al.*,(69) that suggests that the expression of mRNA of P-gp was the highest in ileum exceeding the expression in duodenum and jejunum and was shown to be minimal in the proximal and distal colon. Chianale *et al.*, (70) had also reported similar results in male CF 1 rats. The rate of rho-123 transport in the ileum appeared to be twice that of the transport rate observed in other areas, especially jejunum. It could be hence inferred that P-gp and other transporters like CYP3A with their

overlapping substrate specificity are important in protection against xenotoxins in the intestine (68).

A number of hypothesis have been suggested for P-gp to act as an efflux pump. One of them is the vacuum cleaner hypotheses, where the transporter removes the substrate from the lipid bilayer to the extracellular space by taking it in laterally from within the membrane (71). Another method of efflux is the flippase model. P-gp takes in the substrate from inner to the outer leaflet of the membrane thereby detaching it from the membrane or by changing the membrane lipid composition for better detachment (71).

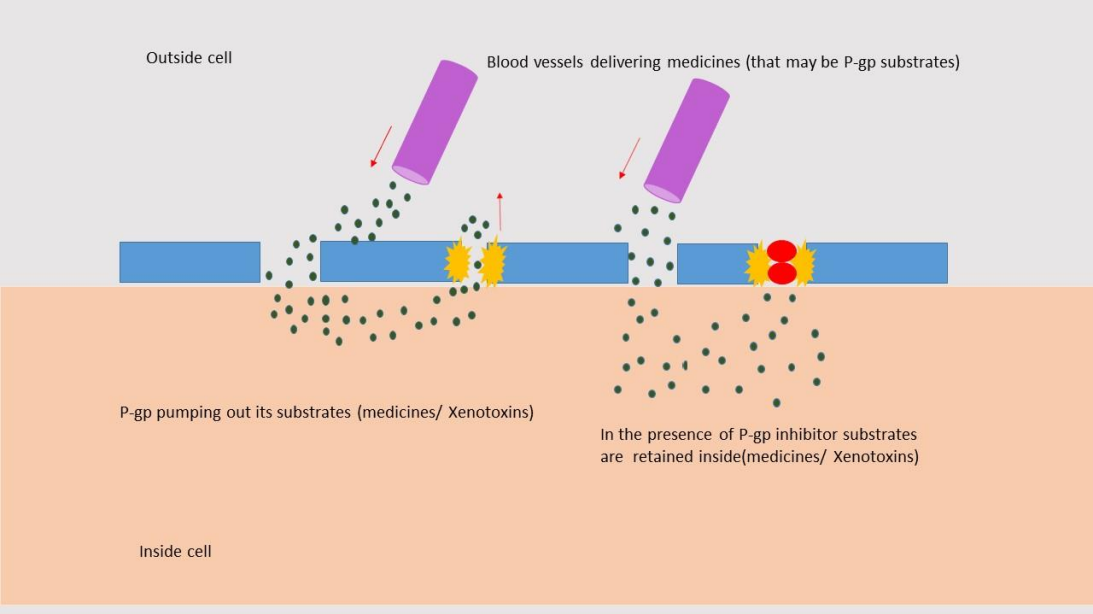

Figure 1.1: P-gp in cell membrane: P-gp effluxes out its substrates that include xenotoxins or any therapeutic drug that enters the cell. This causes an impediment in chemotherapy. In the presence of a repressor for P-gp, the drugs are retained in the cell while inhibiting the transporter to efflux out its substrates. This increases the therapeutic delivery of a given drug.

1.2.6 Understanding the active efflux transporters at the molecular level

The regulation of expression of P-gp at the transcription level may be attributed to the presence of nuclear receptors such as pregnane X receptor (PXR), farnesoid X receptor (FXR) and hepatocyte nuclear factor-4 alpha. PXR is considered to be a major factor in regulating P-gp. They may also be responsible in regulating another intestinal drug metabolising enzyme, CYP3A4. The functions of both these drug metabolising enzymes are complimentary. However, the levels of P-gp and CYP3A4 may not always be directly proportional in patients (48). P-gp, MRP2 and BCRP are under tight transcriptional regulation by nuclear receptors (72). PXR may be induced by a cascade of pharmaceutical agents like rifampicin, clotrimazole, phenobarbital and paclitaxel (73).

The presence of P-gp in the enterocyte brush border limits absorption of certain drugs that interact with CYP3A4. Studies have shown that higher expression of enterocytic P-gp lowers the rate of oral adsorbing drugs in patients with renal transplants. This suggests the importance of P-gp in the drug absorption (48). The mechanisms by which P-gp can limit drug absorption includes reducing the rate of absorption and metabolism from the proximal small intestine to distal intestine thus pumping out the metabolites (48). While observing the expression of transporters on Caco-2 cells, it is mandatory to use cells of the same age and cultivation time as there may be variations between the cells. Seithel *et al.,* demonstrate that a minimal cultivation time of 15-18 days is needed to show high P-gp expression in Caco-2 cells and that functional activity is seen only from day 17 (53). However, previous work had suggested that the expression of P-gp in Caco-2 did not change from day 7-22 according to Western blot analysis (74).

1.2.7 Substrate affinity of P-gp through poly specific drug binding

One of the major reasons for ineffective use of chemotherapy is the presence of P-glycoprotein. P-gp has at least four binding sites within the same efflux protein that efflux drugs from cells (75). The general traits of P-gp substrates are hydrophobicity and partition from the lipid bilayer (71). As discussed before, P-gp has known to have drug binding sites with overlapping substrate specificity. While the anterior

half of the drug binding residues carry hydrophobic and aromatic residues, the posterior end carries polar and charged residues. This suggests that substrates that are both hydrophobic and positively charged with a probability of binding to this half of the drug binding pocket (76). This inward facing conformation is a very important structure in P-gp substrate affinity as it gives way to two portals that can accommodate both non-polar and phospholipid molecules. The portals span the entire width of the molecule and helps P-gp to identify and bind to specific substrates through the inner leaflet (77).

1.2.8 Substrates and Inhibitors

The substrates of P-gp include vinblastine, dexamethasone, cyclosporine, quinidine and ritonavir. The higher the brain-plasma partition coefficient ratio (Kp) the higher the substrate efficiency of P-gp. Kp ratio was studied in wild type mice (Mdr 1a (+/+) and knockout mice (Mdr 1a (-/-)) (78). Some compounds that may be a part of the P-gp substrate complex also modulate the function of P-gp. For example, verapamil and cyclosporine act as substrates for P-gp at low concentrations but at high concentrations saturate the transporter and therefore become inhibitors of activity. Many natural compounds also act as inhibitors of P-gp and these include, components of grapefruit juice, orange juice, and green tea (79). Certain pluronic block copolymers, like P85, that are amphiphilic polymers also have been known to inhibit the function of P-gp. Certain other factors like surfactants can also affect the regulation and activity of P-gp mediated transport. They downregulate the ATPase activity and P-gp expression and alter cell membrane fluidity (80). Proton pump inhibitors (PPIs) such as omeprazole, lansoprazole and pantoprazole that are used in the treatment of acid related diseases are known to act as substrates as well as inhibitors for P-gp (81).

Patients may fail PPI therapy due to high P-gp expression causing smaller quantity of drugs to enter relevant epithelial cells. Some of the inducers of P-gp include clotrimazole, isozafrole, phenobarbitone, reserpine and rifampicin, verapamil, carbamazepine, platelet activating factor and progesterone. Rifampicin has shown to induce intestinal P-gp at high concentrations. Another good inducer of P-gp is

vincristine (82). Some drugs have overlapping substrate specificity with different active efflux transporters and examples of these are shown below.

Table 1.1 : **Comparison of substrate specificity between different active efflux transporters** (83).

Type of Drugs	**Name**	**MDR 1**	**BCRP**	**MRP2**
Anti-cancer	Vinblastine	Yes	No	Yes
	Vincristine	Yes	No	Yes
	Doxorubucin	Yes	Yes	Yes
	Mitoxantrone	Yes	Yes	No
	Topotecan	Yes	Yes	No
Cardiac Drugs	Digoxin	Yes	No	No
	Talinilol	Yes	No	No
	Quinidine	Yes	No	No
Protease inhibitors	Indinavir	Yes	No	Yes
	Ritonavir	Yes	No	Yes
	Nelfinavir	Yes	No	Yes
Antibiotics	Ciprofloxacin	Yes	No	Yes
	Levofloxacin	Yes	No	Yes
Fluorescent dyes	Calcium AM	Yes	No	Yes
	Rhodamine 123	Yes	Yes	Yes

1.2.9 Amyloid beta as a P-gp Substrate

Although most substrates for P-gp are relatively small with a molecular weight under 1.9 kDa, there have been some contradicting data that suggests that P-gp may be able to carry larger substrates (81).

The formation of amyloid beta plaques in the brain is a key component of Alzheimer's disease. The clearance of amyloid beta from the brain is deemed important in the progression of the disease. Efflux by P-gp is a recent mechanism proposed that potentiates its clearance and maybe thought of as a therapeutic target. According to Shibita *et al* and Bell *et al.,* (84, 85) clearance of amyloid beta (Aβ) is a twostep process involving some form of efflux pathway? The Aβ is pushed by the neurons into the brain interstitial fluid, where it may be removed by the proteolytic enzymes like low density lipoprotein receptor-related protein 1 (LRP1). This process is deemed mandatory for the movement of Aβ from the brain into capillary endothelial cells therefore clearing it from the abluminal side (84, 86-89).

There is very little known about the mechanism of removal of Aβ from the capillaries into blood. An underlying process that propels its removal is the presence of ABC transporters such as P-gp. It is present in the luminal side of the brain capillaries and contributes to the formation of the blood brain barrier.

Cirrito *et al.*,(90) have shown that injection of a P-gp inhibitor in human amyloid precursor protein (hAPP) overexpressing transgenic mice lead to an increase of Aβ level. This finding has been supported by other papers that have made similar observations *in-vitro*. Additionally, Hartz *et al.,(91)* suggested that exposure of *in-vivo* brain capillaries of human amyloid precursor protein-overexpressing mice (hAPP) mice to PXR stimulating agents that activate P-gp led to an increased transport of fluorescein attached Aβ-42 in them. In studies using mice models, especially transgenic and hAPP mice, P-gp has been observed to be vital for the efflux of Aβ across the luminal membrane (72, 92, 93)**.**

Accumulation of fluorescein-human Aβ-42 was seen in the lumen of capillaries used as control, but accumulation was significantly reduced in the presence of P-gp

inhibitors like PSC-833 signifying P-gp as a significant factor in the clearance of Aβ (94).

Another advantage of using P-gp as a target mechanism for Alzheimer's disease is the location of the transporter in the brain. Since P-gp is present on the luminal surface of brain capillary endothelial cells and not in the CNS itself, drugs have an easier and higher probability to reach the target rather than having to cross the blood brain barrier (95). Both *in-vivo* and *in-vitro* studies have indicated that the presence of P-gp is the reason for amyloid beta clearance from the brain. Abuznait *et al.*,(96) have studied the uptake of Aβ-40 on LS-180 cell lines. The cells can be induced to express P-gp as they also contain PXR that controls expression of P-gp (92, 97). These cells were treated with P-gp inducers and inhibitors, such as rifampicin and verapamil respectively (98-102).

To understand the mechanism of action of efflux of Aβ by P-gp it is crucial to explore new endogenous substrates of P-gp. Further, as P-gp pumps have not shown efflux of substrates more than 1.9 kDa, proof that P-gp can efflux such large entities would open up the scope of P-gp mediated efflux more generally, with many more potential substrates being explored in the future.

1.2.10 Link between P-gp and antimicrobial activity

Studies have shown that the expression of P-gp is modulated by certain gastrointestinal specific microorganisms. In the study by Omar *et al*.,(103) P-gp expression was observed in the presence of *Helicobacter pylori (H.pylori)*. Under normal circumstances P-gp is expressed at a higher ratio in the duodenum than in the antrum. In the presence of *H.pylori* infection in the antral specimen, the antral to duodenum ratio is increased (104, 105).

In-vitro studies by Neudeck *et al.,* have also suggested that the overexpression of P-gp can protect the cells from invasion of *Listeria monocytogenes*. The protection was proposed in a similar manner to that of *H.pylori*, where the bacterial proteins that is important in invasion being transported in the basolateral to apical direction by P-gp (106). The presence of bacteria is a factor in the development of

inflammatory bowel disease an exacerbation of which could be attributed to functionally deficient P-gp in the apical surface of the enterocyte thus rendering the host susceptible to bacteria (106, 107). It has been previously noted by Crowe and Bebawy., that blocking P-glycoprotein function increased bacterial adhesion with Caco-2 cells. This suggested that P-gp has a role in preventing bacterial attachment (108). This has led to speculations that human gastrointestinal epithelial cells express an antibacterial factor that may act in conjunction with P-gp to prevent bacterial adhesion.

1.3 Antimicrobial peptides

1.3.1 Introduction

Multicellular organisms need to constantly defend themselves against parasitisation by microbes. The most common site of their primary encounter are the epithelial surfaces, that includes the skin, moist surfaces of eyes, nose, airways and the lungs, mouth and the digestive tract , urinary tract and the reproductive system (109).

In their first line of defence against microbes, the host uses innate or adaptive immunity to counteract their action. Over the past four decades, a vast number of antimicrobial substances have been discovered that are secreted by the epithelia and phagocytes and have been characterised based on their size. Hence antimicrobial peptides (AMP) have been defined as polypeptide antimicrobial substances, encoded by genes and synthesised by ribosomes, and generally have fewer than 100 amino acid residues (109). The production of peptides and small proteins with microbicidal activity is an ancient and primary mechanism of immunity.

1.3.2 Characterisation of antimicrobial peptides

Two different classes of these molecules exist. The first class includes gene encoded, ribosomally synthesized proteins, while the second class includes non-ribosomally synthesized peptide antibiotics that are produced by bacteria and eukaryotes. They are characterised as cationic and amphipathic molecules and consist of 12-50 amino acid residues (110). Another significant detail is the presence of arginine and lysine

residues and a substantial portion of hydrophobic residues. They form alpha helices, beta hairpins, extended peptides and loops. Their structure also helps in the formation of transmembrane channels in microorganisms (109). There are certain disadvantages of AMPs that restricts them from drug developments. AMPs are generally unstable and are rapidly affected by pH change and protease degradation, which restricts their oral application. The development of peptides is also not cost effective and hence has been a hindrance to honing their therapeutic ability in the drug industry (111, 112).

1.3.3 Distribution

Human antimicrobial peptides: AMPs are present in tissues that have maximal exposure to microbial organisms or cell types and have been stimulated to act against microbes by the immune system (113).

To understand the antimicrobial peptides present in humans, their tissue distribution will be briefly discussed as this will give the precise method of defensive role that the peptide plays in different cell types.

1.3.3.1 Skin

The follicular structures of skin produce minimal amounts of AMPs that act as a chemical barrier during resting conditions, while after an injury, skin reacts rapidly with AMPs produced from epithelium and those that have been degranulated by recruited neutrophils.

AMPS from the skin are synthesised by eccrine gland cells, keratinocytes, malphigian cells, epidermal cells. Examples of AMPs in skin are listed in table 1.2.

Table 1.2: **List of antimicrobial peptides from skin.**

Antimicrobial peptides	**Molecular weight (kDa)**
Dermicidin	9.8 (114)
Psoriasin	11.4 (115)
RNase 7	14.5 (116)
Lysozyme	13.9 (117)
Lactoferrin	80 (118)

1.3.3.2 Mouth

AMPs are synthesised by the parotid, submandibular and sub Lingual glands, the squamous epithelia of the mouth and the neutrophils E.g.: Histatins (3.0 kDa) (119).

1.3.3.3 Alimentary canal

The intestinal epithelial monolayer is complex, consisting of different cell types and most have a limited lifespan. These cells types are responsible for the synthesis of different AMPs. Paneth cells, macrophages, enterocytes, neutrophils and epithelial cells are some of the examples. Several AMPs are also present in the tissues of the oral cavity, stomach and intestines (120).

Table 1.3: List of antimicrobial peptides from gastrointestinal tract.

Antimicrobial peptide	**Molecular weight (kDa)**
Cathelicidin (LL-37)	4.4(121)
Human neutrophil peptide-1 (HNP-1)	3.4 (122)
Human neutrophil peptide-2 (HNP-2)	3.3 (122)
Human neutrophil peptide-3 (HNP-3)	3.5 (122)
Human defensin-5 (HD-5/α-defensin-5)	3.5 (109)
Human defensin-6 (HD-6/α-defensin-6)	3.7 (109)
Human beta defensin-1 (HBD-1)	3.9 (123)
Human beta defensin-2 (HBD-2)	4.3 (124)
Human beta defensin-3 (HBD-3)	5 (125)
Human beta defensin-4 (HBD-4)	9 (126)
Phospholipase A2	13-15(127)
C-type lectins	30 (128)
Calprotectin	36.5(129)
Lactoferrin	80(130)
Lysozyme	14.3(131)

1.3.4 Antimicrobial peptides of the gastrointestinal tract

The mucosal epithelial cells of the intestine are crucial for defence of the gut against microbes. They have been mentioned in table 1.3 and some of the AMPs have been briefly described below, while emphasis in this review is on the AMPs ranging between 3-5 kDa.

1.3.4.1 Defensins

Defensins are one of the largest families of antimicrobial peptides in mammals. They are generally 30-42 amino acids in length, cationic with a beta sheet conformation and are endogenously produced. They are produced by phagocytes, paneth cells and epithelial cells that are present on mucosal surfaces. Inflammatory bowel diseases show differences in relation to attenuated changes made in the defensins and there have been substantiated data on this link (132, 133). They have six cysteines that interact in intramolecular disulphide bonds with three different linkages.

Based on the cysteine links, they can be differentiated into two different classes, alpha and beta defensins. The genes that encode these defensins are clustered in a segment in the short arm of chromosome 8 in humans (122).

Alpha defensins are one of the major constituents of the mammalian granulocytes. The paneth cells stores a huge portion of the alpha defensins. The four alpha defensins present (human neutrophil 1-4) are found in the azurophilic granules of neutrophilic granulocytes. Half of the protein content is composed of HNP 1-3 found in B cells and natural killer cells (NK cells), while HNP-4 are present in lower quantities. Alpha defensins are involved in the phagocytosis of microorganisms in an oxygen independent fashion. Examples of paneth cell defensins in humans are human defensin-5 and human defensin-6 (HD-5, HD-6) (134). In its precursor form HD-5 is present in the ileal paneth cells but is expressed in villous epithelial cells in its active form. It is also seen in metaplastic paneth cells during chronic inflammation or inflammatory bowel diseases (135).

Human beta defensins are larger than alpha defensins. The three disulphide bridges in beta defensins result in peptides with a triple stranded beta sheet structure and a

hairpin loop containing cationic charged molecules (134). In the stomach and colon, Human beta defensin-1 (HBD-1) is present and expressed constitutively by the gastric epithelia cells. HBD-1 can also be induced progressively by peptidoglycan or lipopolysaccharide (LPS) exposure (136, 137). Human beta defensin-2 (HBD-2) is also called an inducible antimicrobial peptide. HBD-2 is expressed during an inflammation or in the presence of an organism like *H. pylori*. A very relevant study by Gacser *et al.*, (138), suggested that Caco-2 cells can also produce HBD-2 in the presence of fungal infections like candidiasis. Alpha and beta defensins have also shown to exhibit chemoattractant properties especially on cell types such as monocytes, T-lymphocytes and dendritic cells (124).

Mechanism of action

The defensins act through both innate and acquired immunity. Intestinal defensins act on the microbial pathogen by deactivating it and reducing the production of microbial exotoxins in the environment. In the adaptive immune response they regulate the path of several cells like neutrophils, monocytes, T-lymphocytes, dendritic cells and epithelial cells. These defensins act on the cells through G-protein coupled receptor (GPCR). Some examples are human neutrophil peptide 1-3 (HNP 1-3) (139).

Beta defensins act on the memory T cells and dendritic cells via the chemokine receptor 6 (CCR 6). Some studies have suggested that beta defensins could also act as competitors with chemokine ligand 20 (CCL20) due to their structural similarities. Another route to activate the T cells is a coupled effect with TLR-4 and activation of Nf-κB, whilst migrating to the nucleus for activation of a strong immune response. Defensins are responsible for maintaining the intestinal homeostasis, especially alpha defensins as they are produced from the intestinal paneth cells (132). The C-terminal portion of peptide is responsible for their antimicrobial activity. Some of the viral agents that alpha defensins and beta defensins are active against are adenovirus while only minimally against herpes virus, influenza virus and cytomegalovirus (CMV) wherein their activity includes disruption of its envelope, interaction with their glycoproteins and inhibiting the entry of the virus to the cell (140).

1.3.4.2 Cathelicidins

Cathelicidins were first found to be synthesised in neutrophils. The structure of this antimicrobial peptide is comprised of a highly conserved N-terminal region which is called the cathelin domain and a C-terminal region with the active peptide. Cathelicidins have a lot of inter and intra species variability. The cathelicidin gene is located on chromosome 3 in humans and is termed CAMP. This gene encodes the cathelicidin precursor also called the human cationic antimicrobial peptide-18 (hCAP-18) with a total length of 170 amino acids. The C-terminal peptide LL-37 is present in hCAP18, which is diverse with both α-helical and β-hairpin component (139).

The biologically potent peptide LL-37 from hCAP18 can be processed by proteases like serine protease 3 in neutrophils. These proteases are released from azurophilic granules. LL-37 has a linear structure and consists of 37 amino acids. The linear structure can be attributed to no cysteine residues present. Hence, it takes up a conformation of a random coil in a hydrophilic environment and an alpha helical structure in a hydrophobic environment (139, 141-143).

LL-37 is induced by infectious stimuli and exhibits antimicrobial activity against both gram positive and gram negative bacteria (144). Some mammals possess many different cathelicidins. BMAP-28, a bovine AMP has an alpha structure and active against a broad range of bacteria and fungi, while another bovine peptide which is rich in proline residues, Bac 5 appears selective towards some gram negative bacteria (145, 146). LL-37 also acts together with IL-1β to increase the production of cytokines such as IL-6 and IL-10 and some chemokines such as monocyte chemoattractant protein (MCP-1 and MCP-3) (147).

Mechanism of action

Cathelicidin exerts chemo attractive action on monocytes, neutrophils and CD 4^+ T lymphocytes using formyl-peptide receptor like 1 as a conduit which is a GPCR present in these cells. *In-vitro* data have suggested that cathelicidin helps in the transcription and release of chemokines like IL-8 for inducing other cells in the immune system to act as a first line of defence (137, 139, 148, 149). It acts by

binding to LPS and blocking the release of TNF-α. It can also reduce the activity of TNF-α by inhibiting its transcription. Other examples are nitric oxide and tissue factor which are induced by LPS and lipoteichoic acid. Cathelicidins induce degranulation of mast cells and propagates wound vascularisation and re-epithelialisation of skin as a part of the healing process (150). It has also been shown to exhibit antitumor activity (151). These mechanisms confirm cathelicidin as responsible for sepsis and septic shock for gram negative organism induced infections (137, 152).

The peptide has shown susceptibility to *Escherichia coli* and *Staphylococcus aureus* (*E.coli and S.aureus)*. Not all 37 amino acids that constitute LL-37 are responsible for antimicrobial activity.

LL-23, a fragment of LL-37 was shown to have exhibited modulation of chemokine release, while showing minimum antibacterial activity. This can be attributed to the presence of separate hydrophobic surfaces by a hydrophilic serine. However, the major antibacterial domain of LL-37 is GF-17 that maps the amino acid residues 17-32 of the mother peptide, LL-37 (153).

GF-17 by itself has shown antimicrobial activity against *E.coli*, the results being comparable to LL-37. The activity of LL-37 was shown to be reduced considerably when the length of GF-17 was shortened. KR-12 another antimicrobial peptide was formed from GF-17 by removing residues like L31 and V32 from the C-terminus and F17 from the N-terminus, all of which are hydrophobic. As a consequence of it being less hydrophobic, KR-12 does not lyse lipid vesicles, while GF-17 can lyse them**.** This also affects the antimicrobial activity of KR-12 against *E.coli* as opposed to GF-17 (153, 154).

1.3.4.3 Phospholipase A2

Another category of intestinal antimicrobial peptides which are also synthesised by paneth cells is phospholipase A_2. This hydrolyses bacterial membrane phospholipids to generate both free fatty acids and lysophospholipids. Its mechanism of action involves binding of the phospholipase to phospholipids surface. In the intestine, phosphatidyl choline which is hydrophobic protects the surface of the GI tract. This

enzyme acts on anionic phospholipids but not responsive to phosphatidylcholine due to lack of high affinity binding. The anionic phospholipids that phospholipase A_2 does catalyse are phosphatidylglycerol, phosphatidylserine and phosphatidylethanolamine (127).

1.3.4.4 C-type lectins

C-type lectins are present in the enterocytes and paneth cells from intestinal tract. Their mechanism of action demonstrates direct lysis of gram positive microorganisms. They are also present in gastrointestinal tissues (128) .

1.3.4.5 Lactoferrin

Lactoferrin exhibit a multitude of antimicrobial activities with immunotropic properties. It is resistant to the action of proteases in the intestine especially in the alimentary tract. The action on the intestine depends on the presence of specific receptors present on the surface of the brush border cells. While it inhibits the growth of gram negative and pathogenic bacteria, it attenuates the growth of non-pathogenic organisms like *Lactobacillus sp,* and *Bifidobacterium sp* (130). *In-vivo* studies have shown that lactoferrin inhibits bacteraemia and endotoxemia. Its mechanism of action includes impeding the activity of proinflammatory cytokines, nitric oxide and reactive oxygen species. Also, it propagate the differentiation of T and B cells, while ameliorating the activity of NK and lymphokine activated killer cells (NK, LAK)(155) .

1.3.4.6 Lysozyme

Intestinal lysozyme is produced by paneth cells, macrophages and neutrophils. They are active against gram positive bacteria, and show less potency against gram negative bacteria. In the skin they are present in the cytoplasm of epidermal cells and in the malphigian cells in the stratum spinosum layer(156, 157) .

1.3.4.7 Paneth cells

Paneth cells are situated at the base of the intestinal crypts of lieberkuhn. They contain intricate golgi network and endoplasmic reticulum that is an indicator of

extensive secretory activity. They store a number of proteins in their capacity, majority of them being antimicrobial peptides. These AMPs may be transcriptionally induced or expressed constitutively. They are released into the crypt lumen in the presence of an inducer and protect the mucosa of the crypt from the microbial flora of the intestine as well as the colonising pathogens (158-161). Human paneth cells contain α-defensins, lysozyme and secreted phospholipase A_2 ($SPLA_2$). The extensive distribution of these antimicrobial peptides supports the dynamic innate response of the gastrointestinal system. Not only do the paneth cell antimicrobials protect the gastrointestinal system from pathogenic microorganisms but they also help improve the microbiota of the gut (162, 163).

The functioning of the antimicrobials present in the paneth cells is related to controlled granular secretion of the paneth cells. This mechanism can be induced by a variety of stimuli that includes both gram positive and gram negative organisms and components of their cell membrane comprising of lipoteichoic acid and lipopolysaccharides and certain cholinergic agonists (164). Protozoan and fungal products have not proven to be a good stimulus for the secretion. Since the intestine is always subjected to varied bacteria and its products, hence the stimulation of paneth cells happens unceasingly (164, 165). Regulation of this secretory activity is necessary to determine the extent of antimicrobials that are being produced in the intestine.

The most important peptide secreted by the paneth cells are the α-defensins. They are seen in the small intestine. To secrete active, mature paneth cell α-defensins, proteolytic processing is an important step. There is a difference seen in the proteolytic processing in mice to that of primates. In rodents, the matrilysin, also called matrix metallo protease-7 (MMP-7), cleaves the precursor (8.4 kDa) of these α-defensins to produce active peptides (166). MMP-7 is expressed in the paneth cells. Proteolytic cleavage of other α-defensins, such as the pro α-defensin-4 yields a different peptide to that of the α-defensin-5 isolated from the intestinal lumen, suggesting that MMP-7 may not process all the defensins in a similar fashion (167). In humans, the processing of α-defensin 5 takes place in the presence of trypsin (168, 169). According to Ghosh *et al.,* (168) paneth cells accumulate about 0.5 mg of the antimicrobial peptide per cm^2 of the ileal mucosa. Although, other antimicrobial

peptides like the lysozyme and $SPLA_2$ are also present, the proportion of α-defensins is fivefold higher than that of other peptides present. A correlation has been observed between reduced α-defensin-5 and an increase in the ileal Crohn's disease. Paneth cells are also excellent markers for cryptal ontogeny and in studies for differentiation in the epithelium of the intestine (170).

1.3.5 General overview of mode of action of antimicrobial peptide

The mode of action of these AMPs lead to transmembrane pores or extensive membrane ruptures by the formation of ion channels. Adsorption of AMPs onto the bacterial cell membranes by electrostatic attraction and their subsequent aggregation and integration into the lipid bilayer results in the expansion of the outer leaflet that leads to local membrane thinning.

There are three mechanisms proposed for antibacterial activity:

1.3.5.1 Barrel stave model

The hypothesis behind the barrel stave method suggests that hydrophobic peptide regions align with the acyl regions of the membrane lipids, while the hydrophilic inner regions form the inner surface of the pore channel leading to membrane depolarisation and eventually cell death (171).

1.3.5.2 Aggregate Channel model

The peptides are associated over their full-length transmembrane stretch with the lipid head groups and are perpendicularly inserted into the membrane. As a consequence, the lipid monolayer bends continuously through the pore in the fashion of a toroidal hole. The pore is lined with the peptides and the lipid head groups forms membrane depolarisation, micelle formation and cell death (171).

1.3.5.3 Carpet model

Peptides remaining in a parallel orientation to the membrane surface covering it in a carpet like manner. At a critical threshold concentration, the peptides disrupt the membrane finally leading to the formation of micelles or small peptide lipid aggregates (172).

Although most of the human AMPs are between 29-40 amino acids long, studies have shown that smaller artificial peptides can generate pores in membranes by assembling into nanotubes (173).

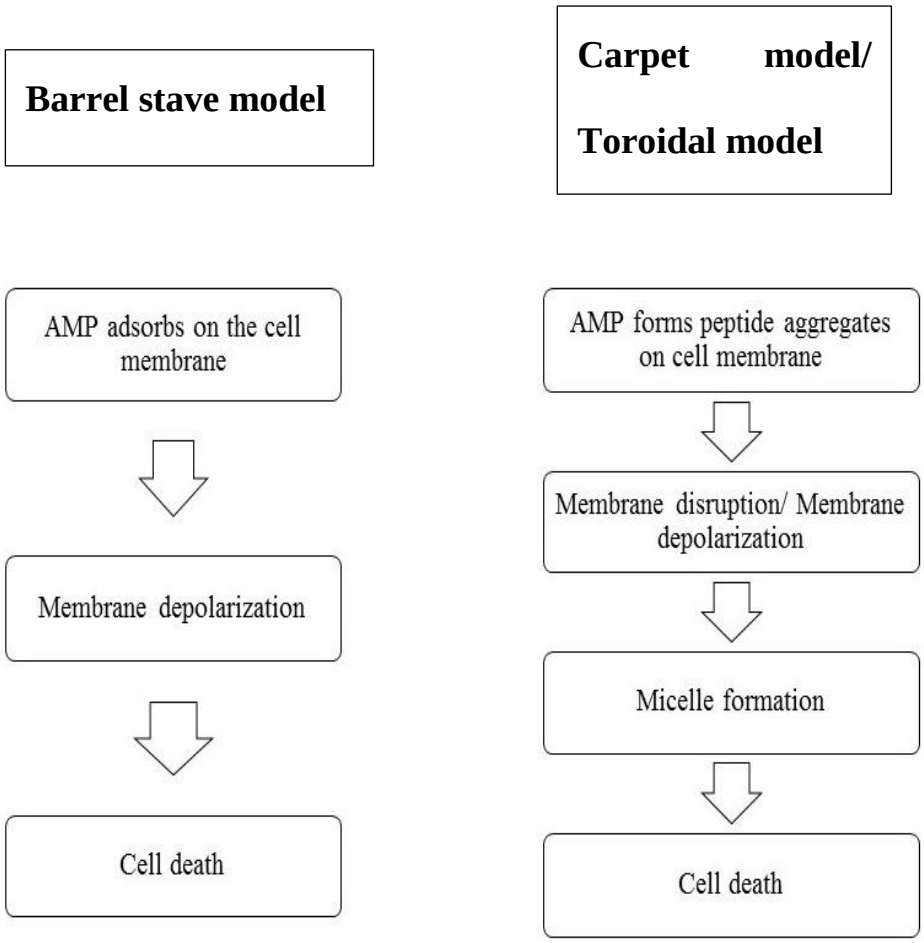

Figure 1.2: Illustration of different modes of antimicrobial activity. AMPs use one of the methods for disruption of the bacterial cells. The carpet and toroidal model follow the same route of cell lysis, difference being membrane disruption or membrane depolarisation.

1.4 The link between peptide structure and antibacterial activity

Cationic antimicrobial peptides are positively charged peptides with lysine, arginine and a significant proportion of hydrophobic residues and are almost always less than 50 amino acids long. The affinity of cationic antimicrobial peptides to microorganisms especially gram negative microorganisms start with electrostatic interactions between the peptide and anionic lipopolysaccharides of gram negative bacteria leading to a disturbance in the membrane by displacing certain cations from the negatively charged LPS (174). These cationic peptides have a better affinity for LPS than some divalent cations like Mg^{2+} and Ca^{2+}. The route to the cytoplasm is facile, which propagates attachment of the peptide to the cytoplasmic membrane, in particular the outer monolayer. This step allows peptides to utilise two pathways, by either entire disruption of the cytoplasmic membrane or peptide translocation into the cytoplasm (175).

1.4.1 Mechanism of action of membrane disruptive peptides

Although all membrane disruptive peptides are of the α-helical class, not every α-helical peptide is a membrane disruptive peptide. The barrel stave model, micellar aggregate model and carpet model that have been explained above in detail are examples of membrane disruptive peptides (174). Regardless of the model system being used the end result is membrane disruption leading to an increase in rate of depolarisation of the bacteria and effective death. The entire process would take few mins for the most potent of the peptides. None of the model systems are better than the other, primarily due to the fact that certain peptides can act on the membrane only in a particular manner (175).

1.4.2 Mechanism of action of non-disruptive peptides

Certain peptides act on the cytoplasm rather than on the membrane itself. This mechanism occurs by translocation across the membrane in a fashion similar to that of micellar aggregation (176). Similar studies have suggested that arginine rich residues translocate across the cellular and nuclear membrane and can also serve as vectors for conjugated compounds. In the microbial cytoplasm, they are thought to have interacted with the nucleic acids of the organism to inhibit or slow down the

synthesis of these components. Other studies have also shown the inhibition of macromolecular synthesis after treatment with peptides of sub lethal concentrations. Certain other peptides have also been shown to have specific inhibition strategies wherein there is inhibition of chaperone assisted protein folding or inhibition of peptidoglycan biosynthesis. Examples include pyrrhocoricin and mersacidin (175).

The conformation of the peptide also defines its antimicrobial activity. β-sheet peptides are more membrane disruptive as they have an affinity towards lipopolysaccharides, while their translocation capabilities may be much poorer. Their route of activity for translocation could be due to micellar aggregation (177). They allow a certain degree of flexibility to their rigid pattern and hence propagate translocation.

The α-helical pattern is characterised by the presence of a small bend in the centre of their molecule that is important for anti-hemolytic activity. An example for α-helical sheet is magainin 2, which uses micellar aggregate model as a template for antimicrobial activity. These magainins interact with negatively charged phospholipids forming pores which allow translocation (178). The method of antimicrobial activity is identified as the formation of a voltage induced channel for disruption of the cytoplasmic membrane. Membrane potential could be the source of voltage (179).

The loop peptides have a single bond formed of disulphide, amide or isopeptide. Thanatin is a good example of this class. Their mechanism of action include targeting structures other than membranes and arrests motility of the bacterium (175).

1.5 Amyloid beta as an antibacterial peptide

Apart from increased brain concentrations of Aβ being linked with the progression of Alzheimer's disease and forms plaques in these patients (180), it has also been shown to exhibit antimicrobial properties. Some of the attributes of antimicrobial activity could be because of its similarity to LL-37 that includes formation of cytotoxic soluble oligomers and insoluble fibrils (181). According to one study (182), Aβ showed activity that is comparable to LL-37. The activity between two

isoforms of Aβ (Aβ-40 and Aβ-42) differed against 6 microorganisms. Aβ-42 showed greater potency compared to Aβ-40 (182).

Although, it is important for AMPs to be cationic to attach themselves to anionic bacterial membranes, Aβ is anionic and therefore not in keeping in line with the normal characteristics of AMPs. Nevertheless, microscopy data from Soscia *et al* (182), shows Aβ attaching to the bacterial membranes of *E.coli, Candida albicans, Staphylococcus epididermis* and *Streptococcus pneumoniae*. This suggests that it binds and disrupts the lipid bilayers and the mitochondrial membranes. Also, structural studies on some anionic membranes have suggested that an overall positive charge is not mandatory for the attachment of the peptide to bacterial membranes, instead there is more concentration on the peptide charge distribution and secondary conformation (183).

To substantiate the hypothesis that Aβ does demonstrate antimicrobial activity, tests were also performed from human brain. An increased antimicrobial activity against *Candida albicans* was seen in the temporal lobe of Alzheimer's patients in comparison to non-AD patients. This finding also opens avenues for reduction of Alzheimer's as there is a constant trigger of the immune system as it is affected by pathogens. The innate immune system utilises the inflammatory pathway to modulate Aβ accumulation (182).

Other researchers also suggest that a range of amyloid proteins have antimicrobial activity. Islet amyloid polypeptide, another toxic amyloid oligomer, has cell penetrating activity and can target mitochondrial membranes (181)

Other amyloids that are antimicrobial proteins include serum amyloid-A which is comprised of apolipoproteins related to high density lipoprotein that shows permeabilisation of bacterial membranes. They have been known to be converted into amyloid like fibrils *in-vitro* causing detoxification of the peptides. Yet another antimicrobial peptide is lysozyme that has also shown an affinity for forming amyloid fibres and deposits (184-186).

Recent reports have also suggested that there is a possible secretion of Aβ-40 and 42 from the most popular model for intestinal epithelium studies *in-vitro*, Caco-2.

According to Puig *et al.,* (187) upon stimulation with lipopolysaccharide (LPS), an increased production of Aβ-42 than Aβ-40 from Caco-2 monolayers was seen. This showed that these peptides could be released *in-vitro,* suggesting a possible increase in the gut more than in the brain, upon stimulation. These reports also open a new avenue for better therapeutic delivery for Alzheimer's. Aβ may also be involved in the modulation of the transport activity of Caco-2 or its tight junction formation

1.6 Gastrointestinal cells

1.6.1 Caco-2 cells

Caco-2 cells are derived from the carcinoma of the epithelium of the colon. They have been increasingly used in pharmaceutical applications and in academic research to study intestinal permeability coefficient. The drug transporters and nutrient transporters that are present in these cells facilitate studies relevant to carrier mediated uptake and efflux mechanisms. The importance of Caco-2 cells as a determinant for oral bioavailability for drug substrates is because of the presence of P-gp and its role as a barrier in the intestinal absorption of drugs (52). P-gp is present in the apical side of the Caco-2 cells and it mediates efflux mechanism from the basolateral to apical side (188). To observe transport studies Caco-2 cells are cultivated for more than 2 weeks on permeable polycarbonate filters where polarised monolayers with apical brush border and well-developed tight junctions are formed (188). The functional polarity and transport activity of Caco-2 monolayer is indicated by the presence of domes that may be achieved after confluence. In comparison to other colon carcinoma cell lines, Caco-2 has shown better morphological and functional enterocytic differentiation at par with the human intestine (189).

Caco-2 cell lines are considered to be an excellent *in-vitro* cell line model for studying transcellular pathway and simulates drug permeation route in the intestine (190). The rate of absorption is much slower in Caco-2 than in the human jejunum though at rates 30-80 times slower. Also, in the Caco-2 cells the rate of drug transport is much lower in comparison to the intestinal epithelium (191). The

discrepancy is related to differences in the permeability ratio of the paracellular pathway and in the absorptive surface areas (190).

1.6.1.1 Transport activity of P-gp in Caco-2 cells

Caco-2 cells are differentiated in a mosaic pattern, where the enzyme activities are present (192). The value of trans epithelial resistance (TEER) tend to be greatest after 3 weeks in culture, although the expression of tight junction associated protein in the mRNA and protein level peaked at 4 weeks in one study (192).

The formation of a functional tight junction may not always be in conjunction with relevant protein expression. As a transporter protein, P-gp activity is increased from day 17-27, when seeded on polycarbonate filters. According to Behrens *et al.,* (193) the seeding densities of Caco-2 cells also acts like a marker for the expression of MDR1. A culture period of two weeks is necessary for the transport studies of major substrates of the active efflux proteins like P-gp, BCRP and MRP. The higher the passage rate, the higher the production of P-gp, although subculturing at late confluency may decrease its rate (194). When Caco-2 cells are cultured in a medium along with an inducer, the rate and expression of these transporters also increase subsequently (49). The induction conditions may increase paracellular permeability, thus producing a much better *in-vitro* model as Caco-2 cells themselves have tighter junctions than the intestinal epithelium they are modelling (195, 196). Also, the expression of P-gp on Caco-2 cells may show variation depending on the culture flasks they are grown in. Polycarbonate flasks have been shown to have higher expression of P-gp than other types of plastic flasks when growing the cells (197). Trypsinisation of the cells before they reach confluency , may also increase P-gp expression levels, however trypsinisation for a long time of over 10-20 mins may not necessarily influence P-gp expression (197).

According to Belliard *et al.,* (198), cytokine stimulation or treatment may modify the localisation of P-gp to nuclear or golgi apparatus membranes and also the cytoplasmic vesicles of multidrug resistance cells, where drugs are transported and concentrated into vesicles. This might explain an alteration in P-gp efflux activity during cytokine treatment (198) .

1.6.1.2 Comparison with the small intestine

With the exception of BCRP, most of the efflux proteins differ by less than 2.5-fold in Caco-2 compared to the intestine as a whole. Only BCRP showed a 100-fold lower expression in Caco-2 than in the human jejunum (49). The differences between this adenocarcinoma cell line and the human intestine are also dependent on the presence of certain transporters like MRP2 and BCRP, while P-gp has shown a great degree of similarity (193). According to Engman *et al.,* (199), there is a good linear correlation between Caco-2 cells and jejunum, which is the prime area for oral absorption. This has been shown through studies conducted in the presence of various different efflux proteins present in Caco-2 cells.

Some of the advantages of using Caco-2 cells as a model for efflux protein activity are that they have abundant P-gp in the cells and they simulate the human enterocytes, with respect to differentiation. Some of the disadvantages of using a Caco-2 cell model are its wide interlaboratory variability and poor CYP3A4 activity (200). Caco-2 cells are modulated by nuclear receptors and ligand treatments. Although, the changes might be seen at the functional level, it may not be prominent at the level of mRNA, indicating that Caco-2 cells are difficult to modulate, suggesting its stability (200).

1.6.2 LS174T cells

LS174T is an adenocarcinoma cell line, which is produced by trypsinising the parent cell line LS180. This variant of the parent cell line comprises of oval to polygonal cells of 20-40 µm in diameter. Giant cells may also be present. The colonies are observed to be loosely packed and separated from each other representing an island. The nuclei are oval with a loosely packed and delicate nuclear membrane. They are more easily sub cultivated than the parent cell line LS180 and produce equal amounts of its most significant marker, carcinogenic embryonic antigen (CEA) (201). They have been used as an *in-vitro* model to confirm the induction of P-gp and other active efflux transporters controlled by the PXR. PXR induction is readily observed in LS174T unlike Caco-2 cells thus making LS174T a better human gastrointestinal cell line for PXR induction related studies, even though LS174T

cells are not capable of generating tight junctions thus preventing bidirectional transport studies (202). Proliferative and invasive studies of LS174T suggest that these cells have a nonpolarised and disorganised morphology also confirming their inadequacy to produce tight junctions. In addition to being models for induction studies, these cells are also known for their mucin production. Mucin is produced by the gastrointestinal cell lines and show effective protection against microbes. The active cell cultures exhibit mucin in their intra cytoplasmic vacuoles. Although, they have shown significant results for mucin production, they show little or very fluctuating trans epithelial electrical resistance (203). The proliferative and invasive properties of LS174T are higher than that of Caco-2. Although this cell line does mimic an intestinal epithelium, the colonic glandular crypt on the surface of LS174T do not appear similar to the intestine, instead having a more malignant phenotype (201).

1.6.3 RKO cells

RKO Cell lines are colon cancer cell lines, with no production of P-gp and minimal production of BCRP and hence can be used as a human intestinal cell line that is negative for P-gp (204). RKO cell lines have been used to study other observations like VEGF induction after hypoxia. VEGF is expressed at a much higher rate in colorectal cancer than metastatic cell lines (205). RKO cell lines have also been used in various studies involving apoptotic assays of medicinal plants (206).

1.6.4 HeLa-MDR1off cell line

The HeLa cell line is a human cervical cancer cell line used as a negative control in this study. It was donated by Professor Gottesman from NIH (Laboratory for Cell Biology), Bethesda, Maryland, USA. It is cloned with the MDR1 gene and hence expresses ABCB1 in high levels. Removal of colchicine and addition of tetracycline for three days, reduces the mRNA levels of P-gp by 300 fold (207). Such transfected HeLa cells have also been used as a good model to demonstrate drug removal from cells using the fluorescent substrate of MDR1, Rhodamine-123 (208).

1.7 Background

Studies have emphasised that a reduced expression of P-gp in the intestine, makes the gut a conducive environment for pathogenic microorganisms. Inflammatory bowel diseases including ulcerative colitis and Crohn's disease are also proposed as some of the disorders in the intestine related to occur when there is reduced expression of P-gp (209). Reduced expression could be attributed to the presence of MDR1 in the 7q chromosome,which may also be linked to IBD and different gene polymorphisms in the MDR1 gene (210). Schwab *et al.,* had first reported a link between the C3435T polymorphism in ulcerative colitis, however, Glas *et al.*, showed that the data may have not been statistically significant (211, 212). Yet another polymorphism that could be seen in IBD was G2677T/A reported by Brant and colleagues (213). However, many studies after this showed inconsistent correlation between this polymorphism and IBD (214, 215).The reason could be credited to different population sizes, selection of the control population and incomplete phenotype description.

Different antimicrobial peptides may be seen to be upregulated or downregulated in the presence of IBD (216). HBD-1, HD 5 and 6 have shown to be reduced in colonic Crohn's disease. However, Cunliffe and colleagues have associated HD-5 in the colonic crypt region of IBD samples, possibly due to Paneth cell metaplasia in inflamed colon (120). In contrast, HBD-2, HBD-3 and LL-37 have shown to be increased in ulcerative colitis (217, 218). The reason for reduced alpha defensins and HBD-1 can also be attributed to mucosal loss in Crohn's disease.

Changes in P-gp expression affecting bacterial attachment have also been indicated in our laboratory, with Caco-2 and LS174T cell lines. In the presence of an elevated P-gp expression, the attachment of most bacteria to the cell membrane is drastically reduced, while in the presence of a P-gp blocker like PSC-833, an increase in the attachment is observed. This could correlate with increased production of endogenous levels of an antimicrobial factor by mammalian cells or the activity could be because of a known AMP in the cells that has unpublished P-gp link.

The largest known substrate for P-gp currently is gramicidin, at 1.9 kDa. However all known antibacterial compounds are larger than this. For this reason, the

suggestion that P-gp effluxes one or more of these antimicrobial compounds in defence of the gastrointestinal system warrants further elucidation.

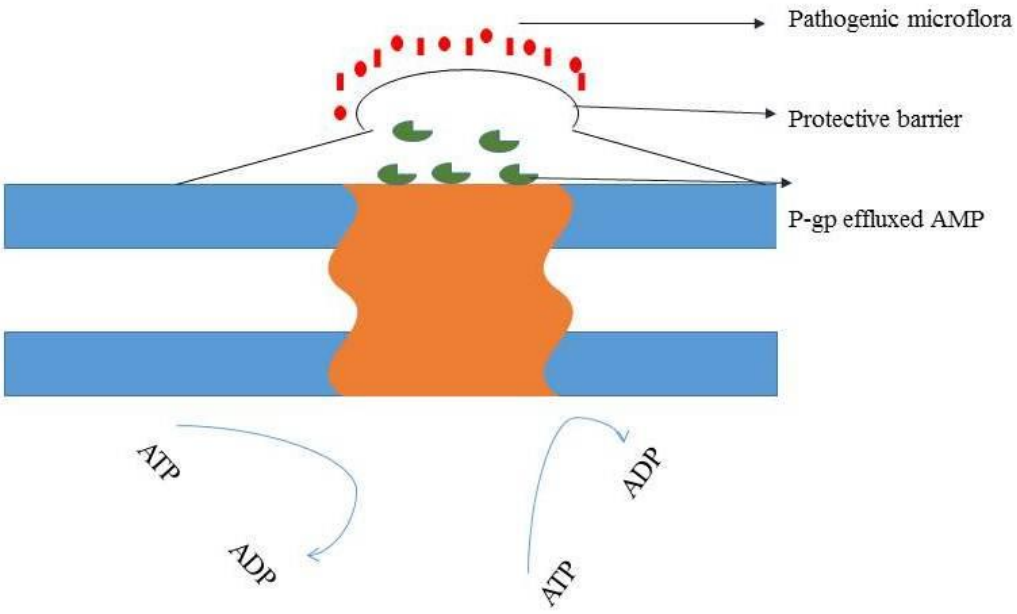

Figure 1.3: P-gp as a barrier against microbes: In the intestinal cells P-gp effluxes out its endogenous substrates that also acts as an antimicrobial peptide protecting the gut mucosa against invading pathogens.

1.8 Aims and objectives

1. **Development of gastrointestinal cell lines in defined media:** For stringent isolation of the low molecular weight antimicrobial factor from conditioned medium, an environment with reduced presence of larger proteins, like albumin is required. Low molecular weight fraction were collected as the hypothesis suggested some antimicrobial activity linked to P-gp efflux and all currently known substrates of P-gp are below 3 kDa. For the purpose of isolation the cell lines were cultured in serum free and reduced serum growth medium along with other growth factors like insulin-transferrin and selenium. Serum contains a large proportion of albumin that adsorbs to low molecular weight peptides. Expression of P-gp was primarily monitored to look for changes that that may occur when cultured in different concentrations of serum.

2. **Evaluation of antimicrobial activity in gastrointestinal cells:** Antimicrobial activity was evaluated in the low molecular weight fraction from cells cultured in growth medium with only 2% FCS. Different methods of separation were included to isolate the factor from a complex sample such as conditioned medium that contains many different proteins. Conditioned medium from gastrointestinal cells was fractionated using size exclusion chromatography and the fractions were also sent for protein sequencing for detecting endogenously produced antimicrobial factors. A comparison between samples that were treated with a P-gp inducer, rifampicin and non-induced samples were studied to demonstrate changes that occur in the activity of P-gp when induced.

3. **Investigation and determination of defined antimicrobial peptides in gastrointestinal cell line:** Although endogenously produced peptides were observed, known antimicrobial peptides in the gastrointestinal tract were not amongst them. In order to comprehend if known peptides would be stimulated in the presence of a microorganism, these antimicrobial peptides were observed in conditioned medium samples from cell lines stimulated with a bacterial stimulus such as lipopolysaccharide.

4. **Substrate efficacy of potential P-gp substrates:** Previous literature and results from the study suggests that Aβ could be a substrate for P-gp even though this is not one of the smaller AMP and is closer to 4.5 kDa than 3 kDa. Therefore studies were performed to illustrate substrate efficacy of P-gp and Aβ through transport experiments conducted on Caco-2 cells.

Chapter 2: Characterisation and development of gastrointestinal cell lines in defined media

2.1 Rationale

There is evidence that P-glycoprotein (P-gp) expression is associated with antimicrobial activity. Studies from Dr Crowe's laboratory have suggested that inhibition of P-gp in Caco-2 and LS174T cell lines are linked to an increase in the attachment of bacteria to the cells. Inducing P-gp saw a dissociation of the microbes from the cell lines (108, 219). As P-gp has not directly shown any known antibacterial activity, a substrate of P-gp with possible antimicrobial characteristics could be responsible for the observed action. In order to isolate the factor responsible, cell lines were grown in reduced Foetal Calf Serum (FCS) and FCS free medium. FCS has variable composition and its usage makes it difficult to isolate effluxed low molecular weight (LMW) proteinaceous active substances from the conditioned medium (CM). This is because of the presence of albumin that is known to adsorb low molecular weight proteins, making their separation from LMW difficult. For stringent isolation of these proteins, replacement of FCS with chemically defined medium was proposed. The basis of the project is to identify and isolate low MW peptides secreted by human gastrointestinal cells and its affinity towards the efflux transporters. Firstly, determining how low a percentage of FCS could be achieved while retaining cellular integrity and expression of P-gp and other transporters was required. To validate the hypothesis, three different gastrointestinal cell lines were chosen that have varying levels of P-gp and breast cell resistance protein (BCRP).

2.2 Introduction

2.2.1 Gastrointestinal cell lines as a model for ABC transporter related activity

Caco-2 cells are derived from the carcinoma of the epithelium of the colon. In post confluence cultures, the cell layer exhibits structural and functional differentiation patterns characteristic of mature enterocytes in which the cell layer is covered by brush border microvilli. Enzymes in the brush border show mosaic expression in confluent Caco-2 cells. They have brush border hydrolase and develops tight layer cells within 2-3 weeks of achieving confluency (220). The cell line undergoes

spontaneous differentiation and expresses many functional and morphological characteristics of an intact small intestine (220).

In comparison to other gastrointestinal cell lines, Caco-2 has shown better morphological and functional enterocytic differentiation. Hence they are routinely considered as an excellent *in-vitro* cell line model for absorptive enterocyte studies (189). They are also used for studying transcellular pathways and simulates drug permeation route in the intestine. The rate of drug transport in Caco-2 cells are positively associated with *in-vivo* absorption for lipophilic drugs (190, 191). However, as tight junctions in Caco-2 cells are stronger than the enterocytes of the gut, hydrophilic drugs that have a paracellular component to their transport are observed to have a relatively lower rate in these cells in comparison to human patients (221).

P-gp and other active efflux transporters are present on the apical side and mediate efflux action from the basolateral to apical side. P-gp acts as an efflux pump by secreting drugs from the cell interior outwards into the intestinal lumen. This activity runs parallel to the absorptive transport of drugs hence restricting absorptive transport. *In-vivo* studies have also reported that in the presence of P-gp inhibitors and in P-gp knockout mice, drugs shown to be substrates of the same had better absorption (222-224).

The efflux transporters are present in both Caco-2 and functional human gastrointestinal tract. The level of P-gp is similar in both, but Caco-2 expresses lower concentrations of multidrug resistance protein (MRP2) and BCRP than the gut. Nevertheless, having lower MRP2 and BCRP makes the Caco-2 cells a good model for investigating the significance of P-gp (225). However, the cell line is heterogeneous. Cultures of Caco-2 cells of different clonal origin contribute to the heterogeneity of the cells. This makes comparison of direct numbers from multiple laboratories difficult, however trends in data are similar (226). Different culture conditions also affect the transport of substrates of Caco-2 cells and modulate certain functions of the cells including proliferation, permeability and differentiation (225). Under routine conditions, the Caco-2 cell line develops numerous features characteristic of intestinal cells. Hence, for studies relating to bioavailability and

biodiversity of drugs, it is important to have a transporter system with the same efficiency as that in the intestine to allow interpretation of *in-vivo* environment (225).

The regulation of P-gp expression is executed by a major transcription factor, pregnane X receptor (PXR). Induction of PXR leads to over expression of P-gp, thereby decreasing the bioavailability of drugs taken orally. A suitable example of a PXR inducer is rifampicin. While Caco-2 maybe used in absorptive studies, variability of P-gp composition and low PXR expression is a limitation when studying regulation. Hence, LS174T cell line can be used for research into regulation and other molecular aspects of intestinal P-gp instead, as its high PXR expression makes it highly suited for P-gp induction with various drugs (3, 227). RKO cells are another colon carcinoma cell line exhibiting no P-gp expression. Therefore, it has been used in the present study as a negative control (219).

Amongst the sources of variability in the activity and differentiation of Caco-2 cells, a dominant component is the medium that also includes FCS.

2.2.2 Influence of Foetal calf serum on LMW peptide isolation

Addition of FCS in the growth medium of cells is an established method for successful growth of cells and helps maintain them in continuous cell culture. FCS is known to provide many growth factors, hormones and attachment factors for the growth and propagation of cells (228). The source of its nutritional ability has not yet been isolated to any single factor even though there have been studies to derive such factors. Being a natural product derived from various conditions, FCS is variable in its composition. Indeed some of the components have not yet been elucidated, reducing even the scope for measuring variability (228). Despite FCS being a major supplement, reports have suggested that its nutritional capacity decreases over time (229).

Reduction in the percentage of FCS in the growth medium is beneficial as it may reduce the variability in the proteins derived from the CM and expense (230). FCS can affect the phenotypic stability of cells and affect the cells at the gene level. The addition of FCS also affects the experimental outcome while using the cells. While

FCS may be a source of growth and attachment it may also be a source of contamination containing endotoxins and other products of microbes and prions during its collection (228).

Isolation of low molecular weight proteins from the secretome has often been futile due to the major large protein present, albumin. Albumin makes up half of the serum proteins. Addition of FCS to DMEM results in albumin adsorbing other proteins present, thereby making their isolation very difficult. Although there have been many chromatographic methods described, binding of albumin to the stationary phase is marginal and hence it is not eluted in one fraction instead being adsorbed to the eluted large proteins.

2.3 Aim

The aim of the present chapter includes:

- Analysis of protein expression of three ABC transporters namely, P-gp/MDR1, BCRP and MRP2
- Determination of mRNA changes of ABCB1 (P-gp) and ABCG2 (BCRP).
- Assessment of TEER values of Caco-2 cells cultured in different concentrations of FCS.
- Evaluation of a representative tight junction protein in Caco-2 cells cultured in different concentrations of FCS.

2.4 Materials

The following materials were procured from Gibco by Life technologies, Victoria, Australia for cell culture maintenance. Dulbecco Minimal Essential medium (DMEM), 1% glutamine, 1% MEM Vitamin solution, 1% MEM Non-Essential Amino Acids (NEAA) and 1% Penicillin/Streptomycin (Pen/strep), Insulin-transferrin-selenium-ethanolamine (ITS-X) and 10% FCS (FCS-001-AU serANA Pty Ltd, Australia). Trypsin was procured from ($Tryp^{LE}$ Express, life technologies, Victoria, Australia). Materials for lysis buffer were obtained from the following: Sodium chloride (Chem-supply, SA, Australia), SDS (Ajax, NSW,Australia), Nonidet P-40 (NP-40), Phenylmethylsulfonyl fluoride (Pmsf) Sigma-Aldrich, Castle Hill, Australia, Tris-Hcl (J.T Baker, NJ, USA) Pierce Protease inhibitor mini tablets (Thermofisher, IL, USA). For preparation of Lowry reagent, the following materials

were obtained from Chem supply, SA, Australia. $CuSo_4.H_2O$, Na-K-Tartarate, Na_2CO_3 and Sodium Hydroxide (NaOH). Folin's reagent was obtained from BDH, Australia. Western blotting was done using the following materials: The following were obtained from Novex Nupage, CA, USA. MOPS, Transfer buffer, Sample buffer, Reducing agent, Antioxidant, 4-12% Bis-Tris gel Tris Hcl was procured from J.T baker, NJ, USA Sodium chloride from Chem-supply,SA, Australia, Tween-20 from Sigma-Aldrich, Castle hill, Australia, Methanol from (VWR chemicals, QLD, Australia). Transfer membrane, immune-blot PVDF, was obtained from Bio-Rad, USA. Primary antibodies for the different transporters were obtained from Santa cruz Biotechnology, Inc, USA: MDR1, G-1 (sc-13131), ABCG2, B-25 (sc-130933) and MRP2 was obtained from Abcam (PA, USA, ab172630). HRP-conjugated secondary antibodies, goat anti-mouse and goat-anti-rabbit were obtained from Jackson antibodies (PA, USA). Monoclonal anti-human β-actin was procured from Sigma-Aldrich (Castle Hill, Australia). Materials for RNA isolation were procured from Sigma-Aldrich for the following: Trizol reagent 1-bromo-3 chloropropane, 75% ethanol (Fisher-biotech, Victoria, Australia) and DEPC-treated water (Thermofisher, Victoria, Australia). High-capacity cDNA reverse transcription kit for cDNA synthesis was procured from (Applied Biosystems, Victoria, Australia). Materials for RT-PCR were obtained from Thermofisher. Primer sequences were procured from Gene works (SA, Australia). Tris base, glacial acetic acid and molecular grade EDTA were obtained from Sigma Aldrich, Castle-Hill, Australia. Molecular grade gel was obtained from Amresco agarose-biotechnology grade and loading dye was obtained from Nengaland Biolab. For confocal microscopy the following material were obtained: Sterile 6 well plates (Nunc, Denmark), Coverslips, 4% paraformaldehyde (Sigma-Aldrich, Castle Hill, Australia), 0.1% Triton-X 100 (Sigma-Aldrich, Castle-Hill, Australia). Goat serum (Life technologies, Victoria, Australia). Primary antibody, Anti-occludin mouse anti-human and Fluorescent Alexa flour 488 anti-mouse IgG (Abcam, PA, USA), 4',6-Diamidino-2-phenylindole-dihydrochloride (DAPI) (Sigma-Aldrich, Castle-Hill, Australia), Vectashield (Vector laboratories, QLD, Australia).

2.5 Methods

2.5.1 Cell line maintenance

The cells were maintained for the first three days in growth medium with 10% FCS at 37ºC, 5% CO_2. Thereafter, the cells were replaced in medium containing 1% glutamine, 1% Vitamin, 1% NEAA and 1% ITS-X. The cells were cultured in FCS free (0%) and reduced FCS medium (2%) separately. ITS-X was added to compensate for the loss of FCS that is normally added to the medium. Nevertheless, the amount of ITS-X added to 0% and 2% FCS medium was the same. Medium was replaced every third day. An incubation time of 24 days was put in place to allow full maturation and differentiation of the cells. Other gastrointestinal cell lines, namely, LS174T and RKO were cultured in a similar way but their incubation time was only 8-10 days due to their inability to form polarised or differentiated cells. The cells were usually confluent in 3 days. After a delayed slow induction of growth, the cells eventually grew to confluency. Trypsinisation of the cells was done at 80% confluency. HeLa–MDR 1 OFF cells was transported with colchicine and with only 1% glutamine, as the cells did not need other supplements to exhibit strong P-gp expression and sustained cell growth. In the current study, colchicine was removed from the medium as by itself it can exhibit antimicrobial properties and would interfere with interpretations from any secreted product in the CM (231). Also as colchicine was only used in the growth medium of HeLa-MDR1 OFF to switch off P-gp expression, and as the cell line was used as a positive control in the study, it was excluded from the medium for all work in this project. All the cell lines were routinely tested for mycoplasma and were always mycoplasma negative (refer appendix I).

2.5.1.1 Cryopreservation of cells

Freezing of cells

Cells were maintained as described above in medium containing 10% FCS. Trypsin was used as the detaching agent for harvesting cells. Cells were incubated with trypsin and sterile calcium and magnesium free PBS at a 1:1 ratio up until the cells were detached completely from the flask. The cells were centrifuged at 335xg for 7

mins in an Allegra^{R} X-12 centrifuge (Beckman, Coulter, NSW, Australia) with growth medium to block any further action of the trypsin. Supernatant was carefully removed by pipetting and the cells pellet was harvested accordingly. For freezing, the cells were suspended in cryo medium (70% DMEM, 20% FCS, 10% DMSO) and transferred into cryogenic vials (Greiner BioOne, Kremsmunster, Austria). The cryogenic vials were placed at -80ºC overnight in a cryoform container (to allow appropriate cooling rates) and then transferred into a liquid nitrogen dewar.

Thawing of cells

Cells in cryogenic vials were warmed by immersing the vial in 37ºC water bath. The thawed cells were then diluted with prepared growth medium in a 15mL conical tube and centrifuged for 7 mins at 335xg to remove DMSO. The supernatant was cleared and cells were suspended in warm complete medium. The cells were seeded in a 25 cm^2 flask and 3 mL of medium was added to the flask. It was incubated at 37ºC, 5% CO_2. The cells were monitored for a period of 24 hours and medium was changed after cells were completely attached to the flask.

2.5.2 Lysis buffer preparation

Table 2.1: Concentration of materials used for lysis buffer preparation

Materials	**Concentration**
Sodium Chloride	150 mM
SDS	0.1%
Nonidet P-40 (NP-40)	1%
Protease inhibitor	1 tablet per 10 mL lysis buffer
Phenylmethylsulfonyl fluoride (PMSF)	0.5 mM
Tris Hcl	50 mM

2.5.2.1 Cell lysis protocol

The cells were grown on 75 cm^2 flasks. The flasks were rinsed with PBS to remove any source of phenol red. 1 mL of lysis buffer (refer table 2.1) was added and the

cells were incubated for 30 mins. The cells were gently passed in and out repeatedly through a serological pipette in the flask and were transferred to a microcentrifuge tube, sonicated and centrifuged at 15000xg for 15 mins at 4ºC. The supernatant was collected in a different microcentrifuge tube. Cell lysates were stored at -20ºC for short term use or -80ºC for long term storage. Protein quantification was done using modified Lowry protein quantification assay.

2.5.3 Modified Lowry protein quantification assay protocol

For a 96 well plate Lowry reagent was prepared as follows:

1 part of CTC reagent

2 parts of nanopure H_2O

1 part of 0.8 M NaOH

10 µL of the sample was added along with 50 µL of water to make a final volume of 60 µL. 120 µL of the Lowry reagent (refer to table 2.2) was added after addition of samples and placed in a shaker for 15 mins. 60 µL of Folin reagent was added thereafter and the plate was placed on the shaker for 30 mins. The samples were read at an absorbance of 750 nm. Standard curve was plotted using Bovine serum albumin (BSA) at different concentrations ranging from 5 µg/mL to 1500 µg/mL.

Table 2.2: Concentration of materials for Lowry reagent

Materials	**Concentration**
CTC reagent- $CuSO_4.H_2O$ Na-K-Tartrate Na_2CO_3	 0.1% 0.2% 10%
Sodium hydroxide (NaOH)	0.8 M
Folin's reagent	0.5 N

2.5.4 Protein analysis by Western blot

Western blot protocol

Cell lysates after protein quantification were subjected to Western blot. 35 µg of the sample along with sample buffer and reducing agent was loaded onto 4-12% Bis-tris gels with MOPS running buffer in a gel tank (Novex) and run at 150 V for 60 mins. The gels were then transferred onto a PVDF membrane (0.2 µm) in transfer buffer containing 20% methanol at 34 V for 90 mins. The blots were blocked overnight with 2% casein in TBS at 4ºC. The blots were washed thoroughly twice the next day with TBST. Primary antibody for P-gp (MDR1) was used at the appropriate concentration and incubated in 9 mL of antibody buffer (1% casein in TBS) for 2 hours at room temperature. The primary antibody (refer table 2.3) was washed with TBST for 5 mins (4 times) each and then incubated with HRP conjugated anti-goat anti-mouse secondary antibody at a concentration of 1:7500 in 9 mL of antibody buffer (1% casein in TBS) at room temperature for 2 hours. The blot was washed with TBST four times for 5 mins each. Blots were exposed in a Chemidoc MP ™ using clarity (Bio-rad) as the HRP based chemiluminescent solution. The bands for MDR1 were normalised with β-actin as the protein control for semi quantitation.

To observe bands for BCRP, the procedure was repeated using BCRP primary antibody at the appropriate concentration (refer table 2.3) in 9 mL of antibody buffer and incubated for 2 hours. After washing with TBST four times for 5 mins each, the blot was incubated with HRP conjugated goat anti-mouse secondary antibody for 2 hours at room temperature. The bands for BCRP were detected and normalised with β-actin as the protein control for semi-quantitation.

To observe bands for MRP2, the procedure was repeated as above using MRP2 primary antibody at the appropriate concentration (refer table 2.3) mentioned in 9 mL of antibody buffer and incubated for 2 hours. After washing with TBST four times for 5 mins each, the blot was incubated with HRP conjugated goat anti-rabbit secondary antibody for 2 hours at room temperature. The bands for MRP2 were detected and were normalised with β-actin for semi-quantitation.

Table 2.3: Dilution of primary and secondary antibodies used in Western blot

Primary Antibody	**Dilution**
MDR1 (G-1), mouse monoclonal antibody	1:220
ABCG2 (B-25), rabbit polyclonal antibody	1:300
MRP2 (EPR10998) (rabbit) primary antibody	1:6500
Mouse anti-human Monoclonal B-actin(Protein control)	1:7500
Secondary antibody	**Dilution**
HRP-conjugated goat anti-mouse- Secondary antibody	1:7500
HRP-conjugated goat anti-rabbit- Secondary antibody	1:7500

2.5.5 RNA isolation

Cells were seeded in accordance to 2.5.1 and RNA was isolated when cells were grown to 80% confluence.

Cells were cultured upto day 24 for Caco-2 cells and for 8-10 days for LS174T and RKO cell lines in 25 cm^2 flasks. 1 mL of TRI reagent was added to the flasks and the cells were vigorously mixed using a 1 mL pipette tip. Care was taken to make sure that there was no cross reactivity as TRI reagent reacts with plastic. The mixture was transferred into a clean RNase, DNase free 2 mL microcentrifuge tube.

For every 1 mL of TRI reagent, 100 µL of 1-bromo-3-chloropropane was used. The microcentrifuge tube was shaken vigorously and allowed to stand for at least 2 mins at RT. The contents were centrifuged at 12000xg for 15 mins at 4ºC. This step is very important as it separates the mix into three phases, the red organic phase (containing protein), an interphase (containing DNA) and a colourless upper aqueous phase (containing RNA).

The upper aqueous phase was transferred to another clean microcentrifuge tube and 0.5 mL (for every 1 mL of TRI reagent) of 2-propanol was added to it. The tube was

allowed to stand at room temperature for 5-10 mins, vortexed and centrifuged at 12000xg for 10 mins at 4ºC. RNA was seen as a precipitate at the bottom of the tube. The supernatant was removed and the RNA pellet was washed by adding 1 mL (for every 1 mL of TRI reagent) 75% ethanol then vortexed and centrifuged at 7500xg for 5 mins at 4ºC. The pellet was left to air-dry after the supernatant was removed and the resulting dry pellet was resolubilised in 20 µL of nuclease free water. An absorbance ratio of $A_{260/280}$ was measured. For viable RNA it is necessary to have the ratio above 2.01. The concentration of cells was also measured and a quantity equal to 1 µg of RNA was subjected to cDNA synthesis.

2.5.6 cDNA synthesis

cDNA was synthesised from 1 µg/µL RNA using Applied Biosystem, high-capacity cDNA analysis kit.

The contents of the kit were mixed with the respective volumes in a clean RNase and DNase free thin-walled microcentrifuge tube and diluted with water to a volume of 20 µL. A cycle of 10 mins at 25ºC followed by 120 mins at 37ºC, followed by another step of 5 min at 85ºC which was later put on hold at 4ºC was done in a thermocycler (Thermofisher). After the cDNA was prepared it was subjected to a PCR reaction or stored in -20ºC for later use (refer appendix II for contents).

2.5.7 mRNA analysis by RT-PCR

RT-PCR was conducted with cDNA synthesised as described in 2.5.6.

The contents (refer table 2.4) were mixed together and diluted with nuclease free water to a volume of 25 µL. The volume of primers used was 1 µL each of the forward (F) and reverse (R) primer from a concentration of 5 µM (refer table 2.5). The samples were put onto a thermocycler (Thermofisher) using the following protocol: Denaturation at 94ºC-1 min, Annealing and extension at 60ºC-30 sx 40 cycles, 72ºC-5 min. The PCR product was cooled down to 4ºC and stored at -20ºC or -80ºC for later use.

Table 2.4: Concentration of materials used for RT-PCR

Materials	**Final concentration**
RT Buffer	1x
dNTP	200 µM
$MgCl_2$	1.5 mM
Taq polymerase	1U/µL

Table 2.5: List of primers used in analysis of gene expression

Gene	Primer sequences (5'-3')	Amplicon size	T_a (ºC)
ABCB1	AAA TTG GCT TGA CAA GTT GTA TAT GG- **F** CAC CAG CAT CAT GAG AGG AAG TC-**R**	84	60
ABCG2	AGA GCT GGC CCT TGT ACT CA-**F** TGC GTT TCA AAC TTG CTC AC-**R**	276	60
GAPDH	ACC ACA GTC CAT GCC ATC AC- **F** TCC ACC ACC CTG TTG CTG TA-**R**	365	60

2.5.8 Gel Electrophoresis

10 µL of the sample and 2 µL of loading dye was mixed together and loaded on to 1.5% agarose gel in TAE buffer that is subsequently stained with gel red (Biotium-gel red nucleic acid stain).

The gel was run at 65 V for 90 mins until the dye front had reached 80% of the gel. Post-run, the gel was viewed using a ChemiDoc MP ™ system (Bio-Rad) (refer appendix IV for concentration of materials used)

2.5.9 Staining of Caco-2 cells for detection of tight junction protein Occludin

Caco-2 cells were seeded at $3x10^5$ cells/well onto clean coverslip placed in 6 well plates.

The cells were cultured according to 2.5.1. On the 7^{th}, 17^{th} and 24^{th} day medium was removed and quickly rinsed twice with 2 mL of PBS. For fixation, 2 mL of warm 4% paraformaldehyde was added and incubated in the dark for 15 mins at RT. The washing step was repeated again twice with PBS. Post-fixation cells were permeabilised using 2 mL of cold 0.1% Triton X-100 (TX-100) dissolved in PBS

was added and incubated for 3 mins. Washing step was repeated. The cells were blocked with 2 mL blocking solution (10% goat serum and 1% BSA in PBS) and incubated at RT for 1 hour. Alternatively, they could be stored at 4ºC overnight. The cell seeded coverslips were then placed carefully in a new 6 well plate. It was incubated with primary antibody at a 1:100 dilution. After incubation of 1 hour the cells were washed with PBS 3 times for 5 mins each. They were then incubated with secondary antibody at a concentration of 1:200 for 1 hour in dark so as to protect the fluorescent tag attached to the secondary antibody from bleaching. After repeating the washing step using PBS 3 times for 5 mins each, 2 mL of DAPI in PBS was added at a concentration of 1:1000 and incubated for 5 mins. It was then rinsed with PBS. The coverslip was then placed with the cell side facing down on mounting media. After excess PBS was evaporated, coverslip was sealed and allowed to dry. Prepared cells were viewed under an inverted (A1+) confocal microscope (Nikon, Tokyo, Japan).

2.5.10 Statistical Analysis

All data are reported as a mean parameter determined ± standard deviation (S.D.) unless stated otherwise. The difference between the groups was determined using Student's t-test. A P-value ≤ 0.05 was considered as statistically significant.

2.6 Results

2.6.1 Morphological characteristics of cells

The cells were cultured in growth medium with 10 % FCS for the first four days for attachment and propagation of cells. Growth medium was then replaced with either 0% or 2 % FCS with an added presence of 1% ITS-X. Caco-2 cells were cultured for 24 days, LS174T and RKO cell lines were cultured for 10 days each. HeLa Mdr1-OFF cell line used as the positive control for expression of P-gp was cultured for 5 days and only in growth medium with 10% FCS. The cells were observed and medium was changed every third day. The cells exhibit minimal phenotypic changes. Caco-2 cells upon differentiation displayed a mosaic pattern of cell growth with well-developed tight junctions that was seen even in cells cultured in 0% FCS. Post-

confluent cells showed dome like appearance that was indicative of its effective polarisation and transport activity. Polarised cells also show brush border at their apical side. As a heterogenous cell line, Caco-2 cells also show different subpopulations of cell growth. LS174T displayed a disorganised morphology with cell colonies that are loosely packed and separated from each other. The cells were non-polarised and did not form tight junctions. Cells cultured in different percentages of FCS showed similarities in their morphology. The RKO cell line is a poorly differentiated carcinoma cell line. They do not appear to form dome like structures or tight junctions. HeLa MDR1 OFF cell line, is a transfected cell cervical cancer cell line with unoccupied cell surface and a condensed nuclear chromatin.

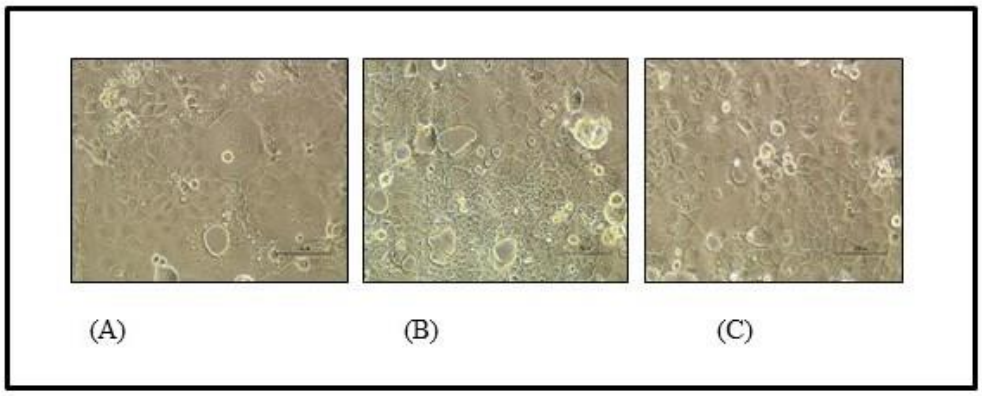

Figure 2.1: Phase contrast microscopic image of Caco-2 cells : Caco-2 cells were cultured in DMEM along with NEAA, Vitamins and glutamine (collectively called growth medium) in different concentrations of FCS. (A) cultured in growth medium with 0% FCS supplemented with ITS-X**.** (B) cultured in growth medium with 2% FCS supplemented with ITS-X. (C) cultured in growth medium with 10% FCS without ITS-X. Images were captured on day 24 using an inverted carl Zeiss phase contrast microscope (Scale bar-250 µm).

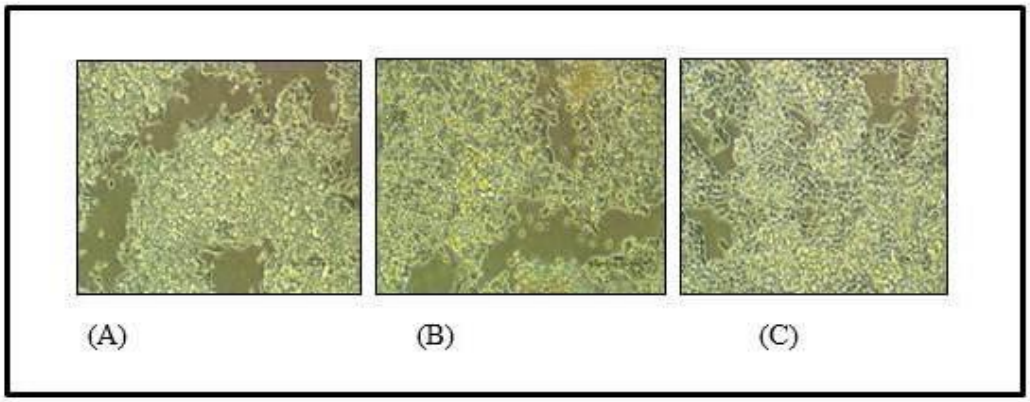

Figure 2.2: Phase contrast microscopic image of LS174T cells : LS174T cells were cultured in DMEM along with NEAA, Vitamins and glutamine (collectively called growth medium) in different concentrations of FCS. (A) cultured in growth medium with 0% FCS supplemented with ITS-X. (B) cultured in growth medium with 2% FCS supplemented with ITS-X. (C) cultured in growth medium with 10% FCS without ITS-X. Images were captured on day 10 using an inverted Carl Zeiss phase contrast microscope (Scale bar-250 µm).

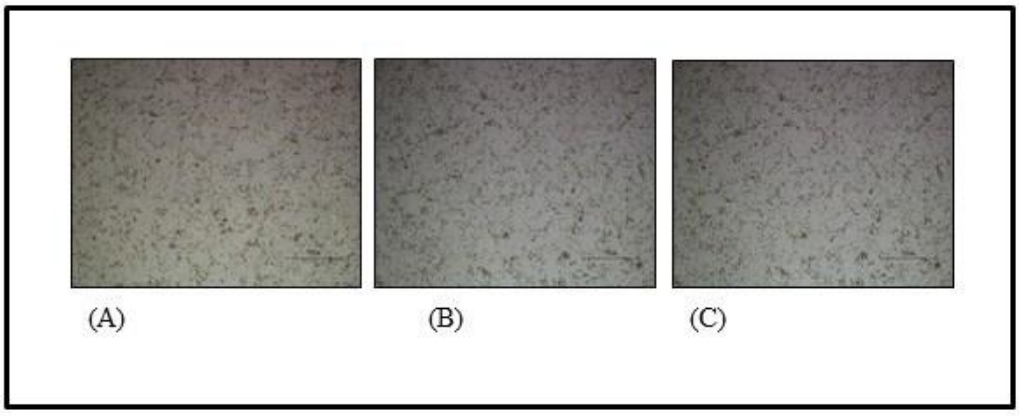

Figure 2.3: Phase contrast microscopic image of RKO cells : RKO cells were cultured in DMEM along with NEAA, Vitamins and glutamine (collectively called growth medium) in different concentrations of FCS. (A) cultured in growth medium with 0% FCS supplemented with ITS-X.(B) cultured in growth medium with 2% FCS supplemented with ITS-X. (C) cultured in growth medium with 10% FCS without ITS-X. Images were captured on day 10 using an inverted Carl Zeiss phase contrast microscope (Scale bar-250 µm).

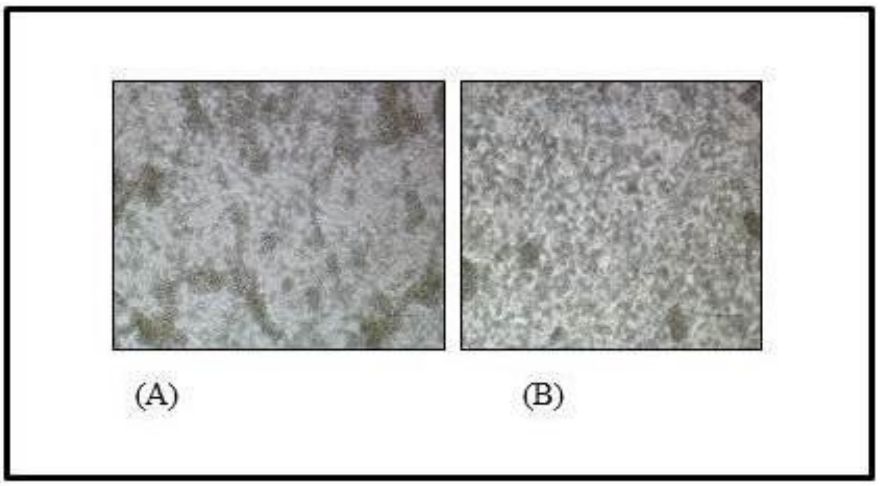

Figure 2.4: Phase contrast microscopic image of HeLa MDR1 OFF cells : HeLa-MDR1 OFF cells were cultured in growth medium with 10% FCS along with glutamine (with and without the addition of colchicine). (A) cultured without colchicine. (B) cultured with colchicine. Images were captured on day 5 using an inverted Carl Zeiss phase contrast microscope (Scale bar-250 µm).

2.6.2 Protein analysis of ABC transporters

35 µg of the cell lysate from various passages of Caco-2 cells (P-68 to P-81), LS174T (P-65 to P-76) and RKO cells (P-15 to P-25) were loaded onto 4-12% Bis-tris gels.

2.6.2.1 Expression of P-gp

Expression of P-gp in Caco-2 cells was reduced in medium with 0% FCS compared to cells cultured in 2% FCS. Late passages were used as the lab had already shown that cells from early passages (cells were purchased from ATCC at passage 20) were P-gp poor (232). β-actin was used as a protein loading control (Figure 2.5 (A)). Irrespective of the passage number, cells cultured in growth medium with 0% FCS showed nil or minimal expression of P-gp. Early passage cells expressed minimal P-gp expression in both 0% and 2% FCS. Maximal P-gp expression was seen at P-81 when cells were cultured in 2% FCS. The images were analysed using image lab software and compared to HeLa-MDR 1 OFF cells (Figure 2.5 (B)). LS174T cells

were only affected minimally by reduced FCS percentage in the growth medium. The highest expression of P-gp was seen in P-76, indicating that both the cell lines showed improved expression of the transporter past P-75. The expression of P-gp in LS174T cells was lower than in Caco-2 cell line at P-80. RKO cells did not express P-gp in any condition and was hence used as a negative control cell line.

2.6.2.2 Expression of BCRP

The percentage of FCS in growth medium did not affect the expression of BCRP. Low passage Caco-2 cells (P-68 cultured in 2% FCS) only showed 65% of expression in comparison to the control (Caco-2 cells cultured in 10 % FCS), but high passage cells (P-81 cultured in 2% FCS) demonstrated similar expression to the control. The expression of BCRP in LS174T cells was lower than in Caco-2 cells at P-80. The difference in expression between cells cultured in 0% FCS and 2% FCS was only minimal at all passages (Figure 2.6 (A)). Since P-gp and BCRP are both regulated by PXR, the expression levels may possibly follow the same course. The images were analysed using image lab software and compared to Caco-2 cells used as the control (P-80, cultured in 10% FCS) (Figure 2.6 (B)).

2.6.2.3 Expression of MRP2

The expression of MRP2 was not detected in LS174T cells. Only Caco-2 cells expressed MRP2. Low passage cells in growth medium with both 0% and 2% FCS did not show a high expression of MRP2 (P-68), while cells cultured in growth medium with 2% FCS at P-81 showed maximal expression of MRP2. Similar to the other transporters, MRP2 also showed best expression beyond P-80 (Figure 2.7 (A)). However, the cells did not express MRP2 as much as the control (Caco-2 in 10% FCS) and was only 55% of the control. The images were analysed using image lab software and compared to Caco-2 cells (P-80, cultured in 10% FCS) (Figure 2.7 (B)). Caco-2 cells cultured in 0% FCS did not show a regular pattern of expression of transporters. For P-gp expression, no band was seen in cells of P-68 (cultured in 0% FCS), while for MRP2 no band was seen in cells of P-76 (cultured in 0% FCS).

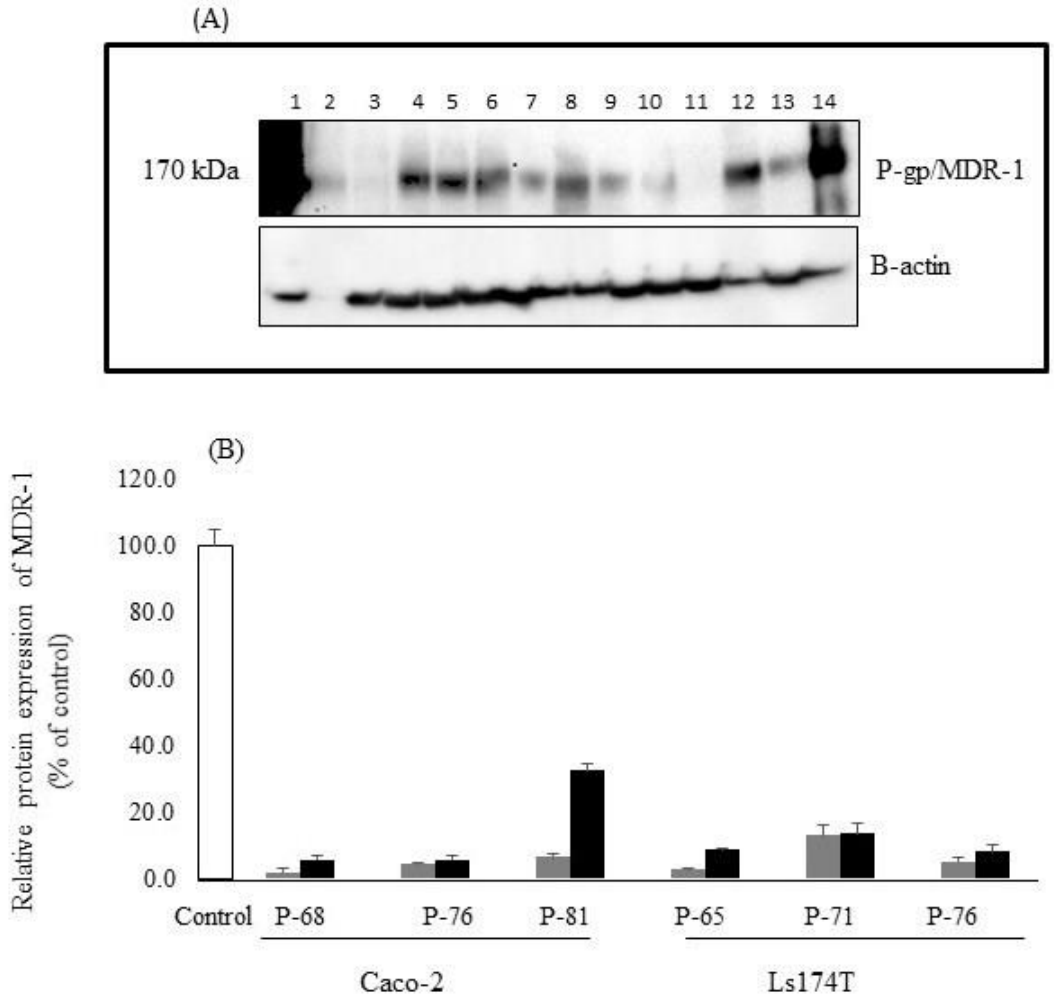

Figure 2.5: Western blot images of P-gp/MDR1 (170 kDa) in both Caco-2 and LS174T cell lines. (A) represents protein expression by Western blot.**1**-HeLa-MDR1 OFF (P-10) with colchicine (positive control). **3-8** represents LS174T cells cultured in growth medium and different concentrations of FCS. **3-4** Passage-65 cultured in 0% FCS and 2% FCS, respectively. **5-6** Passage-71 cultured in 0% FCS and 2% FCS, respectively. **7-8** Passage-76 cultured in 0% FCS and 2% FCS, respectively. **9-14** represents Caco-2 cells cultured in growth medium and different concentrations of FCS. **9-10** Passage-68 cultured in 0% FCS and 2% FCS, respectively.**11-12** Passage-76 cultured in 0% FCS and 2% FCS, respectively. **13-14** Passage-81 cultured in 0% FCS and 2% FCS, respectively. β-actin was used as the endogenous control (42 kDa) **(B)** Densitometry analysis of protein expression of P-gp/MDR1.The values were normalised to β-actin and compared to the positive control Caco-2 (P-80,10% FCS at 100%). Data represents the average expression of MDR1 (P-gp) ± SD (n=3).

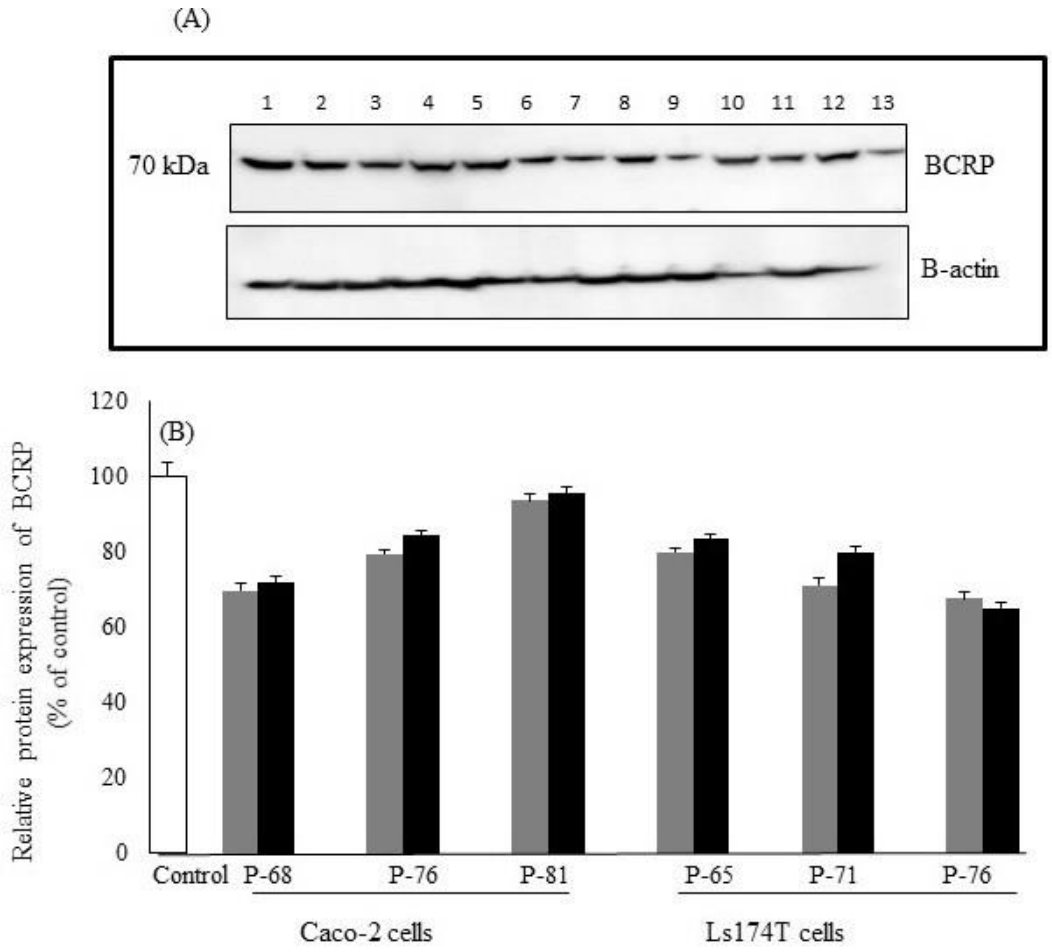

Figure 2.6: Western blot images of BCRP (70 kDa) in both Caco-2 and LS174T cell lines. **(A)** represents protein expression by Western blot. **1**-Caco-2 (P-80) with 10% FCS (positive control). **2-7** represents Caco-2 cells cultured in growth medium and different concentrations of FCS. **2-3** Passage-68 cultured in 0% FCS and 2% FCS, respectively. **4-5** Passage-81 cultured in 0% FCS and 2% FCS, respectively. **6-7** Passage-76 cultured in 0% FCS and 2% FCS, respectively. **8-14** represents LS174T cells cultured in growth medium and different concentrations of FCS. **8-9** Passage-65 cultured in 0% FCS and 2% FCS, respectively. **10-11** Passage-71 cultured in 0% FCS and 2% FCS, respectively. **12-13** Passage-76 cultured in 0% FCS and 2% FCS, respectively. β-actin was used as the endogenous control (42 kDa). (B) Densitometry analysis of protein expression of BCRP. The values were normalised to β-actin and compared to the positive control Caco-2 (P-80, 10% FCS at 100%). Data represents the average expression of BCRP ± SD (n=3).

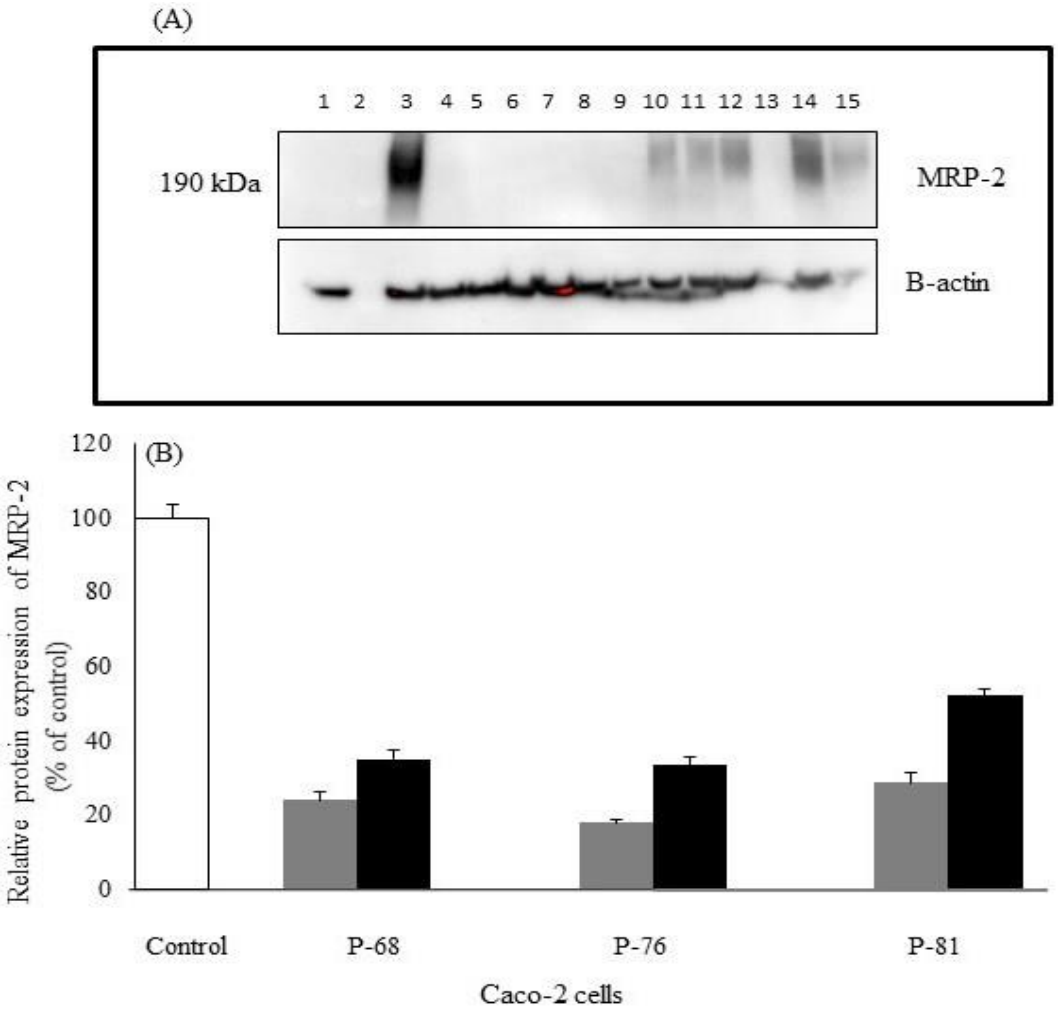

Figure 2.7: Western blot images of MRP2 (190 kDa) in Caco-2 and LS174T cell lines. **(A)** Represents protein expression by Western blot. **1**-RKO (P-18) with 10% FCS (Negative control). **3**-Caco-2 (P-80) in 10% FCS (Positive control). **4-9** represents LS174T cells cultured in growth medium with different concentrations of FCS. **4-5** Passage-65 cultured in 0% FCS and 2% FCS, respectively. **6-7** Passage-71 cultured in 0% FCS and 2% FCS, respectively. **8-9** Passage-76 cultured in 0% FCS and 2% FCS, respectively. **10-15** represents Caco-2 cells cultured in growth medium with different concentrations of FCS. **10-11** Passage-68 cultured in 2% FCS and 0% FCS, respectively. **12-13** Passage-76 cultured in 2% FCS and 0% FCS, respectively.**14-15** Passage-81 cultured in 2% FCS and 0% FCS, respectively. β-actin was used as the endogenous control (42 kDa). **(B)** Densitometry analysis of protein expression of MRP2. The values were normalised to β-actin and compared to the positive control Caco-2 (P-80, 10% at 100%). Data represents the average expression of MRP2 ± SD (n=3).

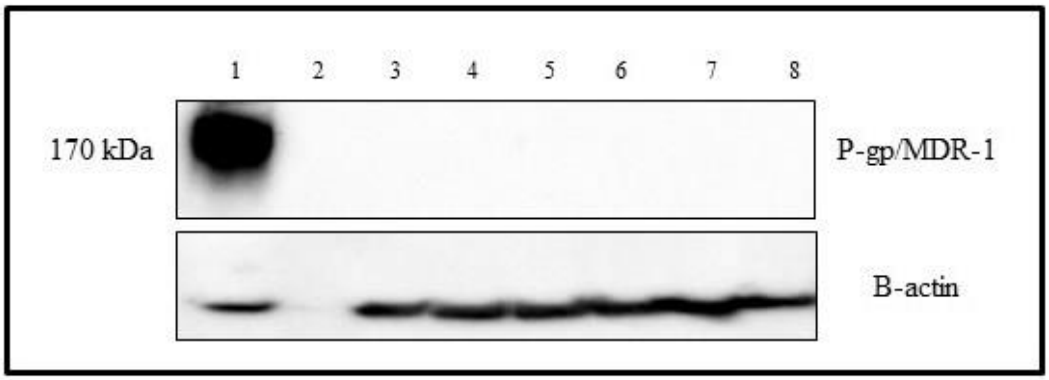

Figure 2.8: Western blot images of P-gp/MDR1 in RKO cell line. **1**- Caco-2 (P-80) in 10% FCS (Positive control). **3-8** represents RKO cells cultured in growth medium and different concentrations of FCS. **3-4** Passage-15 cultured in 0% FCS and 2% FCS medium, respectively. **5-6** Passage-20 cultured in 0% FCS and 2% FCS medium, respectively. **7-8** Passage-25 cultured in 0% FCS and 2% FCS medium, respectively.

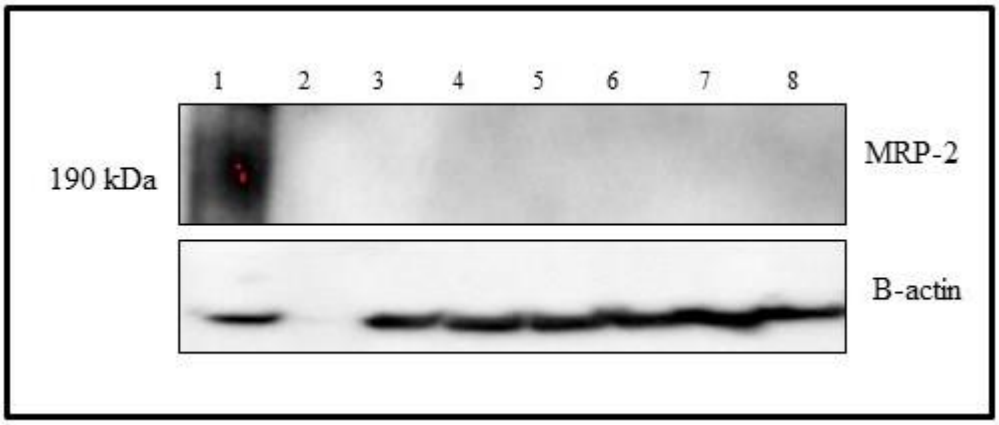

Figure 2.9: Western blot images of MRP2 in RKO cell line. 1- Caco-2 (P-80) in 10% FCS (Positive control). **3-8** represents RKO cells cultured in growth medium and different concentrations of FCS. **3-4** Passage-15 cultured in 0% FCS and 2% FCS medium, respectively. **5-6** Passage-20 cultured in 0% FCS and 2% FCS medium, respectively. **7-8** Passage-25 cultured in 0% FCS and 2% FCS medium, respectively.

RKO cell lines did not show an expression of both P-gp and MRP2 as indicated in Figure 2.8 and 2.9. Passages (P-15 to P-25) were used. The cell line was used as the negative control in our study as it did not express P-gp.

2.6.3 Gene expression of ABC transporters

The Caco-2 cells were cultured until the 24th day and RNA was isolated from the cells, cDNA was synthesised and subjected to RT-PCR using the appropriate primers for qualitative analysis of different gene expression. 10 µL of the PCR product was mixed with 2 µL of the loading dye and loaded onto 1.5% gel in TAE buffer. The bands were exposed to 286 nm on a transilluminator within a ChemiDoc MP ^{TM} dark cabinet. While the gene expression of ABCB1 was unchanged in the LS174T cell line (Figure 2.10 (A)), there appeared to be a constitutive reduction of its expression in the Caco-2 cells (Figure 2.10 (B)). The expression in cell lines grown in 0% FCS free showed minimal or no gene expression, while cells grown in DMEM with reduced FCS (2%) and 10% FCS showed significant expression of ABCB1. There were no changes seen in the expression of ABCG2 in either Caco-2 or LS174T cell lines irrespective of the FCS percentage.

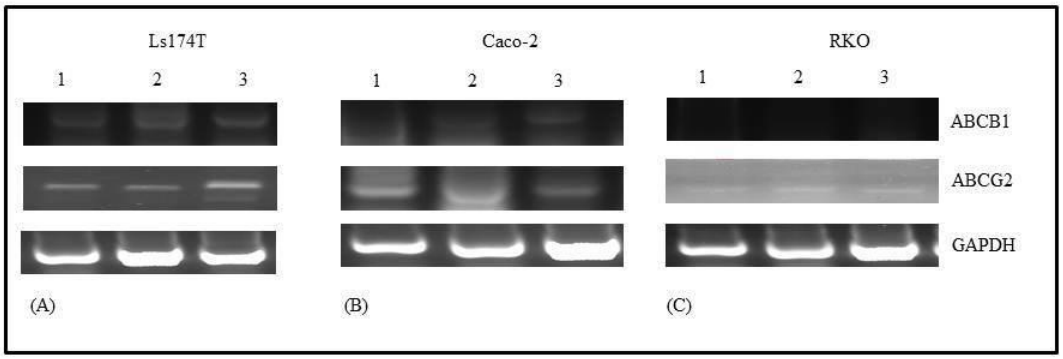

Figure 2.10: mRNA expression of ABCB1 (84 bp), ABCG2 (276 bp) in three different gastrointestinal cell lines. **(A)** Represents LS174T cells cultured in growth medium with different concentrations of FCS. **1-**cultured in 0% FCS. **2-**cultured in 2% FCS. **3-** Cultured in 10% FCS. **(B)** Represents Caco-2 cells cultured in growth medium with different concentrations of FCS. **1**-cultured in 0% FCS. **2**-cultured in 2% FCS. **3-** Cultured in 10% FCS. **(C)** Represents RKO cells cultured in growth medium with different concentrations of FCS. **1-**cultured in 0% FCS. **2-** Cultured in 2% FCS. **3**- Cultured in 10% FCS. GAPDH is used as the endogenous control (365 bp).

2.6.4 Bidirectional transport studies using a known P-gp substrate

Initially a bi-directional transport assay using millicell inserts was attempted to understand efflux of a known P-gp substrate (Rhodamine-123) in chemically defined medium. Caco-2 cells were grown on 0.6 cm^2 inserts (Millipore,Victoria, Australia) at 65,000 cells/ cm^2. The cells were initially grown in culture media with 10% FCS for attachment and propagation for three days. They were then replaced in DMEM with reduced FCS percentage (2%) or no FCS (0%) along with ITS-X for both conditions and compared to standard control (cells cultured in growth medium with 10% FCS, without ITS-X). On the 21^{st} day before the cells were used for experiment, their Trans epithelial electrical resistance (TEER) values were measured using an endohm meter. Only inserts that showed TEER values above 300 Ω cm^{-2} were to be used as evidence of tight junction presence. Cells that were growing in growth medium with 0% FCS showed low TEER values, none of which that reached 300 Ω cm^{-2}. Cells cultured in DMEM with 2% FCS showed improved expression in comparison to cells cultured in 0% FCS, although they did not exceed a TEER value of 300 Ω cm^{-2} on days 21.

As tight junctions were not seen in the experimental cells (cells cultured in 0% and 2% FCS), this suggested that cells need FCS for better formation of tight junctions. Only cells growing in DMEM with 10% FCS showed values that could be used for experiments (above 300 Ω cm^{-2}). In order to know the transition of the TEER value between cells growing in different concentrations of serum, the values were continuously measured from day 7 to day 24.

TEER values showed a more rapid increase on day 17 for cells cultured in growth medium with 2% (>300 Ω cm^{-2}) and 10% FCS. However experiments cannot be conducted on the same day as P-gp maturation is highest after 3 weeks of incubation (day 21) (233). On day 21, the TEER value of cells cultured in 2% FCS decreased while cells cultured in 10% FCS still increased. At no time point did the TEER value of cells cultured in 0% FCS increase from an initial value of 90 Ω cm^{-2} (Figure 2.11).

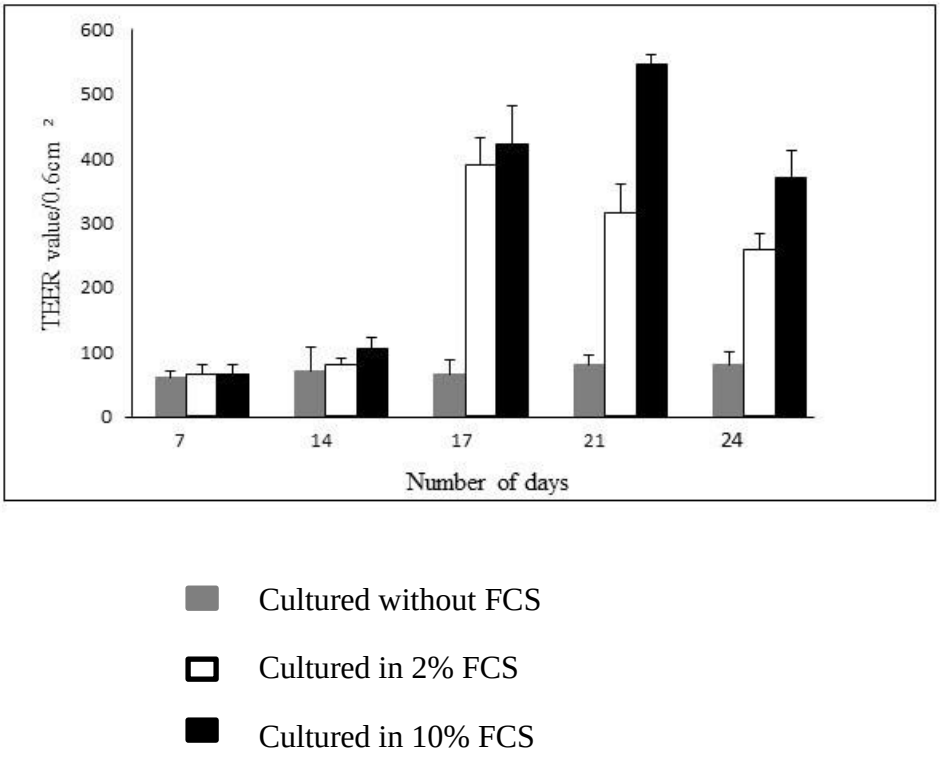

Figure 2.11: TEER value calculation of Caco-2 cells. Comparison of TEER values on different days between Caco-2 cells cultured in growth medium with different concentrations of FCS. The values were calculated with an endohm meter. Data represents the average TEER value ± SD (n=3).

2.6.5 Tight junction visualisation using immunofluorescence

Occludins are membrane proteins that contribute to tight junction formation, so to investigate the low TEER value detected above, occludin was examined immunohistochemically in the Caco-2 cell monolayers. Immunofluorescence was conducted on the 7th, 17th and 24th day on Caco-2 monolayers. All cells were seeded at a density of 3x10^{5}cells/ well. On the 7th day, cells grown in growth medium with 0% and 2% FCS showed comparable expression of the tight junction protein occludin to control cells (cultured in growth medium with 10% FCS). On the 17th day, images taken revealed that cells that were cultured in growth medium with 0% FCS showed a very low expression of occludin, while its expression in 2% FCS was high and comparable to cells cultured in growth medium with 10% FCS. On the 24th day, cells growing in 0% FCS medium continued to show minimal expression of occludin, however cells cultured in 2% FCS also showed a reduction in expression

of occludin. Thus, the results suggested that as the cells matured in a reduced FCS environment, they could not maintain the occludin expression to that of 10% FCS. Maximal expression of occludins was seen in cells cultured in 10% FCS as revealed by the fluorescence intensity.

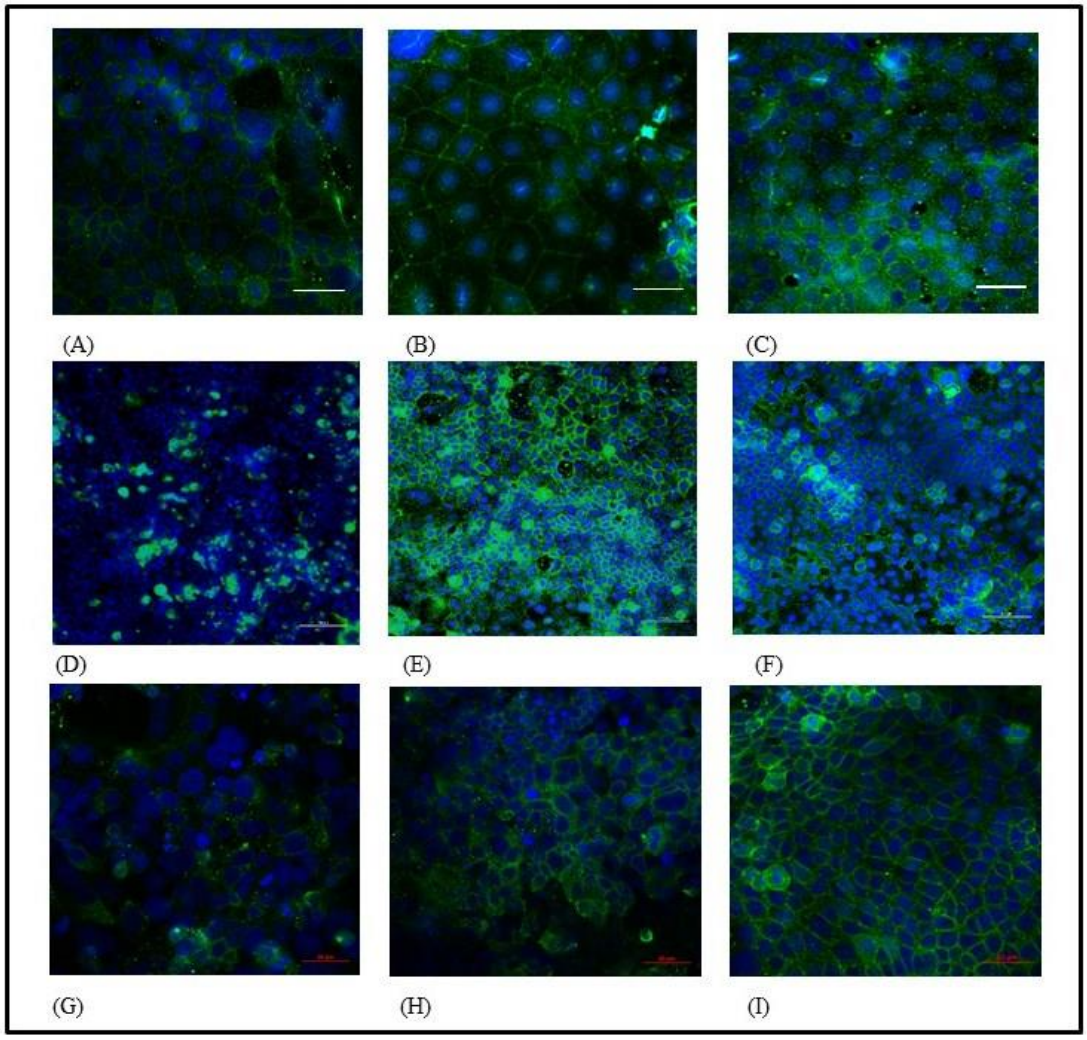

Figure 2.12: Immunofluorescence of Occludin (green) and the nuclear stain DAPI (blue). Caco-2 cell line cultured in growth medium with different concentrations of FCS. **(A-C)**: **Study done** on day 7 in 0, 2 and 10% FCS respectively**. (D-F): Study** done on day 17 on 0, 2 and 10% FCS respectively. **(G- I):** Study done on day 24 on 0, 2 and 10% FCS respectively. Scale bar at 50 µM.

2.7 Discussion

The present study aimed to develop a study model of gastrointestinal cell lines, Caco-2 and LS174T, cultured without FCS. Serum is an important component of complete tissue culture medium, a lack of which may show differences in expression of various transporters and peptides. The requirement for serum free medium was primarily to isolate low molecular weight proteins or peptides from CM without interference from albumin in their collection. Other nutrients such as vitamins and non-essential amino acids (NEAA) were also made available to the medium as it had been the standard protocol used in our laboratory. Various hormones like insulin, glucagon and cortisol have been identified as a part of the serum complex of proteins but haven't been substantiated enough to be present at similar concentrations in every batch of serum that have been collected for *in-vitro* use.

Ferruzza *et al*., have already attempted to create a defined medium for Caco-2 cells and they found ITS-X adequate to replace FCS (230).

In the present study, initially the cells were cultured in growth medium containing 10% FCS for attachment and better propagation. They were then replaced in growth medium with 2% FCS or 0% FCS for three weeks to demonstrate genotypic and phenotypic characteristics. This is in support with other studies that have also utilised similar conditions implementing ITS-X to replace FCS, although they do not show any experimental evidence of protein analysis of specific transporters (230, 234).

According to Jumarie and Malo., ITS-X supplemented medium did not show any difference in confluency and total protein content when quantified in comparison to cells cultured with FCS (229). The present study also demonstrated that cells growing in medium supplemented with ITS-X in a culture with 0% FCS and 2% FCS did not show any difference in confluency and growth of cells along with protein quantification in comparison to cells growing without ITS supplemented in 10% FCS. The influence of basal membrane proteins on the growth and differentiation of cells have also been demonstrated in their study. They had previously noted that rat epithelial intestinal cells do not grow without the presence of basal membrane proteins. Jumarie and Malo., has also shown that Caco-2 cells did not grow well in a FCS free medium for the first two days indicating that FCS

contains certain attachment factors necessary for the growth of cells (229). Similar to these results the present study also showed that the Caco-2 cell line growing in growth medium with 0% FCS for the first two days had a sparse population. Hence 10% FCS was necessary for attachment and propagation of cells after which it is replaced by growth medium with 0% FCS or 2% FCS.

According to Huschtscha *et al.*, LS174T cells are a moderately differentiated tumor cell line that requires either FCS or other growth supplements like transferrin for its growth (235). They have also shown that cells cultured in medium with low FCS showed a higher expression of the oncogene (p53) which may be linked to induction of stress like conditions and poor growth characteristics of these cells.

Although, cells that were growing in defined medium (0% and 2% FCS) were phenotypically similar to cells growing in 10% FCS without ITS-X supplementation, their protein expression characteristics were not the same. Fully defined medium showed only low expression of the ABC transporters. The experiment was conducted over multiple cell passages, some of which did not show any band specific for P-gp in cell lysates cultured in 0% FCS medium (Figure 2.5). In the Caco-2 cell line, P-gp showed higher expression in late passage (P-81) consistent with the data previously published from our laboratory (232). LS174T cell lines showed a better expression in cells growing in P-76 in comparison to cells growing in P-71 and the difference between cells cultured in both conditions was not as stark as in the Caco-2 cell line. Engman *et al.*, have also suggested that Caco-2 cells of higher passages have shown better monolayer integrity and consistent expression of P-gp despite treatment with Vitamin D_3 that decreases the permeability of these cells (199).

BCRP in Caco-2 cells also showed a better expression in late passage cells than in younger passages, although the difference in expression was not very significant in the passages as a whole. In LS174T cells, the expression of BCRP was lesser than its expression in Caco-2 cells (236). Fully defined medium did not affect the expression of BCRP by a great margin, indicating that for the expression of this transporter the unique growth factors in FCS were not mandatory for its expression and could be

compensated for with the addition of defined growth factors such as ITS-X (Figure 2.6).

In our studies, MRP2 was only detected in Caco-2 cells, therefore relative expression changes could not be compared to other cell lines. There was a difference in its expression between the lysates (0% FCS and 2% FCS) in all three passages with maximum expression in P-81 (Figure 2.7).

To observe if there was a similarity between protein expression and gene expression, mRNA changes were observed in all three cells lines for ABCB1 and ABCG2. The ABCB1 mRNA has been shown to be prevalent homogenously throughout the intestine (199). Changes were seen in cells cultured in different percentages of FCS. There were no bands for certain passages of Caco-2 cell lines cultured in growth medium with 0% FCS (data not shown) while a significant reduction in band intensity was seen with lower than normal percentages of FCS. ABCG2 was also examined for changes in the three cell lines in different percentages of FCS. No significant difference was observed between the cell lines. The endogenous control used was glyceraldehyde 3-phosphate hydrogenase (GAPDH). Since no comparative study was done for mRNA changes in different passages, significant difference between the passages was not demonstrated. Hence a representative figure has been included (Figure 2.10).

Reports have indicated that the mRNA level of P-gp (ABCB1) is reduced in patients with ulcerative colitis with additional correlation of reduced levels of PXR and ABCB1 mRNA (104, 237). Blokzijl and colleagues, have shown that although there may be a correlation in the mRNA levels of ABCB1 and PXR the protein levels of PXR did not reduce with the decrease in the mRNA levels in the presence of ulcerative colitis. The protein levels remained consistent, suggesting that the decrease in the mRNA levels of P-gp is not dependent on the PXR levels or vice versa (238).

In the present study, the Caco-2 cells were lysed on day 24 for all experiments (239, 240). In contrast, Goto *et al*., suggested that the expression profile of P-gp is better in the cell proliferative stages rather than in the cell differentiation stage. They have

shown that the half-life and transcription rate of mRNA of ABCB1 is maximal on day 3 rather than day 9 (196). It has also been shown by others that mRNA levels of ABCB1 in Caco-2 was much higher in the proliferation stage rather than in the differentiation stage, while the protein expression levels in Caco-2 did not differ much suggesting a time lag in the reduction of MDR1 protein after the decrease of its mRNA level. Similar levels of protein and mRNA was also seen for MRP2 (241).

In the intestine, P-gp, BCRP and MRP2 are present at the apical surface of the epithelial cells of the intestine. While, the expression of P-gp increases from the duodenum to the colon, BCRP is present throughout the small intestine and MRP2 expression is highest in the duodenum while tapering towards the ileum and colon (28, 242-244). Previous studies have shown that the expression of BCRP in Caco-2 cells increased 3 fold with an increase in the number of passages (245).

Tight junction maturation in the Caco-2 cell line is an important factor responsible for differentiated polarised epithelial cell phenotype. A change in the TEER values of these cells is a good indicator of the tight junction efficacy of Caco-2 cells.

According to Ranaldi *et al.*, Caco-2 cells grown in DMEM with FCS displayed maturation of the tight junctions, when these cells were grown on permeable filters (246). On day 24 TEER values were approximately 510 Ω cm^2, while cells cultured in defined medium never reached adequate tight junction formation.

The present study also showed that Caco-2 cells cultured in serum free medium were unable to generate adequate tight junctions. While the minimal TEER values for experiments needed to be 300 Ω cm^{-2}, the cells showed much lower values (approximately 90 Ω cm^{-2}). The control group of cells grown in 10% FCS without ITS-X supplementation showed values up to 750 Ω cm^{-2} (Figure 2.11).

It is necessary for cells cultured in DMEM without FCS to be monitored regularly especially with regards to permeability characteristics of Caco-2 cells as differentiation is a pivotal trait and hence one of the major reasons to be used as an *in-vitro* model for absorptive studies and transport experiment (194, 226, 247, 248).

Even though it has been suggested that cells cultured in ITS-X could exhibit better differentiation and show TEER values that may be used for experimental reasons, the same group have also stressed that cells cultured on permeability filters with FCS in the basolateral compartment only showed most efficient and functional characteristics required for tight junction layer (230). The activity of MDR1/ P-gp was measured using transport experiments with digoxin as a substrate and verapamil as an inhibitor for basolateral and apical directions. The net apical efflux of digoxin for cells growing in ITS-X was $5.33x10^{-6}$ cm/s, while for cells cultured in medium with FCS in both compartments (apical and basolateral) was $9.33x10^{-6}$ cm/s. The efflux ratio was also much lower in cells growing in ITS-X in comparison to cells growing in the conventional 10% FCS, with a result of 4.14 and 14.1 respectively (230). Having stated that the TEER values were reduced in Caco-2 cells cultured in serum free medium, they have also suggested that it maybe simulating *in-vivo* studies as the TEER values of Caco-2 cells cultured in FCS is much higher than the human small intestine mucosa (64).

In order to substantiate the calculated TEER values in Caco-2 cells, the cells were stained with occludin. It is a tight junction protein and necessary for formation and regulation of directional cell migration, especially in epithelial cells (249). They were the first tight junction protein discovered and are known as the most important tight junction protein limiting paracellular transport. They are also responsible for maintaining apical-basolateral polarity in epithelial cells (250-252). As with other receptors in the epithelial cells, occludins may also be affected by levels of available FCS. Complete absence of serum in the medium, causes endocytosis of occludins which are rapidly lost from the plasma membrane (253).

Du *et al*., showed similar results with MDCK cells which while being serum starved for 30 minutes showed no expression for occludins on immunoprecipitation analysis. On stimulation with 10% serum for 10 mins, a high expression of occludin was observed (249).

Staining for occludins was done on the 7th, 17th and 24th for Caco-2 cells. The cells were cultured in growth medium with 10% FCS for four days and then transferred to DMEM without FCS or DMEM with 2% FCS, with the addition of ITS-X as described in 2.5.1. On the 7th day, cells growing in growth medium with 0% FCS or 2% FCS medium showed the presence of occludins which was almost comparable to

cells cultured in growth medium with 10% FCS. As these cells were cultured in medium with 10% FCS for the first four days, the formation of this tight junction protein was not affected by the absence of FCS. On the 17th day of cell growth, the cells were again stained for occludins. Cells grown in medium with 0% FCS showed minimal expression of the tight junction protein, while cells cultured in medium with 2% FCS showed a higher expression. Tight junction formation was decreased with time in defined conditions with 0% FCS and 2% FCS (Figure 2.12). Even though, the formation of occludins can be seen on the 7th day in DMEM without FCS, there is a rapid decrease in their expression, as the lack of FCS does not support the expression of tight junction proteins occludins.

Although, occludins are deficient in Caco-2 cells in a serum starved condition, it does not necessarily influence barrier cell function. Schulzke *et al.,* had suggested that a deficiency in occludins by themselves are not as deleterious as a deficiency in another tight junction protein, claudin-1(254). In the absence or functional incapability of claudin in animals, they have indicated that there was a high mortality rate especially in mice due to loss of electrolytes and water. Also as tight junctions may not be formed by just one protein but rather by a heteropolymer of other tight junction proteins and the loss of the less efficient proteins can be substantiated by the more efficient ones such as Claudin-1 (254). Nevertheless, the present study shows a positive correlation between TEER values and occludin presence. As the gastrointestinal cell line symbolises the first line of defence against ingested drugs or other xenobiotics good understanding of how tight junction proteins are altered would be advantageous into the future.

As Barnes and Sato suggested, it is unexpected that all of the characteristics of cell lines grown in serum free medium were similar to cells cultured in medium with conventional serum (255).

2.8 Limitations

Tight junction efficacy has been determined and data shows that bidirectional transport cannot be done on cells that are cultured in reduced percentage of FCS, as the phenotype is too different from cells growing in 10% FCS on cell inserts. Although occludin has been studied here, other tight junction proteins like claudin-1 and cadherin have not been tested. Tight junction itself is formed by a combination

of all tight junction proteins. While, occludin that were observed after 3 days of defined medium was encouraging, the data from the present study has shown that 10% FCS needs to be used as long as possible before removing to collect CM for peptide /factor isolation.

2.9 Conclusion

This chapter helps in the determination of expression of ABC transporters in growth medium used as defined medium with ITS-X without FCS (0%) or with reduced percentage of FCS (2%). It was established that FCS is necessary for the attachment and propagation of the cells, even if they were replaced with defined additives after the first 3 days. Although phenotypically the cells do not look different from cells growing in a full culture medium, the expression of specific ABC transporters changes when grown in a medium devoid of FCS, proving the necessity of FCS as an agent for better expression of transporters.

The method of using FCS free culture also shows evidence of poor tight junctions of Caco-2 cells. The formation of tight junctions and polarised layer is one of the pivotal characteristics of Caco-2 cells, for which they have been used as the *in-vitro* model for permeability studies. However, the growth additives in the medium may influence the cells in their tight junction and polarisation formation thus affecting P-gp expression. Bidirectional studies cannot be performed on cells cultured in defined medium as the TEER values suggested that tight junctions are not formed adequately. As albumin in FCS interacts with other components of the medium, especially low molecular weight proteins that were effluxed from the cells causing difficulty in its isolation, partially defined medium was chosen as the method for isolation of low molecular weight proteins.

Chapter 3: Assessment and evaluation of antimicrobial activity in gastrointestinal cell lines.

3.1 Rationale

In 2011, our lab had shown the presence of antimicrobial repression in the gastrointestinal cell lines, Caco-2 and LS174T (108, 219). To further prove the presence of the activity, conditioned medium (CM) was also taken from these cells that were treated with rifampicin, a P-gp inducer, where microbial control was observed. This chapter employs various methods involved in isolation of the particular factor or peptide that caused antimicrobial activity. To further prove the link between P-gp induction and increased antimicrobial activity only relatively low molecular weight (LMW) molecules, ideally less than 3 kDa were screened as any putative substrates of P-gp should be present in filtrate after 3 kDa cut off filters were employed. The rationale being that even 3 kDa was 50% larger than the largest substantiated P-gp substrate, gramicidin is only 1.9 kDa (40, 108) and it did not seem possible to push expectations of P-gp affinity to molecules any larger than this.

3.2 Introduction

Low molecular weight (LMW) proteins (< 3 kDa) were screened for antimicrobial activity and an array of methods were applied for the isolation of these peptides. The necessity for screening of the LMW fraction is linked to the ability of any putative antimicrobial peptide to be a substrate for P-gp (219).

For isolation of LMW peptides (< 3 kDa) from CM of gastrointestinal cells such as, Caco-2 and LS174T cells, various techniques including precipitation, size exclusion chromatography (256) and proteomics were employed. Proteomics involves the study of global expression patterns, molecular interactions and the functional states of proteins present in a cell (257). Proteomics is important to understand the changes in protein expression patterns and hence to identify the potential proteomic biomarkers that may be linked to potential antimicrobial activity. Adequate sample size is necessary for analysis and interpretation of proteins especially the low abundance ones that may be diluted in the presence of the highly abundance proteins. For this purpose, extraction, enrichment and pre-concentration of the analytes was required to improve the quality of analysis (257, 258).

Before analysis of the samples, it was necessary to reduce the sample complexity. Easily recognisable fractions are required in order to identify the samples. Also because the culture medium used a small amount of FCS which contained some albumin and other high abundance proteins, it was imperative to deplete them before subjecting the samples to further analysis. Hence, molecular weight cut off filters (<30 kDa and <3 kDa) were used to reduce a large percentage of the albumin present in the CM while retaining the LMW fraction for analysis. Even so, some unknown target proteins maybe retained by the albumin-sponge effect, thereby reducing the sample load of the LMW fraction (259).

To further separate the larger proteins, precipitation was implemented using the organic solvent acetonitrile. The smaller proteins were released into the supernatant, while larger and high abundance ones such as albumin were precipitated (260). Precipitation was done after the usage of molecular weight cut off filters as only a relatively smaller amount remained from the starting volume of the CM sample. This helped in an effective way of precipitating larger proteins as they should have been theoretically reduced.

In the present study an AKTA purifier (P-900) was used as our fractionating device using the principle of SEC. This method also allows enrichment of cross linked peptides, increasing the yield of structural information from cross linking experiments (259, 261). The fractions can be detected with three different wavelengths which are 215, 254 and 280 nm thus providing a range of proteins/peptide that can be detected. Usage of a Bio-Rad P-6 column allows a size exclusion limit in the range of 800 Da to 6 kDa resulting in a smaller fraction with the desirable size of peptides. Some disadvantages of using SEC were the elution volumes that dilute the sample and the necessity of using high injection volumes (259).

Proteomics uses technologies to determine the different properties of proteins secreted from cells, tissues and body fluids. Analysis of the targeted secreted proteins by very sensitive methods is crucial as they may be contaminated by various other proteins and could be well below detection limits.

Challenges were associated with the analysis of relatively low protein content. Because of the complexity of the samples such as being within the CM, it was necessary to have more than one technique to resolve the samples.

3.2.1 Mechanism of action of antimicrobial peptides

Antimicrobial peptides (AMPs) use different methods for killing microorganisms, e.g., barrel stave model and carpet model (171, 172). The barrel stave model is based on the theory that suggests that after binding with the particular microorganism, the AMPs with linear, amphipathic or helical conformation form transmembrane pores in the planar lipid membranes, thus creating a leaky membrane in bacteria, which eventually die. For helical peptides, a minimum of 22 amino acids are required to form pores, while with sheeted structures, a minimal of 8 are needed (262, 263). Barrel stave is a useful model to demonstrate non-cell selective lytic peptides, while peptides that are selectively lytic to certain bacteria follow the carpet model of bacterial lysis. The carpet model requires an appropriate balance between hydrophobicity and a net positive charge in the AMPS as well as their ability to co-assemble in the membrane-bound state. Some other properties include well controlled enzymatic degradation, stability in body fluids such as blood and serum and effective changes in their sequence that make them better equipped for treatment of infectious diseases (264).

3.2.2 Relationship between peptide structure and antimicrobial activity

There are several parameters that identify antimicrobial activity in peptides (175). Some of the parameters involved are liposome leakage through a large unilamellar vesicle (175). The degree of liposomal leakage and its kinetics has also been monitored as a measure for the activity of AMP that includes leakage kinetics, aggregation kinetics and fusion kinetics. Leakage kinetics corresponds to the degree of liposomal leakage. Aggregation kinetics corresponds to the degree of liposomal aggregation and fusion kinetics corresponds to fusion of the membrane with the peptide. The α-helical structure also serves as a parameter in determination of the antimicrobial activity in peptides. None of the parameters is a sole criterion for confirmation of any membrane destroying activity of the peptides, but together they

provide compelling evidence of suitable peptides for antimicrobial activity (111, 175).

3.2.3 Endogenous peptides used as substrates of P-gp

P-gp is known to efflux many small molecules to varying degrees and has also shown affinity for selected peptides, especially circular ones such as cyclosporine A (265, 266). The ability of P-gp to interact with different substrates can be attributed to the presence of many internal binding sites or different overlapping regions of a flexible binding site within the P-gp transporter that may be large enough to accommodate multiple small compounds simultaneously or even a moderately sized peptide (267).

3.3 Aim

The aim of the chapter includes:

- Enrichment of the LMW fraction from the conditioned medium of gastrointestinal cells, Caco-2 and LS174T
- Evaluating antimicrobial activity in the LMW fraction against gram positive and gram negative microorganisms which include *Escherichia coli, Salmonella typhimurium and Staphylococcus aureus*.
- Assessment of putative antimicrobial factors by mass spectrometric analysis in the LMW fraction.

3.4 Materials

The molecular weight cut-off filters, Amicon Ultra centrifugal filters were procured from Millipore (Victoria, Australia), Acetonitrile was procured from VWR chemicals (QLD, Australia). Centrifugation was done in JA-20 rotor using Beckman coulter centrifuge (NSW, Australia). Oakridge tubes were procured from Nalge Nunc, Denmark. Silver staining kit was procured from Invitrogen (Silverquest, and Silver staining kit (Victoria, Australia). 4-12% bis-Tris gels were procured from Novex Nupage, 1.5mm 15 well gels (Life technologies, Victoria, Australia) and MES buffer was obtained from (Novex Nupage, 20X, (Life technologies, Victoria, Australia). Bio-Rad P-6 columns were procured from Bio-Rad laboratories (CA,

USA), AKTA purifier (P-900) (GE, Uppsala, Sweden). Nylon 0.45µm filters (Millipore, Victoria, Australia). Microbial strains were used from stocks held within the Microbiology laboratory of the School of Pharmacy, Curtin University. These were *Escherichia coli (E. coli* ATCC 9637*), Salmonella typhimurium (S.typhimurium* ATCC 7823) *and Staphylococcus aureus (S.aureus* ATCC 6538*).* Other materials used were Nutrient agar plate, Sterile 96 well plate (Nunc, Denmark), Enspire multi-mode Plate reader-2300-9040 (Perkin-Elmer, Victoria, Australia)

3.5 Methods

3.5.1 Tangential filtration/ Centrifugation using molecular weight cut off filters

Cell lines (Caco-2, LS174T) were cultured in accordance with section 2.5.1. After the first four days, DMEM with 10% FCS was replaced with 2% FCS and ITS-X without antibiotics.

Conditioned medium from the Caco-2 cells was collected between days 16 to 28 at an interval of three days. For collection of medium from Caco-2 cells treated with P-gp inducer rifampicin, 5 µM rifampicin was added to the medium on day 22 and removed on day 25 when new medium (devoid of rifampicin) was added and was collected on day 28. A washing step with phosphate buffered saline (PBS) was done to ensure that all surface rifampicin was removed, as rifampicin itself has antimicrobial properties (268). This was done so as to compare antimicrobial activity in the medium with and without induction of P-gp. LS174T cells were induced with rifampicin from day 4 to day 7 and new medium devoid of rifampicin was added to the cells thereafter. The medium was collected on day 10 for experiments. The medium collected was spun through molecular weight cut off filters (30 kDa at 4000 g for 30 mins at 4ºC and the filtrate was subsequently passed through 3 kDa (4000 g for 1 hour at 4ºC). The medium was serum deprived after the first four days as, in addition to FCS being an expensive commodity, different batches of FCS contain diverse constituents thus making any peptide associated interpretations from CM difficult.

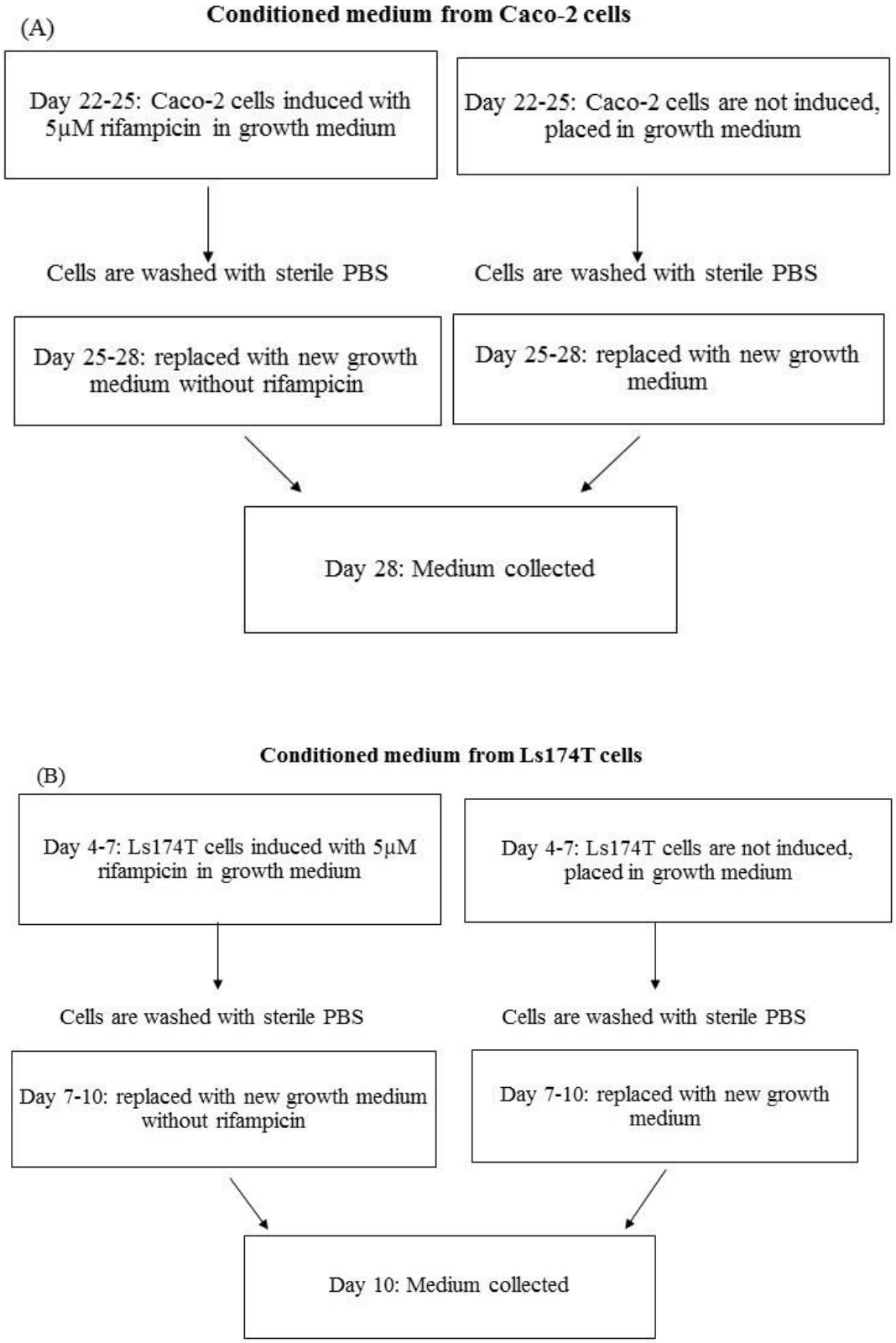

Figure 3.1: Illustration of process of collection of conditioned medium from (A) Caco-2 cell line and (B) LS174T cell line.

The filtrate was processed immediately for precipitation of LMW proteins or stored at -20ºC for later use.

3.5.2 Comparison of precipitation techniques of low molecular weight proteins

Two different techniques were employed for precipitation of LMW proteins from the CM collected; methanol precipitation and acetonitrile precipitation. Methanol precipitation included the use of four volumes of methanol with one volume of the sample, vortexed and centrifuged for 5 mins at 10,000 g (269). Methanol was removed from the top layer and 4 volumes were added again, centrifuged at 10,000 g for 15 mins. The precipitate was reconstituted in nanopure H_2O (>17Ω) and subjected to protein quantification (section 2.5.3). Unfortunately, only low levels of protein were observed using this method. Hence acetonitrile precipitation was employed for better restoration of LMW weight proteins. One volume of acetonitrile was added to one volume of the sample, left at room temperature (RT) for 30 mins. It was then centrifuged at 15,000 g for 15 mins at 4ºC in Oakridge tubes.

Half the volume was pipetted out and frozen at -80ºC for lyophilisation overnight. The lyophilised sample was reconstituted in nanopure H_2O and observed for protein quantification (Lowry method in accordance to section 2.5.3).

3.5.3 Silver staining

40 µg of the lyophilised sample was loaded onto 4-12% Bis Tris gels and subjected to SDS-PAGE electrophoresis at 200 V for 40 mins. MES buffer was used as the running buffer. It was then subjected to silver staining procedure (refer table 3.1 for components).

The gel was fixed with 25 mL of fixative for 20 mins with gentle rotation. It was decanted and washed in wash buffer for 10 mins. The wash buffer was decanted and 25 mL of sensitising solution was added for 10 mins. The gel was washed again for 10 mins with wash buffer and then replaced with nanopure H_2O for 10 mins. The gel was then incubated in 25 mL of staining solution for 15 mins and then washed with

nanopure H_2O for 1 minute. Overly extensive washing will lead to the loss of silver ions, thereby reducing the sensitivity. The gel was then incubated for 10 mins in 25 mL of developing solution until the band intensity was developed optimally after which stopping solution was added with the gel still immersed in developing solution. The gel was then agitated for 10 mins till a colourless solution was formed. The stopper solution was decanted and the washing step was repeated.

Table 3.1: Different constituents used in silver staining

Solution	**Components**
Fixative	Ethanol-40% Acetic acid-10% Nanopure H_2O-50%
Wash buffer	30% ethanol
Sensitising solution	Ethanol-30% Sensitiser-10% Nanopure H_2O -60%
Developing solution	Developer-10% Enhancer-0.5% Nanopure H_2O-89.5%
Staining solution	Stainer-1% Nanopure H_2O-99%

3.5.4 Size exclusion Chromatography

To further purify CM from the cell lines and to fractionate them based on their size, 500 µL at approximately 100 µg/mL protein was loaded onto Bio-Rad P6 columns and eluted with 50 mM PBS (pH 7.4). The buffer was filtered using 0.45 µm filter paper. The column was washed twice and eluted with the same buffer. Fractions were collected at 0.5 mL/min with two column volumes. Further concentration of the fractions was usually avoided as the chances for loss of proteins is increased with every sample preparation step.

3.5.5 Antimicrobial activity

E.coli, S.aureus and S.typhimurium were cultured on nutrient agar plates overnight and incubated at 37ºC. Freshly cultured organisms were collected by a sterile loop, diluted in 10 mL sterile PBS and compared to 0.5 McFarland standard. Alternatively, they were also subjected to photometric analysis at 625 nm reading between 0.08-0.1 after centrifuging at 3500 g for 5 mins. The pellet was reconstituted in 200 µL sterile PBS. The microbial inoculum was added to samples at a dilution of 1:100 to give a final bacterial load of approximately 50 colony forming units (CFU), as it is the least volume of inoculum for causing infection. Samples were diluted with DMEM without antibiotics and other additives at a ratio of 1:2. The experiment was done in a 96 well sterile plate.

The samples were measured for microbial growth at various time points spectrophotometrically, using one plate per time point. After each point the 96 well plate was centrifuged at 285xg for 5 mins. Supernatant was transferred to a clean 96 well plate and measured at 560 nm to observe changes in DMEM that may have been caused by bacteria. The absorbance values show a decrease when the sample turns acidic (colour change to yellow).

3.5.6 Mass spectrometry

The fractions procured from size exclusion chromatography (AKTA purifier) were submitted to Proteomics International Pty Ltd for LC/MS/MS analysis and processed according to the protocol by Bringans *et al* (270).

The CM samples containing proteins/peptides were digested with trypsin for peptide extraction according to their standard protocol. The peptides were analysed by electrospray ionisation mass spectrometry using the Shimadzu Prominence nano HPLC system (Shimadzu) coupled to a 5600 TripleTOF mass spectrometer. The digested samples were then loaded onto an Agilent Zorbax 300SB-C18, 3.5 µm (Agilent Technologies) and eluted with a linear gradient of nanopure H_2O: acetonitrile with 0.1% formic acid (v/v). The spectrum was then analysed by Proteomics International Pty Ltd to identify any proteins of interest matched with Mascot sequence software (Matrix Science) with MSPnr100 database for humans.

3.5.7 Statistical Analysis

All data are reported as a mean parameter determined ± standard deviation (S.D.) or standard error of mean (SEM). The difference between the groups was determined using Student's t-test. A P-value ≤ 0.05 was considered as statistically significant.

3.6 Results

The CM collected from gastrointestinal cells were centrifuged through molecular weight cut off filters (30 kDa and 3 kDa) and the filtrate was subjected to acetonitrile precipitation. Centrifugation was done at 15,000 g for 15 mins after which the supernatant was lyophilised in order to concentrate for further use. The lyophilised sample was then reconstituted with a small volume of nanopure H_2O to concentrate the initial volume about 30-fold higher than originally. Modified Lowry's method was used to measure total proteins in the sample. A high yield (460 µg/mL) of proteins was obtained in comparison to methanol precipitation (130 µg/mL).

3.6.1 Silver Staining

After quantification, 40 µg/mL of total protein in the samples was loaded on the gels and subjected to SDS-PAGE and consequently silver stained. Samples from passages 80-89 was used for the Caco-2 cell line and passages between 70-80 were used for the LS174T cell line. With an increase in the incubation of cells, the corresponding medium collected as described in section 3.5.1, showed a thickness in the band corresponding to < 5 kDa, with maximal band thickness between days 19-22. CM from LS174T cell lines was collected on day 10 as they mature faster than Caco-2 cells. A similar observation was made, whereby the cells increased in band thickness with increase in age of cells. Comparative studies between the passages were not shown as most passages showed similar results.

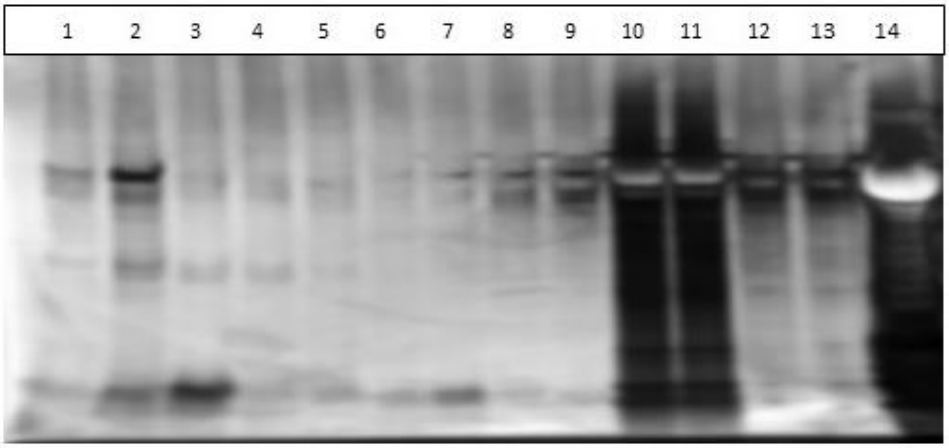

Figure 3.2: Silver staining of Caco-2 and LS174T cells. The lanes as represented as follows: **1**- Control medium (growth medium in 2 % FCS and ITS-X without antibiotics). **2**-Caco-2:16-19D (P-81). **3**-Caco-2:19-22 D (P-81). **4**-Caco-2:22-25D (P-81). **5**-Caco-2:25-28 D (P-81 non-induced). **6**-Caco-2:16-19D (P-83). **7**-Caco-2:19-22D (P-83). **8**-Caco-2:22-25D (P-83). **9**-Caco-2: 25-28D (P-83 induced). **10**-LS174T: 7-10 D (P-70). **11**-LS174T: 7-10 D (P-75 induced). **12**-LS174T: 1-4D (P-70). **13**-LS174T: 1-4D (P-75). **14**- 2% control medium that has not been passed through filters and ACN precipitation. Induced represents medium collected after incubation with 5 µM rifampicin for 3 days (a P-gp inducer).

Different passages were used for rifampicin induced and non-induced CM, so that various passages were represented and to demonstrate that the difference in induction between the passages did not differ considerably.

3.6.2 Size exclusion chromatography

Size exclusion chromatography was performed using AKTA purifier (P-900) system that is connected to a fractionator and the fractions were monitored at 214, 254 and 280 nm. 254 nm and 280 nm were considered ideal for detection of peptides from body fluids (261, 271). 214 nm was also included as all proteins would be detected at that wavelength and there will be less risk of losing any proteins present.

Bio-Rad P-6 columns were used for low molecular weight isolation. P-6 separates samples between 6000 Da and 800 Da. 100 µg/mL of proteins were loaded at a volume of 500 µL. Different buffers were used for elution of the LMW fraction, e.g., 0.1% SDS, 10 mM PBS and 50 mM PBS. 50 mM PBS (pH 7.4) showed better

separation of the samples yielding higher protein content in the fractions while not interacting with microorganisms thereby any subsequent results of antimicrobial activity were not affected. Although, acetonitrile was shown to elute the LMW fraction of proteins it was not recommended, as with all organic solvents it would affect the results of antibacterial tests and show difficulty in evaluating antimicrobial activity.

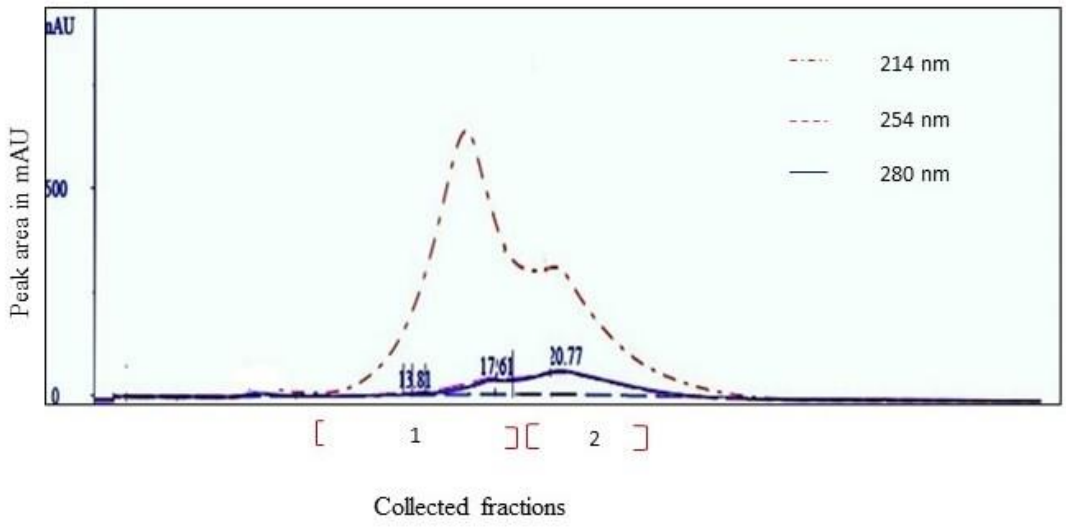

Figure 3.3: Size exclusion chromatography of control. Growth medium in 2% FCS and ITS-X without antibiotics was used as the control and 0.5 mL/min fractions were collected and pooled together to create two broad groups as shown above (1 and 2).

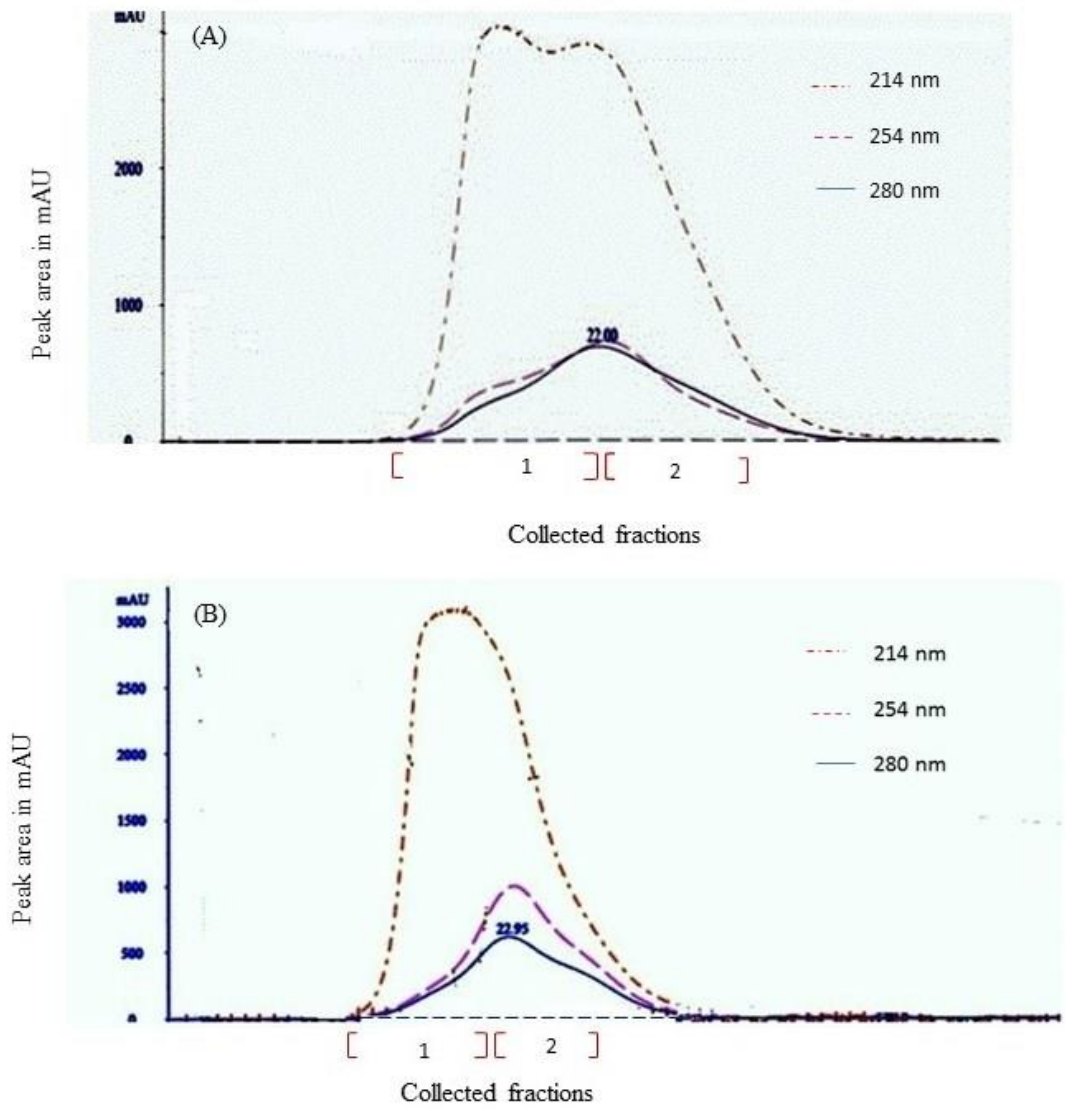

Figure 3.4: Size exclusion chromatography of Caco-2 cells. Conditioned medium from the Caco-2 cell lines collected between days 25 to 28. 0.5 mL/min fractions were collected and pooled together to create two broad groups as shown above (1 and 2) **(A)** The cells were not induced with rifampicin. **(B)** The cells were treated with 5 µM rifampicin between days 22 to 25.

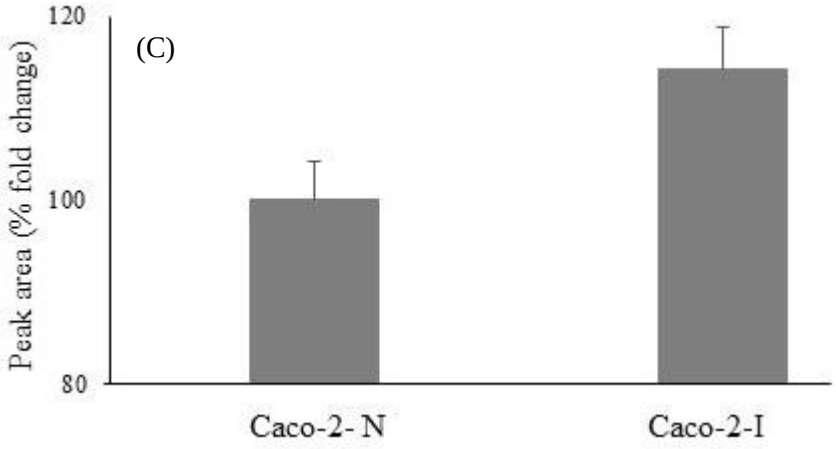

Figure 3.4(C): Comparison of peak area of conditioned medium taken from induced versus non-induced Caco-2 cells. The ratio is calculated with area of the samples to area of control (growth medium in 2% FCS with ITS-X without antibiotics). N represents normal against which the graph is plotted and I represents Induced. Data represents average peak area for each group of cells ± SEM (n=3).

The samples loaded were monitored at 214, 254 and 280 nm with 214 nm showing the highest sensitivity compared to 254 nm and 280 nm. After induction with rifampicin, the cells were replaced with fresh medium as described in section 3.5.1 and this was loaded on the AKTA. Also medium from the same passage of cells was collected without induction of rifampicin. Medium collected from cells induced with rifampicin showed a higher area under the curve as opposed to medium from non-induced cells and has been illustrated by densitometry analysis (Figure 3.4, $p-<0.05$).

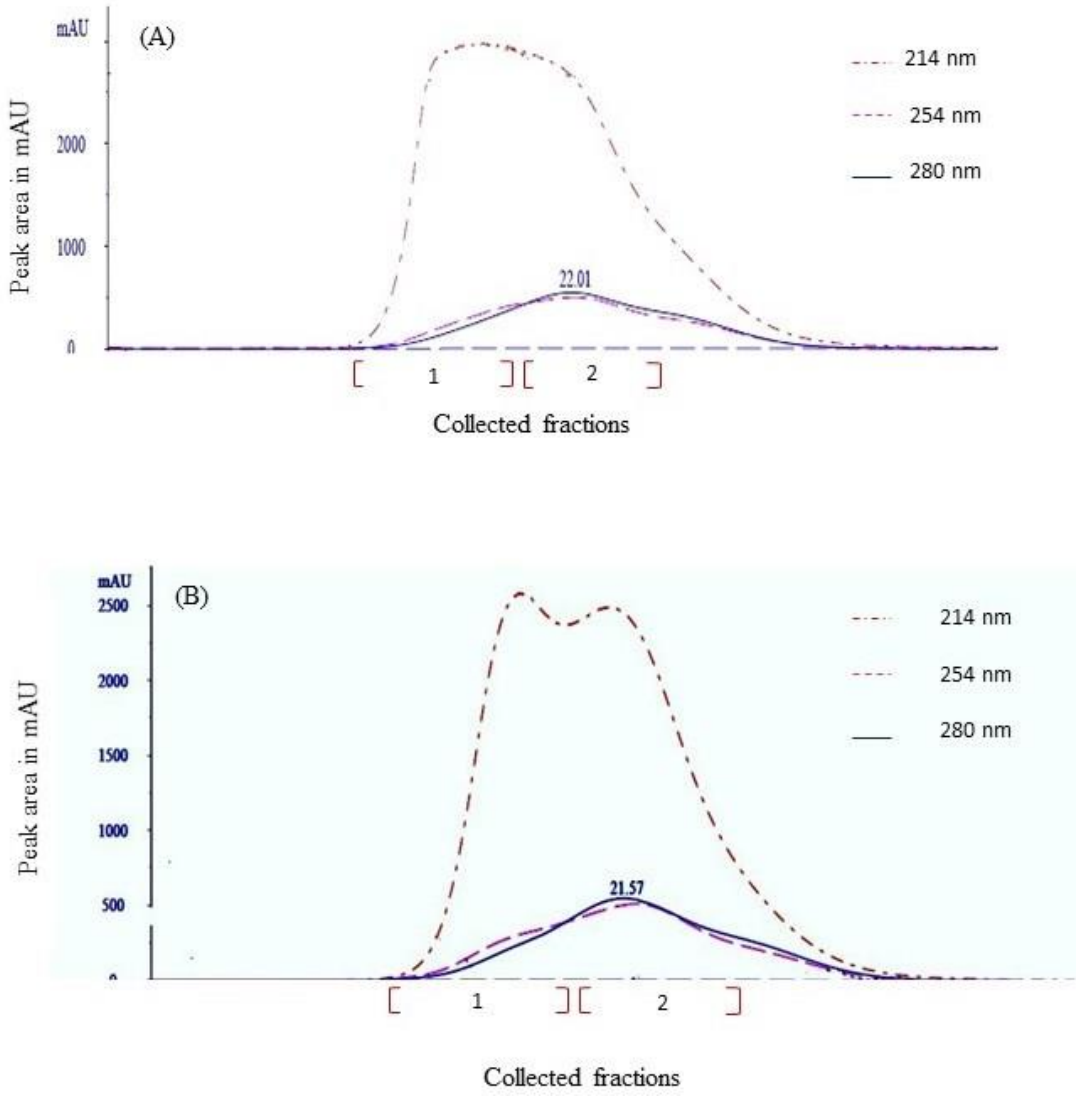

Figure 3.5.: Size exclusion chromatography of LS174T cells. Conditioned medium from the LS174T cells collected between days 7 to 10. 0.5 mL/ minute fractions were collected and were pooled together to create two broad groups as shown above (1 and 2) **(A)** The cells were not induced with rifampicin. **(B)** The cells were treated with 5 µM rifampicin between days 4 to 7.

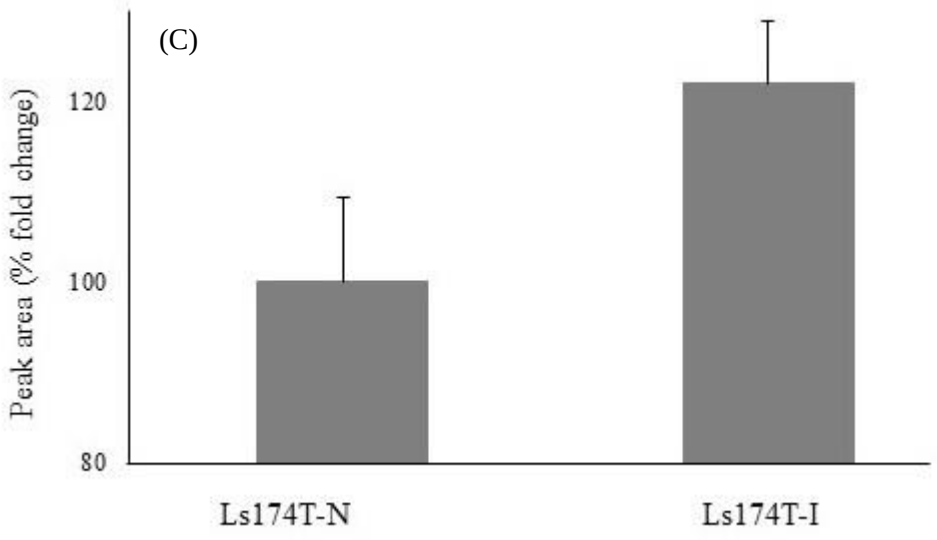

Figure 3.5 (C): Comparison of area of conditioned medium taken from induced cells versus non-induced LS174T cells. The ratio is calculated with area of the samples to area of control. The control used was growth medium in 2% FCS with ITS-X without antibiotics. N represent normal against which the graph is plotted and I represent Induced. Data represents average area of LS174T induced and non-induced cells ± SEM (n=3).

As discussed for Caco-2 cells, medium collected from LS174T cells also showed a similar pattern. Medium from cells induced with 5 µM rifampicin showed a higher area under the curve (mAU) in comparison to medium collected from non-induced cells and has been illustrated by densitometry analysis but was not statistically significant, $p>0.05$ (Figure 3.5).

The difference in area of induced versus non-induced LS174T cells was lesser in comparison to difference in the area of induced versus non-induced Caco-2 cells.

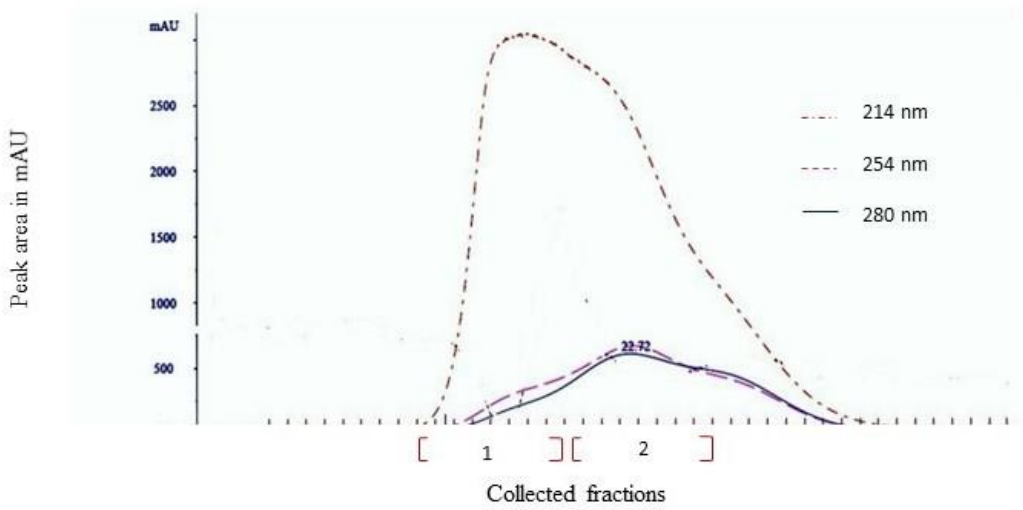

Figure 3.6: Size exclusion chromatography of RKO cells. Conditioned medium from RKO cells were collected between days 7 to 10. 0.5 mL/ minute fractions were collected and were pooled together to create two groups as shown above (1 and 2).

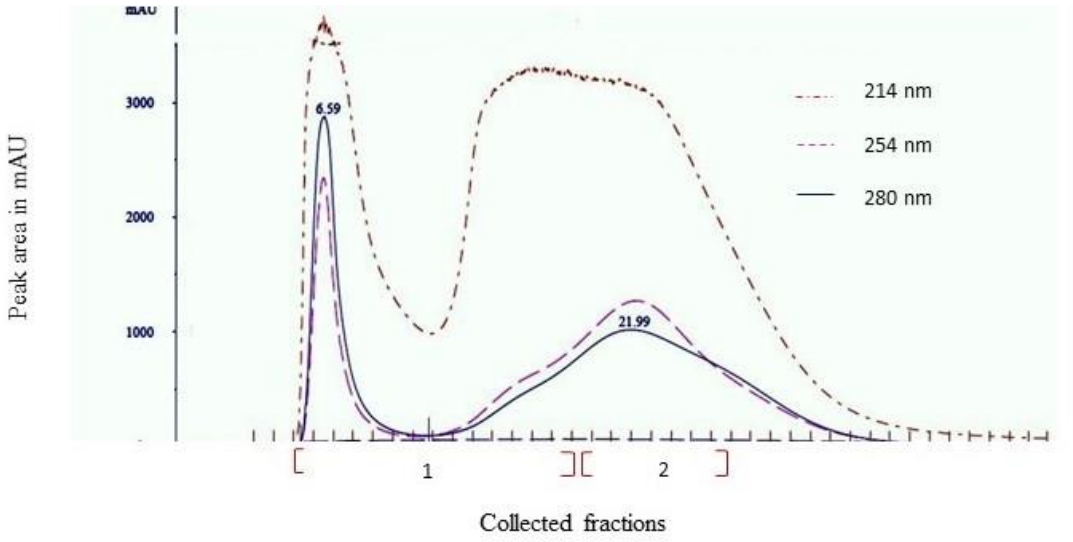

Figure 3.7: Size exclusion chromatography of HeLa MDR1 OFF cells. Conditioned medium from HeLa MDR 1 OFF cells were collected between days 3-5. 0.5 mL/ minute fractions were collected and were pooled together to create two groups as shown above (1 and 2).

3.6.3 Antimicrobial sensitivity test

The fractions collected from the AKTA purifier (P-900) were pooled together and divided into three pools of sample. The first pooled sample contained fractions collected from the first tube of the peak to the 6th tube just before an increase in the area under the curve (AUC) at 22 mins approximately. The second pooled sample contained tubes starting from the 7th fraction to 12th fraction that covered all of the fractions under the increase in the AUC starting from the 22nd minute. The third pooled sample contained fractions from the 13th tube to 20th tube. This fraction was not used for antimicrobial sensitivity test as the 13th tube started showing trace amounts of phenol red. As the molecular weight of phenol red is only 354.4 Da, the samples may not be ideal for observation of antimicrobial activity since most AMPs ranged between 1200-4000 kDa. Each fraction had approximately 3 mL of the sample. The fractions were then subjected to antimicrobial sensitivity test as discussed in 3.5.5. Antimicrobial activity of the sample was monitored at different time points (6, 16 and 24 hours).

3.6.3.1 Antibacterial activity against E.coli

Activity of the samples against microorganisms was seen as early as the 6th hour from the time of incubation. The growth of *E.coli* by the non-induced samples of the first pooled fraction-1 in both cell lines (Caco-2 and LS174T) was suppressed significantly. The non-induced sample of fraction-2 in Caco-2 cells also showed significant suppression in comparison to the induced sample. However, the non-induced sample of the second fraction of LS174T did not show a statistically significant difference in the suppression of *E.coli* at 6 hours in comparison to its induced counterpart. At 16 hours, the non-induced sample of fraction 1 from Caco-2 cells showed a statistically significant inhibition ($p<0.001$) compared to the matched induced sample. The second fraction did not show a difference between its induced and non-induced counterparts. The non-induced sample of fraction 1 in LS174T showed better antibacterial action, than the induced sample. However, fraction-2 which showed an increase in the AKTA-purifier (P-900) AUC, which implied more protein matter in the fraction displayed better inhibition of growth of *E.coli* by the induced fraction-2 ($p<0.0001$). At 24 hours the non-induced sample of fraction-1

continued to show statistically significant inhibition of *E.coli* that was more prominent than its matched induced fraction. However, in the Caco-2 derived induced fraction-2 there was better antibacterial activity than the non-induced sample of the same fraction ($p<0.0001$). This sample also showed better activity than both the non-induced and induced samples of fraction-1. Moreover, LS174T cells also showed a similar pattern, where the induced sample of fraction 2 (with increased AKTA-purifier AUC) showed better inhibitory activity. The growth curve of *E. coli* suggested that maximum inhibition was demonstrated by Caco-2 fractions rather than LS174T. Also their growth was inhibited most at the 16^{th} hour. Furthermore, although the non-induced sample of both fractions showed considerable activity at the 6^{th} hour, non-induced fraction-2 did not sustain this activity for the rest of the experimental period (Figure 3.8 (A) and (B)).

3.6.3.2 Antibacterial activity against S.typhimurium

Antibacterial activity against *S.typhimurium* appeared to be more potent than against *E.coli*. At 6 hours, inhibitory activity against the organism was seen by both fractions in Caco-2 cells, although the difference in the activity of induced and non-induced samples was not significant. However, in LS174T cells, the non-induced samples of pooled fraction-1 showed better inhibition than the matched induced sample, while the induced sample of fraction-2 displayed better inhibition of the organism than their matched non-induced samples. The difference was statistically significant ($p<0.0001$). At 16 hours, the difference in activity between the induced and non-induced samples of pooled fraction-1 in Caco-2 cells displayed similar levels of inhibition, while the induced sample of pooled fraction 2 (with increased AKTA-prime AUC) demonstrated better activity than its non-induced sample. However, in LS174T, the induced sample of fraction-1 showed better activity than the non-induced sample. Fraction-2 did not show any significant inhibition of the microorganism. 24 hours showed only minimal inhibition of the organisms. However, the induced samples of fraction-2 of both cell lines displayed better inhibitory activity than their non-induced samples for both cell lines. At 24 hours, the pattern of inhibition appeared to be similar for both Caco-2 and LS174T (Figure 3.11 (A) and (B)).

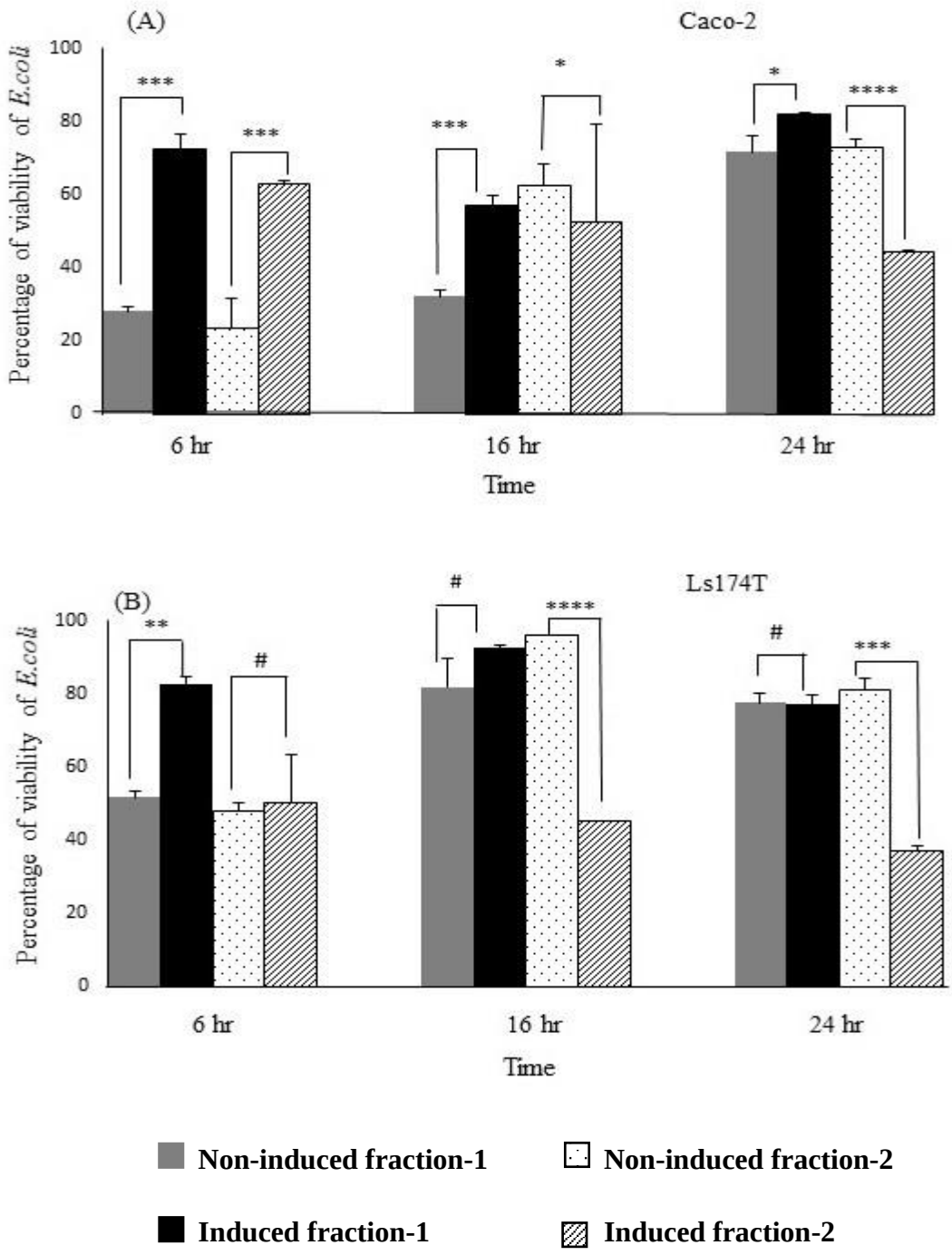

Figure 3.8: The percentage of viable *E.coli* at various time points. Non-induced and induced fractions of Caco-2 and LS174T samples were compared against *E.coli* viability. Measurements were done at 3 time points after ongoing exposure to CM. (A) represents non-induced and induced samples of Caco-2 cell line. (B) Represents non-induced and induced samples of LS174T cell line. # -non-significant; *-P ≤ 0.05; ** - P ≤ 0.01; *** - P ≤ 0.001; **** - P ≤ 0.0001. Data represents the average viabiltiy of *E.coli* ± SEM (n=4). Data was compared to positive control (growth medium in 2% FCS with ITS-X,without antibiotics).

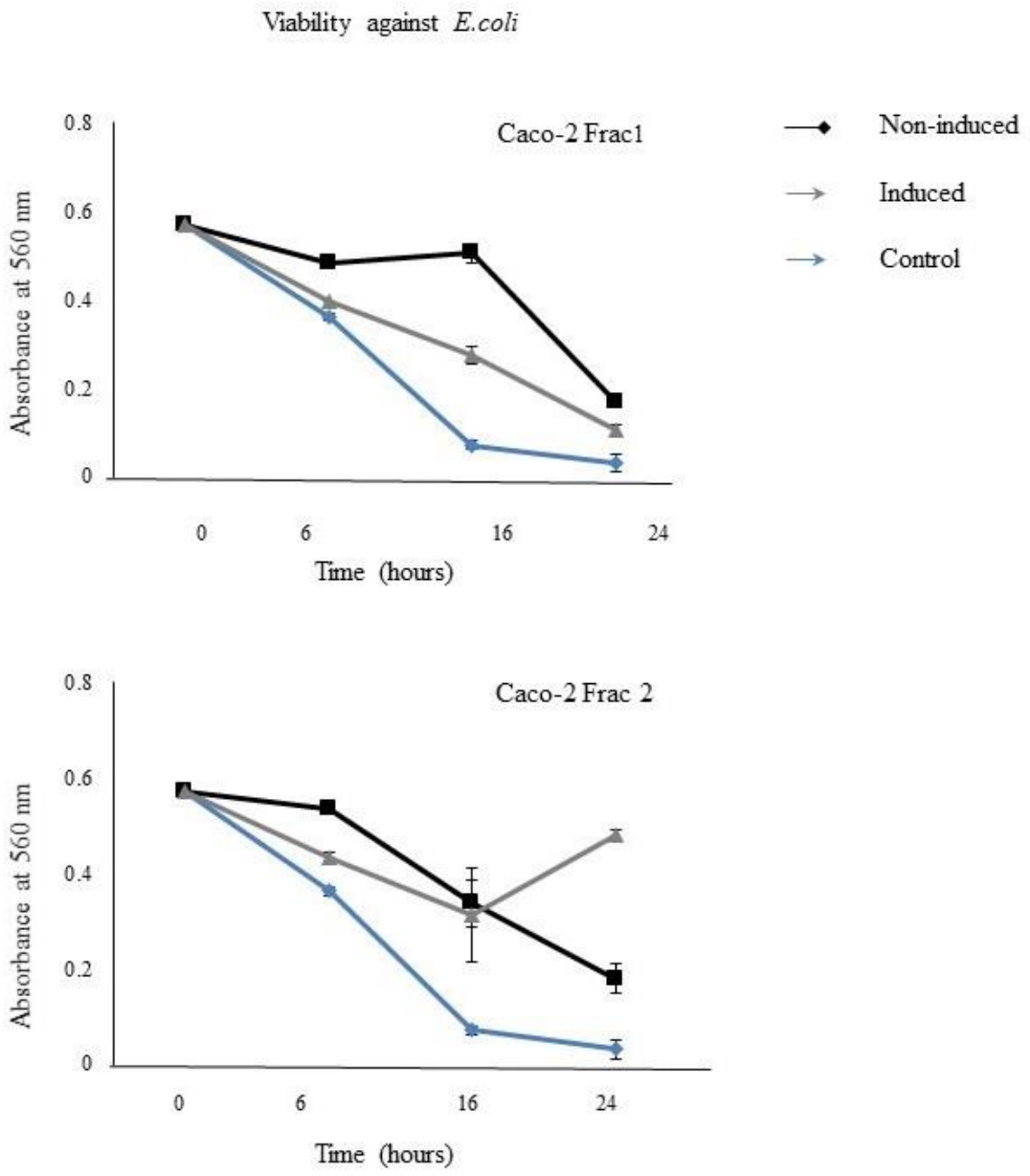

Figure 3.9: Absorbance values of the conditioned medium samples against *E.coli* at different time points. Different fractions of Caco-2 cell line was plotted against the control sample (2% growth medium without antibiotics). The absorbance values were taken at different time points (6,16,24 hours). A decrease in the absorbance values represents an acidic change in the medium, caused possibly by the bacterium. Data represents average viability of *E,coli* ± SD (n=4)

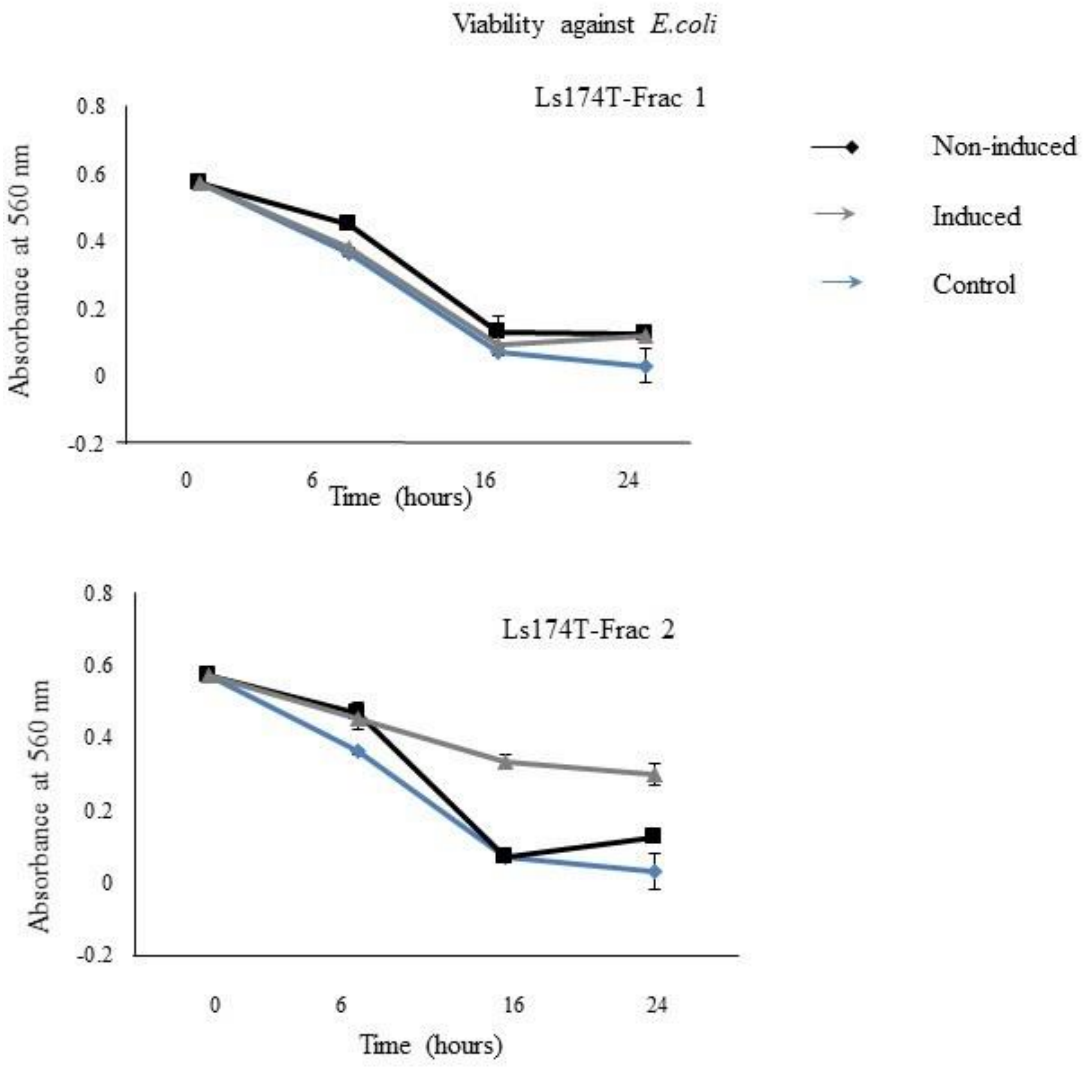

Figure 3.10: Absorbance values of the conditioned medium samples against *E.coli* at different time points. Different fractions of LS174T cell line was plotted against the control sample (2% growth medium without antibiotics). The absorbance values were taken at different time points (6,16,24 hours). A decrease in the absorbance values represents an acidic change in the medium, caused possible by the bacterium. Data represents average viability of *E,coli* ± SD (n=4)

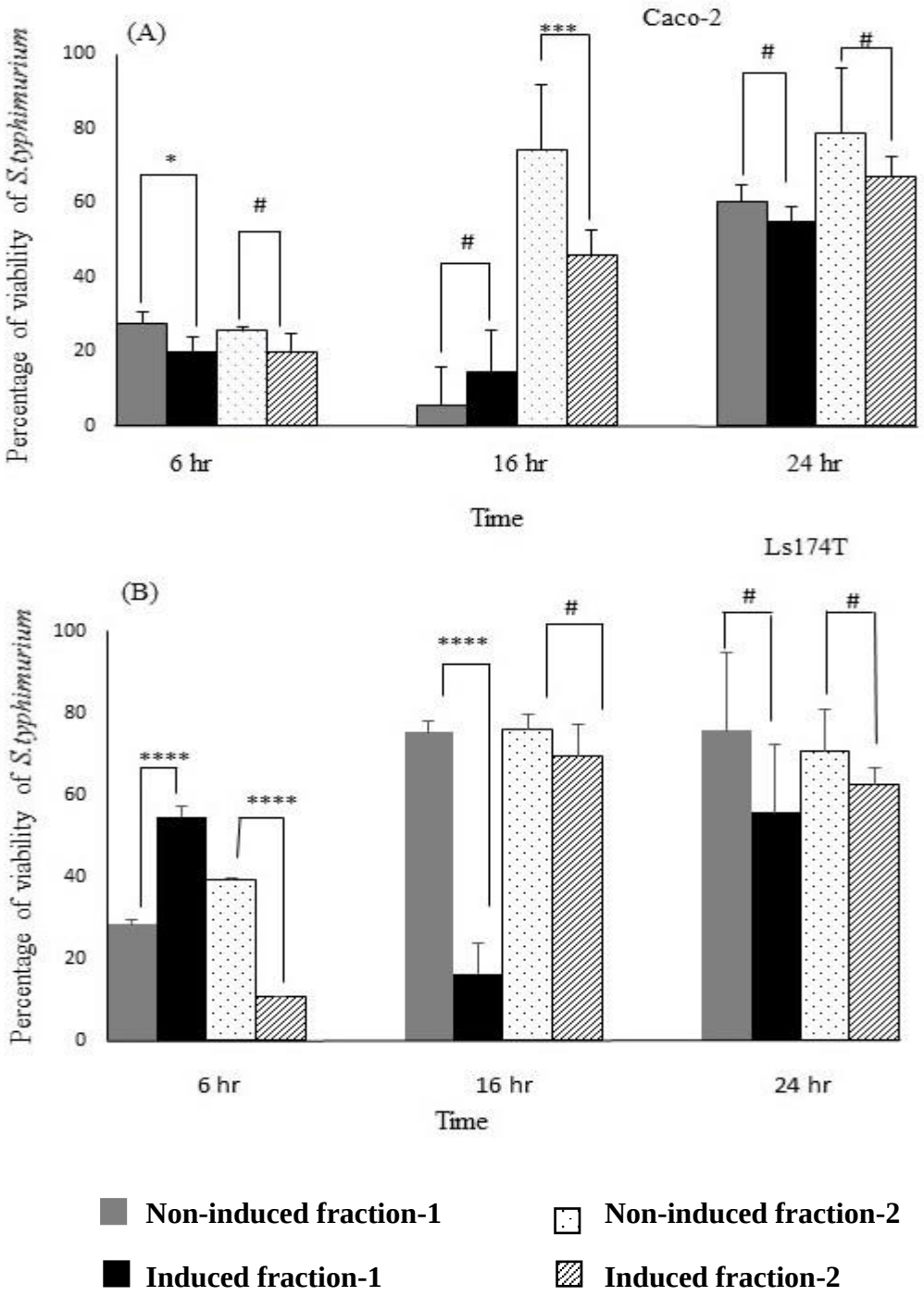

Figure 3.11: The percentage of viable *S.typhimurium* at various time points. Non-induced and induced fractions of Caco-2 and LS174T samples were compared against *S.typhimurium* viability. Measurements were observed at 3 time points after ongoing exposure to CM. (A) represents non-induced and induced samples of Caco-2 cell line. (B) represents non-induced and induced samples of LS174T cell line. # -non-significant; *-P ≤ 0.05; ** - P ≤ 0.01; *** - P ≤ 0.001; **** - P ≤ 0.0001. Data represents the average viabiltiy of *S.typhimurium* ± SEM (n=4). Data was compared to positive control growth medium in 2% FCS DMEM with ITS-X,without antibiotics).

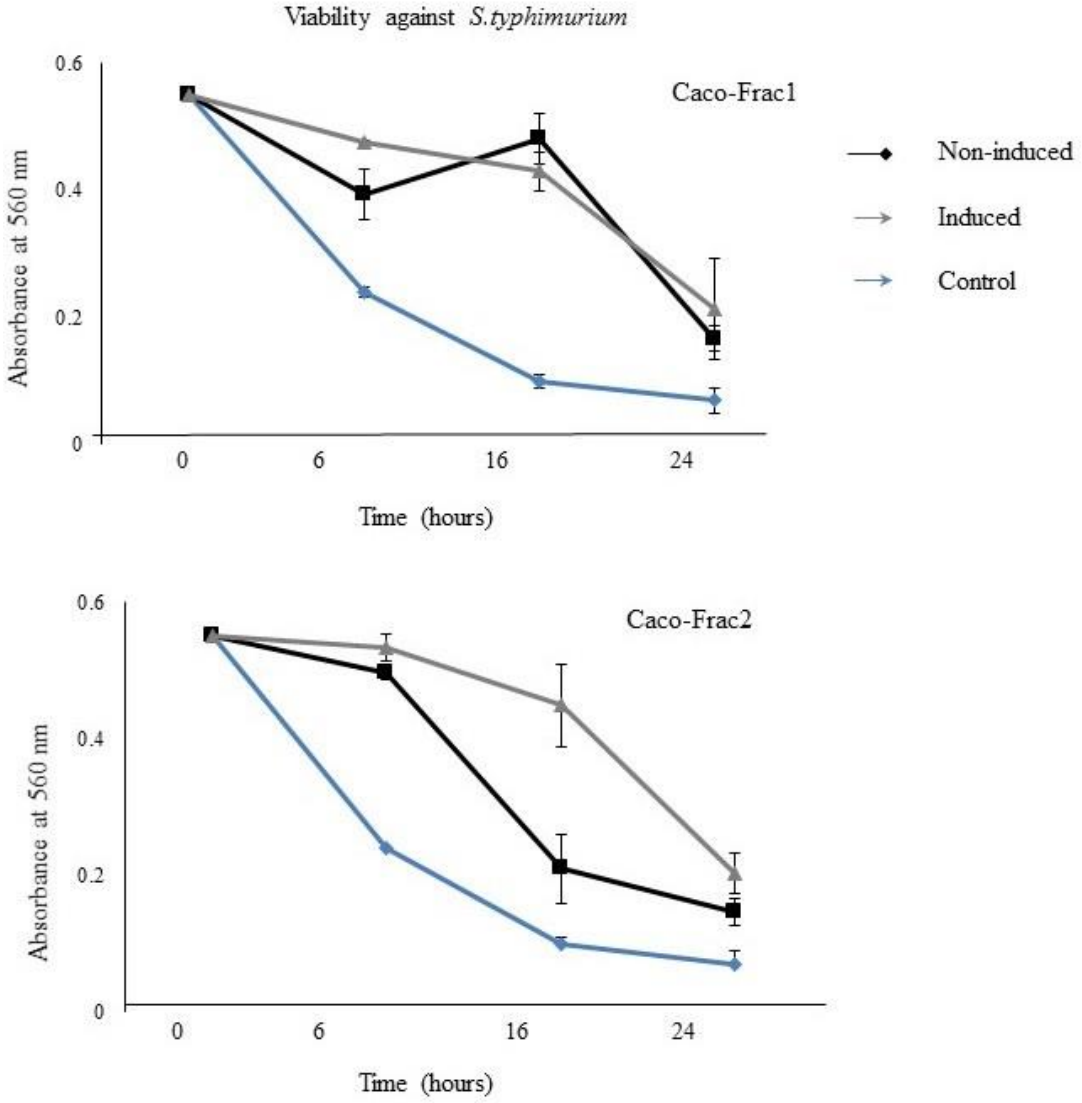

Figure 3.12: Absorbance values of the conditioned medium samples against *S.typhimurium* at different time points. Different fractions of Caco-2 cell line was plotted against the control sample (2% growth medium without antibiotics). The absorbance values were taken at different time points (6,16,24 hours). A decrease in the absorbance values represents an acidic change in the medium, caused possibly by the bacterium. Data represents average viability of *S.typhimurium* ± SD (n=4).

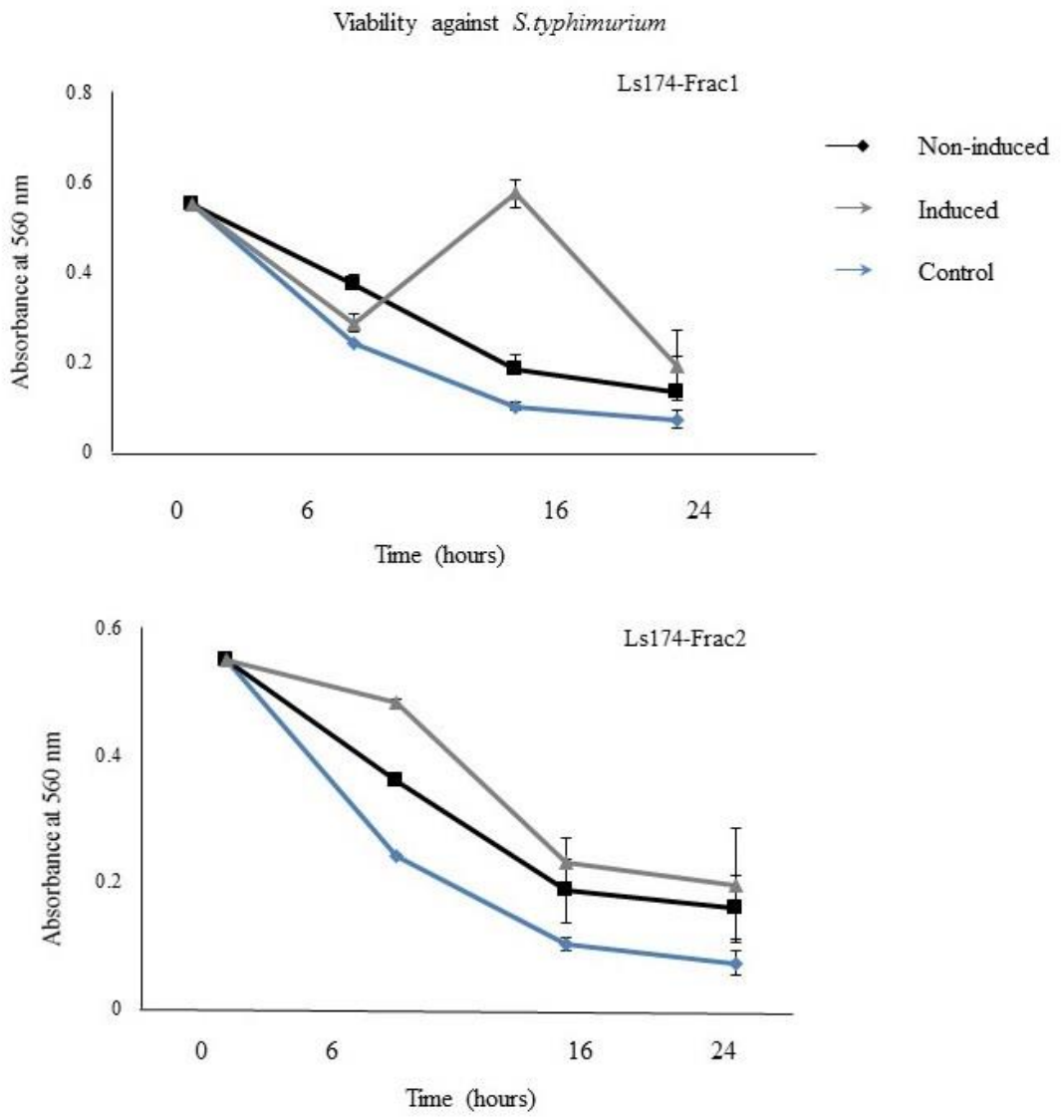

Figure 3.12: Absorbance values of the conditioned medium samples against *S.typhimurium* at different time points. Different fractions of Caco-2 cell line was plotted against the control sample (2% growth medium without antibiotics). The absorbance values were taken at different time points (6,16,24 hours). A decrease in the absorbance values represents an acidic change in the medium, caused possibly by the bacterium. Data represents average viability of *S.typhimurium* ± SD (n=4).

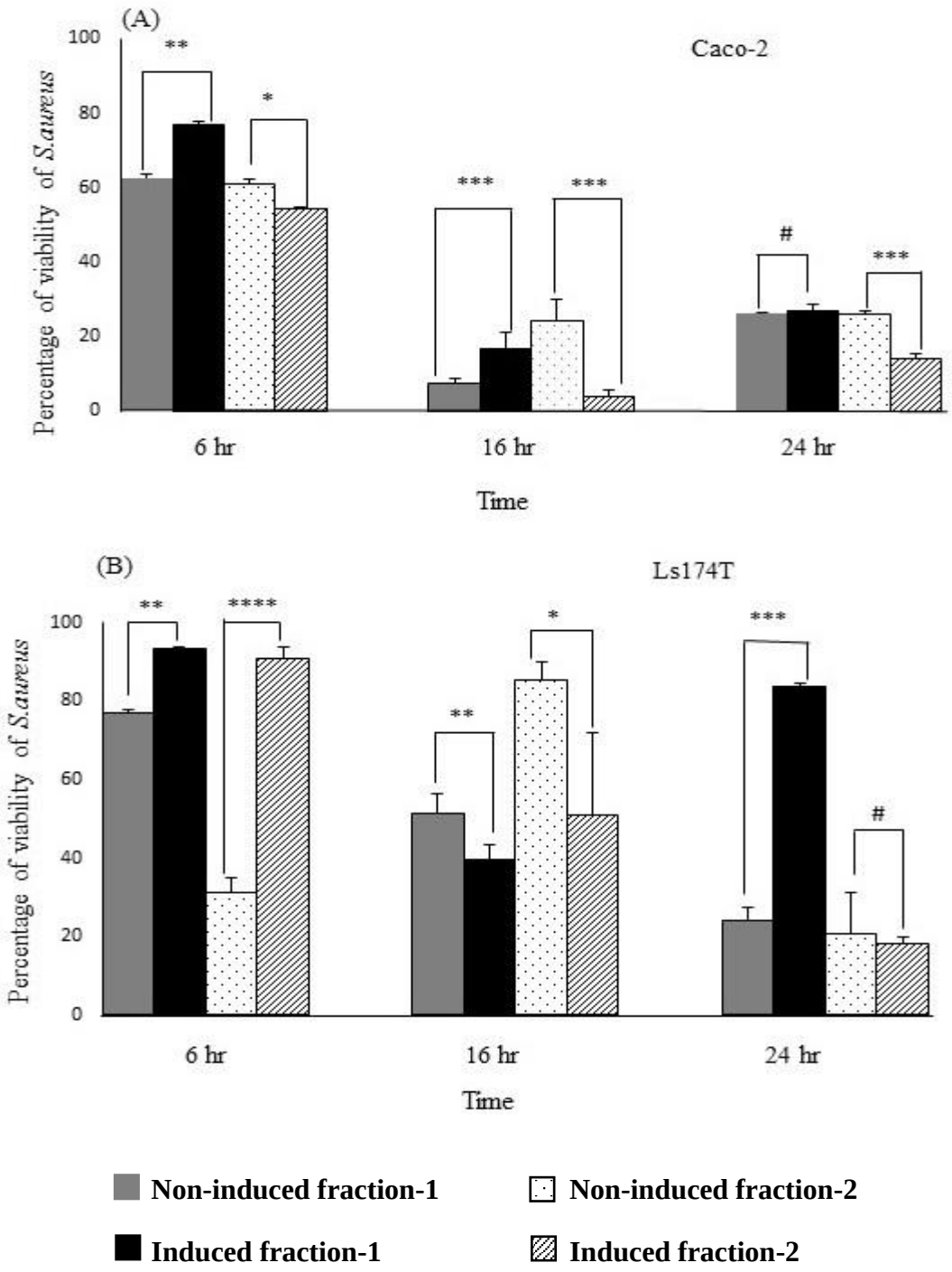

Figure 3.14: The percentage of viable *S.aureus* at various time points. Non-induced and induced fractions of Caco-2 and LS174T samples were compared against *S.aureus* viability. Measurements were observed at 3 time points after ongoing exposure to conditioned medium. **(A)** represents non-induced and induced samples of Caco-2 cell line. **(B)** represents non-induced and induced samples of LS174T cell line. # -non-significant; *-P ≤ 0.05; ** - P ≤ 0.01; *** - P ≤ 0.001; **** - P ≤ 0.0001. Data represents the average viabiltiy of *S.aureus* ± SEM (n=4). Data was compared to positive control (growth medium in 2% FCS DMEM with ITS-X,without antibiotics).

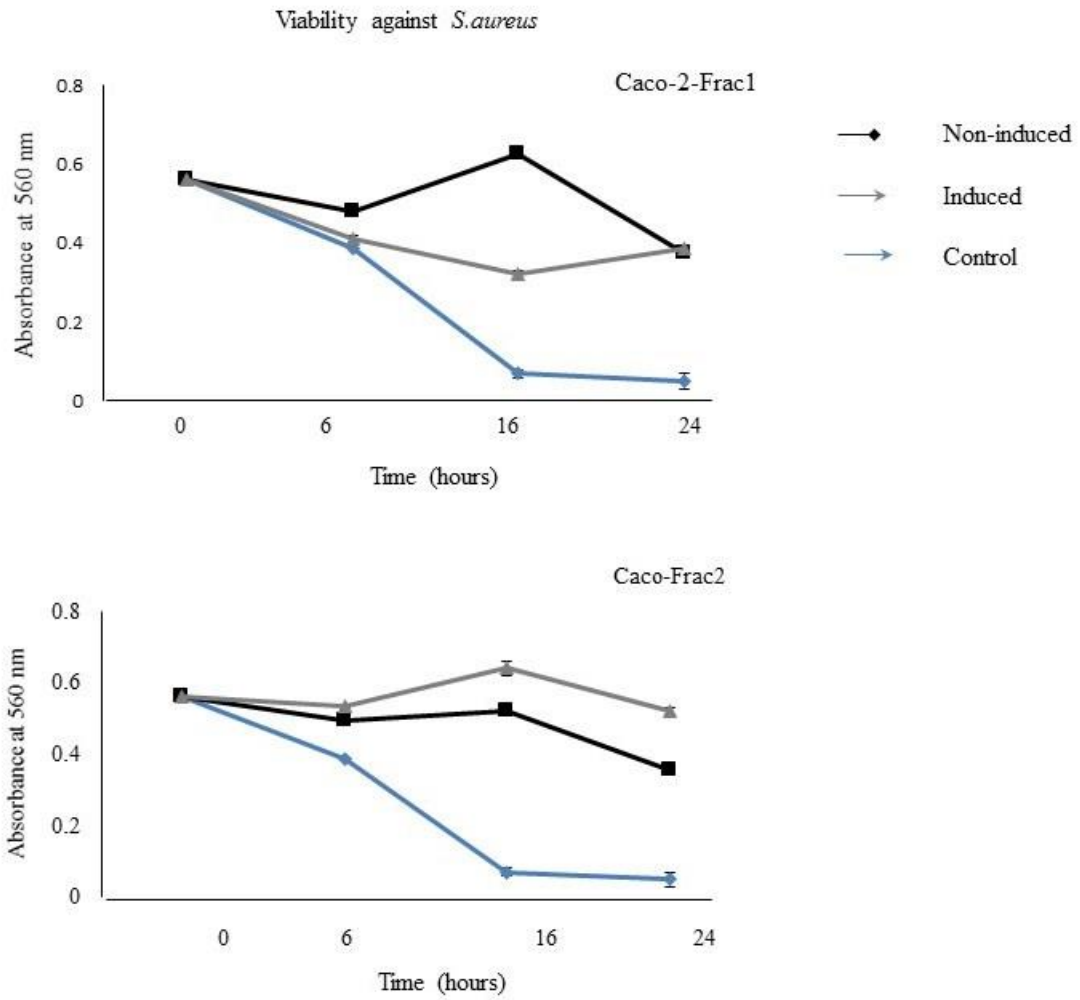

Figure 3.15: Absorbance values of the conditioned medium samples against *S.aureus* at different time points. Different fractions of Caco-2 cell line was plotted against the control sample (2% growth medium without antibiotics). The absorbance values were taken at different time points (6,16,24 hours). A decrease in the absorbance values represents an acidic change in the medium, caused possibly by the bacterium. Data represents average viability of *S.aureus* ± SD (n=4).

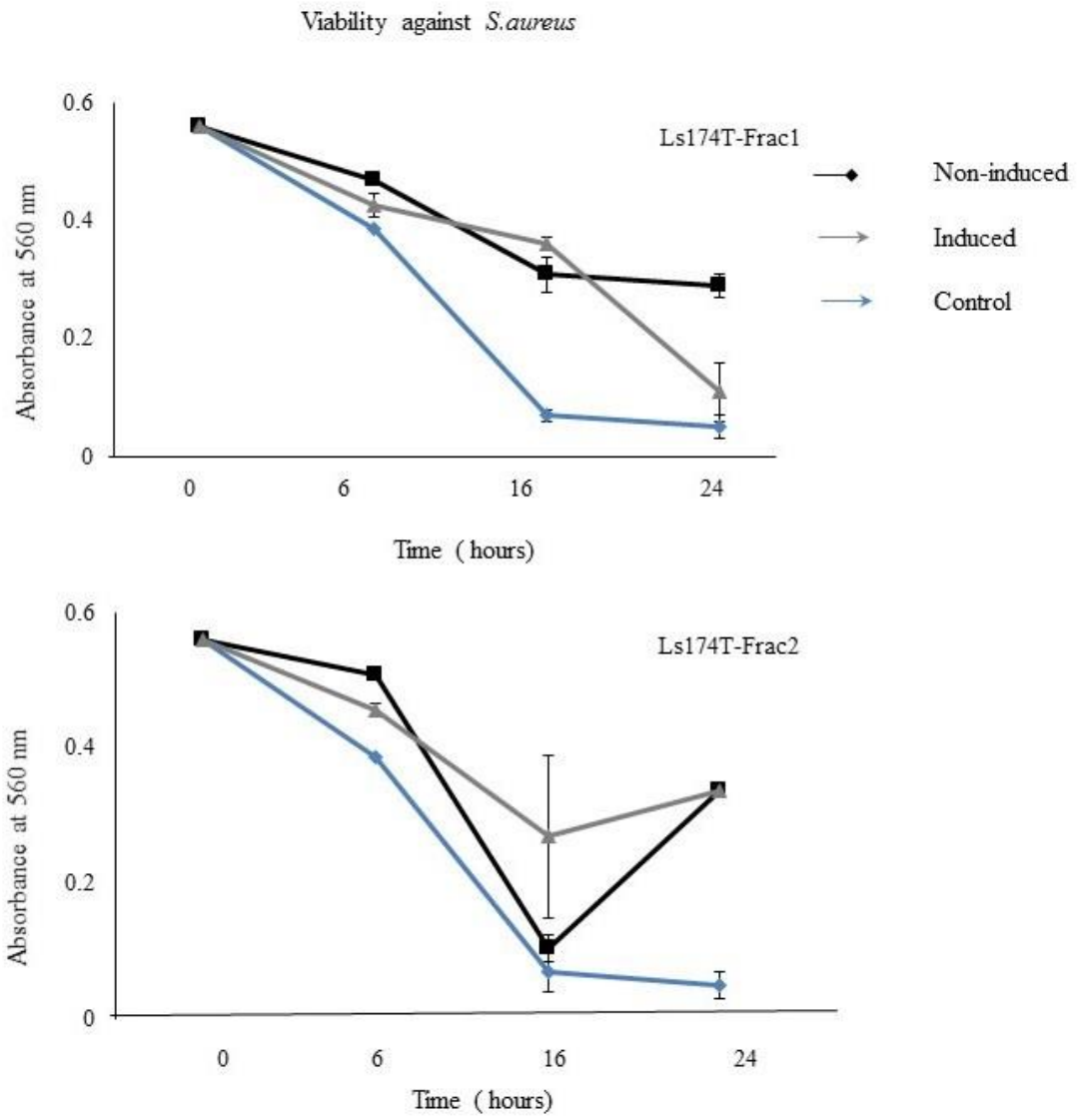

Figure 3.16: Absorbance values of the conditioned medium samples against *S.aureus* at different time points. Different fractions of LS174T cell line was plotted against the control sample (2% growth medium without antibiotics). The absorbance values were taken at different time points (6,16,24 hours). A decrease in the absorbance values represents an acidic change in the medium, caused possibly by the bacterium. Data represents average viability of *S.aureus* ± SD (n=4).

3.6.3.3 Antibacterial activity against S.aureus

S.aureus exhibited a different inhibitory pattern in comparison to the gram negative organisms (Figure 3.14 (A) and (B)). At 6 hours reduced inhibition of the organism was seen. Induced sample of fraction-1 of Caco-2 cells and LS174T cells showed less inhibitory activity than its matched non-induced sample. Induced fraction-2 of the Caco-2 cells showed better inhibition than its non-induced sample ($p<0.05$),however, in LS174T cells, the non-induced sample of fraction-2 showed better inhibition than its induced fraction ($p<0.0001$). At 16 hours, the non-induced sample of fraction-1 of Caco-2 cells showed better inhibition than the induced sample. The induced sample of pooled fraction-2 showed better activity than their matched non-induced sample. However, LS174T showed better inhibition in the induced samples of both pooled fractions (1 and 2). Overall at 16 hours, Caco-2 appeared to show better activity than LS174T amongst both fractions. At 24 hours, the induced samples of fraction-2 (with increased AUC in the AKTA purifier) of Caco-2 demonstrated better activity than its non-induced counterpart ($p<0.001$), while no significant result was seen in the induced and non-induced samples of fraction-1. In contrast, in LS174T cells, the non-induced sample of pooled fraction-1 showed a increasingly potent activity against *S.aureus* than the induced sample ($p<0.001$) and no significant difference in activity was seen between the induced and matched non-induced samples of pooled fraction-2.

Taking account of all samples from the two gastrointesinal cell lines, it could be concluded that the induced sample of fraction-2 (increase in AUC) displayed better inhibitory activity than the non-induced samples against all three microorganisms.

In conclusion, by 24 hours, although inhibition was not as robust as that of 16 hours, the induced samples of second fractions of both cell lines showed better suppression of growth of both gram positive and gram negative organisms. Overall, the activity of secreted products from Caco-2 cells were much better than that of medium from LS174T cells against all three microorganisms as mentioned above.

3.6.4 Mass spectrometric analysis of samples

The samples were subjected to mass spectrometry analysis by Proteomics International Pty Ltd. Results from sequencing of these samples showed many protein types were to be found in the conditioned medium, which is not surprising given that 2% FCS was still present in the medium. The sequences retrieved from Fraction 1 are as following:

Table 3.2: List of proteins/ peptides identified by LC/MS/MS and matched with MSPnr100 against the human database from Caco-2 non-induced samples

Protein name	NCBI Accession number	Peptide match	Sequence covered	Mascot Score	Mass
Hypothetical protein	CAA24950	2	2	63	13606
Insulin like growth factor 2	Q9NZ18	2	2	22	63441
Histone 2B	B4DR52	1	1	19	18030
Human cDNA clone UTERU 3016789	Q6ZTK4	3	2	18	50339
Anti-HIV Ig heavy chain	AEM61932	1	1	14	14128
Ig light chain	AAC36609	3	1	19	10994
Thioredoxin reductase-1	Q16881	1	1	33	59750
Hcg-1813002	EAW99833	2	1	32	19016

Table 3.3: List of proteins/ peptides identified by LC/MS/MS and matched with MSPnr100 against the human database from Caco-2 induced samples

Protein name	NCBI Accession number	Peptide match	Sequence covered/% of coverage	Mascot Score	Mass
Peroxisomal biological factor	AAH11963	1	1	42	28441
Hypothetical protein	CAB55945	23.1	80%	37.8	23546

Table 3.4: List of proteins/ peptides identified by LC/MS/MS and matched with MSPnr100 against the human database from LS174T non-induced samples

Protein name	NCBI accession number	Peptide match	Sequence covered	Mascot Score	Mass
Hydroxy lysine kinase	A2RV492	3	1	17	18770
Collagen α-3 chain isoform	XP_016883155	1	1	15	45155

Table 3.5: List of proteins/ peptides identified by LC/MS/MS and matched with MSPnr100 against the human database from LS174T induced samples

Protein name	NCBI Accession number	Peptide match	Sequence covered	Mascot Score	Mass
Hcg 2009783	EAW91866	2	1	22	100064
Human Ras protein like activator	AO24RBK8	1	1	19	900069
Collagen α-3 chain isoform	XP_016883155	1	1	15	45155

The second fraction that covers the area under the curve was submitted to Proteomics International Pty Ltd.

Table 3.6: List of proteins/ peptides identified by LC/MS/MS and matched with MSPnr100 against the human database from Caco-2 non-induced samples

Protein name	**NCBI Accession number**	**Peptide match**	**Sequence covered**	**Mascot Score**	**Mass**
Haemoglobin α 1-2 hybrid	ABF56145	3	3	59	15262
Histone H4	QoVAS5	1	1	57	11307
Ig heavy chain variable region	ACS6810	1	1	39	14400
Leucine rich repeat serine/threonine-protein kinase2	XP_016874277	4	4	28	145969
Human trinucleotide repeat containing gene	Q 9UPQ9	14	13	25	193883
Human rho GTPase-activating protein	Q9P2N2	4	4	14	82008

Table 3.7: List of proteins/ peptides identified by LC/MS/MS and matched with MSPnr100 against the human database from Caco-2 induced samples

Protein name	NCBI Accession number	Peptide match	Sequence covered/% of coverage	Mascot Score	Mass
Histone 2b J	EAX03082	12	2	89	8644
Serine/threonine kinase 31	AAK31978	5	5	35	115657
Serine/threonine kinase Nek11	B4DDN2	7	5	29	53130
Hypothetical protein	CAA09865 CAA 66560	1 2	1 2	33 18	28329 54537
Chain A , Atrial natriuretic peptide receptor isoform C complexed with brain natriuretic peptide	IYKI A	24.8	68%	24.8	3080

Table 3.8: List of proteins/ peptides identified by LC/MS/MS and matched with MSPnr100 against the human database from LS174T non-induced samples

Protein name	NCBI Accession number	Peptide match	Sequence covered/ % of coverage	Mascot Score	Mass
Salivary acidic proline rich phosphoprotein	NP_001278244	16	8	62	20840
Nuclear pore complex protein Nup 205	NP_055950	3	3	34	227775
Hypothetical protein	30023	6	4	16	69302
Ubiquitin carrier protein	B42856	24	47 %	24	17000
Hypothetical protein	CAB55945	23.1	37.8	80%	23546

Table 3.9: List of proteins/ peptides identified by LC/MS/MS and matched with MSPnr100 against the human database from LS174T induced samples

Protein name	NCBI Accession number	Peptide match	Sequence covered/% of coverage	Mascot Score	Mass
Ig heavy chain variable region	CEF92617	2	2	32	13559
Hypothetical protein product	BAG57205	23.1	33%	23.1	28760
Histone 2B	B4DR52	1	1	19	18030
Chain A, structure of atrial natriuretic peptide receptor complexed with atrial natriuretic peptide	IYKOA	24.8	68%	24.8	3080

Table 3.10: List of proteins/ peptides identified by LC/MS/MS and matched with MSPnr100 against the human database from Fraction-1 RKO cell line

Protein name	NCBI Accession number	Peptide matched	Sequence covered	Mascot score	Mass
G protein coupled receptor	AAL30811.1	20	2	38	693805
Hypothetical protein	CAI56716	7	6	16	203420

Table 3.11: List of proteins/ peptides identified by LC/MS/MS and matched with MSPnr100 against the human database from Fraction-2 RKO cell line

Protein name	NCBI Accession number	Peptide matched	Sequence covered	Mascot score	Mass
Human tubulin alpha 1C	F8VS66	1	1	66	14130

Table 3.12: List of proteins/ peptides identified by LC/MS/MS and matched with MSPnr100 against the human database from Fraction-1 HeLa MDR 1 OFF cell line

Protein name	NCBI Accession number	Peptide matched	Sequence covered	Mascot score	Mass
Transglutaminase-6	EAX10600	5	5	46	101923
Human translocating chain associated membrane protein	A8K032	4	4	42	43132

Table 3.13: List of proteins/ peptides identified by LC/MS/MS and matched with MSPnr100 against the human database from Fraction-2 HeLa MDR1 OFF cell line

Protein name	NCBI Accession number	Peptide matched	Sequence covered	Mascot score	Mass
Transgelin	AAH02616	1	1	40	22530
T-cell receptor alpha chain variable region	ANO54159	1	1	34	9510

Other sequences that have not been repeated but have been detected in the samples are represented in Table 3.14.

Table 3.14: List of protein/ peptide identified by LC/MS/MS and matched with MSPnr100 against the human database from Fraction 2 of Caco-2 induced sample

Protein name	NCBI Accession number	Peptide matched	Sequence covered	Mascot score	Mass
Cystatin 'C	325651800	20	1	73	3388

Various proteins of a much larger molecular weight was matched in the sample that should ideally contain only very LMW proteins. Having observed that, the proteins sequences that matched to these large proteins constituted only an approximate of 12-15 amino acids in length. Therefore, it could be suggested that the larger proteins may have cleaved during the fractionation process, thereby producing its cleavage products in the filtrate.

To ensure that the process of fractionation did not damage or only brought minimal degradation of proteins present in the sample, a known protein sample (Amyloid beta-42) was also submitted to Proteomics International. LC/MS/MS was conducted on the samples as the primary protocol. The peptide was not detected even at a very high concentration and all charged states were looked for. The sample could be detected only through MALDI-TOF analysis, suggesting that certain known peptides may not be detected through an LC/MS/MS (Figure 3.11).

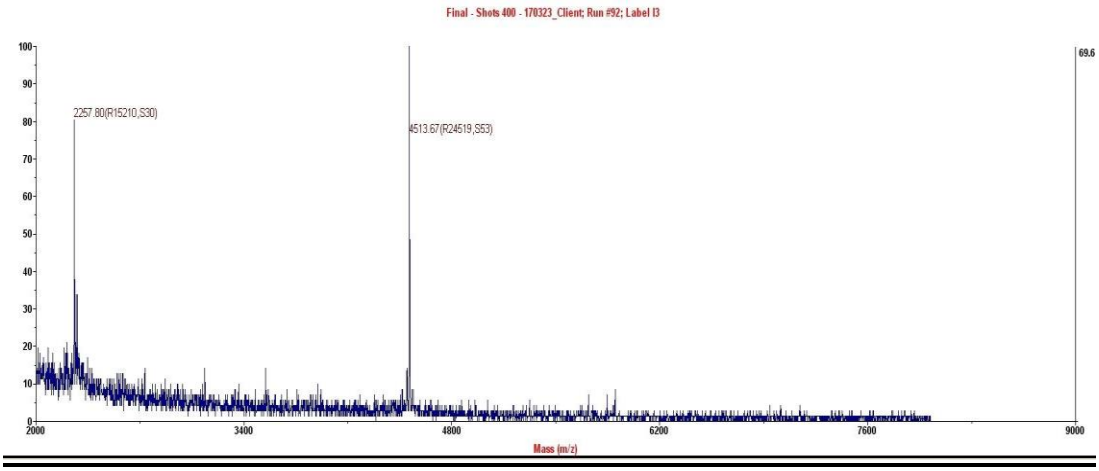

Figure 3.17: MALDI-TOF analysis of amyloid beta-42 by Proteomics International. Major ion present is 4513.67 and minor ion for MH_2^{2+} 2257.80 matching the theoretical mass at 4513.20.

3.7 Discussion

This chapter illustrates antimicrobial activity in CM of gastrointestinal cells by employing different methods for isolation of LMW peptides that potentially exhibits this antimicrobial activity.

The rationale for screening LMW fraction is attributed to previous experiments from our lab suggesting P-gp involvement in reducing bacterial adhesion (108). The previous results had shown that bacteria would associate less with the cells when P-gp expression was increased and more when the activity of P-gp was blocked. Subsequent unpublished data from Dr Crowe's lab showed CM from the cells impeding the growth of multiple gram positive and gram negative organisms. Therefore P-gp may play a role in innate immunity by effluxing the antimicrobial factor into the extracellular space.

Initially, molecular cut-off filters were used for fractionation of induced and non-induced CM of both the cell lines. The cells were incubated with 5 µM rifampicin, as it is a well-known inducer of P-gp (272). Based on previous experiments, cell lines induced with rifampicin showed higher antimicrobial activity (108). The filtrate from molecular weight cut off filters (30 kDa and 3 kDa) was precipitated by acetonitrile. The precipitation technique was performed after filtration with molecular weight cut off filters, so that a smaller volume of the filtrate is precipitated for stringent isolation. Different organic solvents can be used for precipitation of proteins, depending on the size of proteins that need to be eluted. Acetonitrile is used for adsorption of larger proteins like albumin, thereby releasing the smaller proteins in the supernatant (273). Usage of other organic solvents like methanol and ethanol displayed poor recovery of LMW proteins when quantified by Modified Lowry's test. Ahmed *et al.,* also showed that use of acetonitrile was a good method for precipitation of larger proteins, especially animal proteins like albumin present in serum (274).

Silver staining was used as a broad indicator for confirmation of LMW protein present in the final prepared samples. It is one of the most sensitive methods and hence used widely. Silver ions present in the solution allows for the detection of very low quantity of proteins (275).

Silver staining of post filtered and precipitated samples of CM showed very clean bands below 5 kDa. The bands varied in thickness between the days collected. With an increase in incubation time of cells (in days), thickness of the band also increased, suggesting a possible efflux of proteins by the gastrointestinal cell lines. Caco-2 showed an increased thickness of the band between days 19-22, while LS174T showed better band thickness between days 7-10 (Figure 3.2).

The LMW fractions were further processed by AKTA purifier which is based on the principle of SEC (276). Bio-rad P-6 column was used for the purpose. Theoretically, the cut off range of the column was between 800 da to 6 kDa. The samples were eluted with 50 mM PBS (pH 7.4), as the optimal strength for elution. Chellapandi *et al*., used 20 mM PBS for elution of salivary peptides with antimicrobial properties from reptiles using AKTA purifier for size exclusion chromatography (277).

Since, the peptides in our samples were likely to be at a lower concentration than their study an increased concentration of 50 mM PBS was used (278, 279). Also in the current study, concentrations below 50 mM did not show effective elution of proteins. Other mobile solvents used such as 0.1% SDS, methanol or acetonitrile, showed cross reactivity when the samples were subjected to antimicrobial sensitivity assay, hence invalidating their use as the elution solvent.

Elution of samples with PBS was initially done with three column volumes. Since, most of the elution was completed within two column volumes, collection of the eluent was restricted to two column volumes worth of material. After an equilibration time of 15 mins, the samples were loaded and monitored at 215, 254 and 280 nm (261, 271). CM contains of an array of different proteins and peptides. Some of these proteins and peptides are thought to cross link with each other (261, 280).

The samples collected were pooled together and divided into two fractions. First fraction contained tubes 1-6 of the peak. The second fraction consisted of tube 7-12 that occupies the increase in AUC. Both the induced and non-induced samples of both cell lines were loaded with approximately equal concentration of proteins (100 µg/mL). The samples were run using the same method of detection. The area under the curve at approximately 22 mins

showed a change when the column was loaded with induced sample of CM. Both cell lines were induced with 5 µM rifampicin, a P-gp inducer. Induced medium used for antibacterial activity experiments was collected at day 28, while induction itself was done between days 22-25. This was done so as to make sure that all traces of rifampicin was removed as rifampicin would produce an antibacterial effect (268). It is known that P-gp induction remains elevated for more than 48 hours after removal of rifampicin (281). This environment ensure the possible benefit of seeing increased efflux of a P-gp substrate while removing a potential contaminant from the environment.

Antibacterial activity of the samples was conducted against *E.coli, S.aureus and S.typhimurium.*

E.coli is the most common contaminant of the mammalian gastrointestinal tract and is also distinguished as a pathogen. They have an innate ability to attach to the intestinal epithelium and cause destruction of the microvilli. The mucus layer in the intestine protects against pathogens. The presence of certain intestinal specific microorganisms has also been known to cause an increase in the mucin production by goblet cells of the intestine (282).

S.typhimurium causes gastroenteritis, which may be both typhoidal and non-typhoidal. The difference between the two is caused by the influence of microbiota in the intestinal system. *S.typhimurium* has been known to induce pathogen control by causing inflammation e.g a complex GIT microbiota reduces *S.typhimurium* infection (283, 284).

The fractions showed antibacterial effect against both gram positive and negative organisms. At the 6^{th} hour where overall growth is not high due to the lag phase of growth, significant inhibition of growth of *E.coli* and *S.typhimurium* were detected. In the present study, at the 6^{th} hour the non-induced samples of both fractions of Caco-2 and LS174T cell lines indicated better bacterial inhibition than the induced fractions, whereas the induced samples of both fractions of Caco-2 showed better inhibition of *S.typhimurium*. Although, *E.coli* and *S.typhimurium* are both gram negative organisms, selective inhibition could be seen. *S.aureus*, is a skin pathogen and a gram positive microorganism. Although significant inhibition was seen by the induced sample of fraction-2, other samples did not show evident activity against the bacterium.

By 16 hours, the inhibitory activity against microorganisms had considerably improved against *E.coli*. The induced samples of fraction-2 of Caco-2 and the induced sample of fraction-1 of LS174T cell line showed better inhibition against *S.typhimurium* and *S.aureus*. This was the only fraction that showed inhibitory activity against *S.typhimurium* at 16 hours in LS174T cell line. At 16 hours the microorganisms used in the study would be in the exponential phase of growth. A similar pattern of inhibition was seen amongst both the gram negative organisms. Comprehensive inhibitory activity was also demonstrated against *S.aureus*, which is also a skin pathogen

By 24 hours, the induced samples of fraction-2 from both Caco-2 and LS174T showed better inhibition against all three organisms. Good inhibition was also seen in the non-induced sample of fraction-1 of LS174T. Overall, the inhibitory activity was less at the 24th hour mark than it was at 16 hours. Although, the induced samples have shown better inhibition than the non-induced samples, a general decrease in the activity of the samples were seen. A possible explanation is attributed to the factor of interest in the CM being bacteriostatic and not bactericidal, thereby restricting the growth of microorganisms for a short period of time.

HBD-2 has shown bacteriostatic activity against gram positive organism *S. aureus* at a very low concentration of 100 µg/mL from other researchers (285). Lactoferrin a large AMP in bovine samples has also shown bacteriostatic effect against septic causing organisms such as *S.epidermidis* (286). Human beta defensin (HBD-1), HBD-2 and Human defensin (α-defensin) HD-5 and lactoferrin from human preterm breast milk at day 7 has demonstrated significant bacteriostatic effect against *E.coli*, *S.aureus* and *S.epidermidis* by over 90%. The concentrations of defensins in milk on were 94 ng/mL, 10 ng/mL and 135 pg/mL respectively, although it was likely to change with milk production over time. In comparison to Lactoferrin the activity of defensins may have been negligible as the concentration of Lactoferrin in breast milk was 4.5 mg/mL (286). Another plausible explanation is at 24 hours, the microorganisms may have entered the stationary phase making all inhibitory activity redundant against the microorganisms (287, 288).

Other bacterial associated antimicrobial peptides have also shown to be bacteriostatic that work to form a matrix around bacteria without killing them, thus only giving a temporary

relief from the bacteria (289). The gram positive organism *Staphylococcus epidermidis* are present on human skin (290). The phenol soluble modulins (PSM) from this organism (ò-toxin) helps in the neutralisation of the pathogen (291). Cogen *et al.,* have shown that in whole blood the presence of this toxin provides a bacteriostatic effect against group A streptococcus (GAS) at a concentration between 2-4 µM (292). *In-vivo* studies from the same lab also supported this finding. A wound from mice was pretreated with the toxin and incubated with GAS after 30 mins of treatment. The wound was harvested and a decrease in the presence of the pathogen was seen in comparison to its control (wound pretreated with PBS).

Yet another explanation may be degradation of the peptide during the incubation period because of a potentially unstable environment. The described α-defesins peptide structure may not be in its tertiary folded structure and that could make them prone to protease degradation. Their activity would be still intact for a short period of time though. This is possible because of regulation by the proteasomes that has been induced by ubiquitination and other proteasome inducing pathways (293). Also, variability in the nonconserved domains of the peptide maybe observed (294-297).

The three microorganisms showed different level of inhibition of their growth curve against the same sample. Although uniform methods were used for separation of pooled fractions certain differences in separation of fraction or external addition (spiking of CM) of microorganisms may have led to this pattern.

The samples have been compared to growth activity in the control sample (growth medium with 2% FCS without antibiotics).

From the present study, it could be inferred that the LMW peptides that causes antibacterial inhibition are evident in the fractions of the CM. The exact mechanism and the exact fraction of the antibacterial activity has not been elucidated in the study. Cationic antimicrobial peptides that possess smaller amino acid chains are very important in the field of antibacterial therapy, as they have a broad spectrum of activity and different modes of action (298).

The fractions were also screened for sequencing by Proteomics international Pty Ltd. In addition to the sequences documented from table 3.2 to table 3.13, other sequences that were observed in the samples are cystatin C and several extracellular matrix proteins. They included laminin, collagen, and fibrinogen and these were indeterminate in the extracellular matrix. Cystatin C has only been detected once in our samples and its presence could be due to technical problems that may originate from improper cleaning of the column that was used for sequencing and inaccurate filtering of samples. Similarly the larger proteins that were observed in a supposedly <3 kDa filtrate also could have been observed owing to this.

Khamis *et al.*, performed a study in their laboratory that developed a novel *in silico* method to interpret AMP characteristics (299). They described α-defensins, which were a key gastrointestinal AMP, containing increased cysteine, arginine and tyrosine, while there were decreased lysine and glycine residues. Arginine appears to be an important amino acid for demonstration of antibacterial activity as it causes electrostatic interactions that ruptures the microbial envelope. A replacement of the arginine with lysine brings about a decrease in antibacterial activity. β-defensins appeared to have an increased proportion of valine, arginine and phenylalanine compared to α-defensins (299). Chandrababu *et al.,* also proposed that cysteine, glycine and arginine as the most important amino acids for increase in antimicrobial activity. Cathelicidins have a high proportion of proline which may be related to its antimicrobial activity (300).

In the current study some of the hypothetical proteins showed sequences that may exhibit possible antimicrobial activity based on the concept of the key amino acids being important. Y.KGGTTPNLRLLCSITGGTS.P is one such sequence with tyrosine and proline at the C terminal and N terminal ends. Proline, arginine, cysteine, phenylalanine and increasing amounts of glycine were also seen in the sequence.

Although some groups have shown through computational methods that cysteine and arginine were the most important amino acids that contribute to antibacterial properties, other groups have shown that lysine rich histones exhibited effective antibacterial activity (301-303). An examples is M.PEPAKSAPAPK.K (Histone 1 2Bj) and H.AVSEGTKAVTKYTSS.K (Histone 2B), that show an increasing number of lysine residues in them.

In line with this view, histones 2B and histone 4H were detected in the CM from this study. Histones being a part of the chromatin binding of cells are not unusually detected but also have been known to display other mechanisms, namely antimicrobial activity (304). They are divided into multiple groups based on their amino acid constitution. Histone H1, H2A and H2B are shown to have lysine residues, Histone H3 and Histone H4 have shown to be rich in arginine residues (305).

The inhibitory concentration of histones was shown to be in the similar range as that of other AMPs such as defensins. In the study by Tagai *et al.*,(305) the inhibitory concentrations of histones from calf thymus were deduced. The lowest IC_{50} value was shown by Histone 2B at 3.8 µM, which was still too high to be present in the CM samples of the current study. In the present study, the concentration of histones could not be inferred as the fractions did not contain just one single antimicrobial peptide, but rather a mixture of peptides.

Histones such as histone 1A, 2A and 2B have exhibited bacteriostatic effect against gastrointestinal pathogens. They were shown to be produced constitutively in the intestinal villi epithelial cells and in the placental epithelial cells (302, 306). Supernatants of the cultured human intestinal epithelial cells and wound fluids also have exhibited antimicrobial activity and in the presence of high salt concentration (302, 307, 308). Histone derived peptides such as buforins and parasins have also demonstrated antibacterial activity (301, 309). They have also shown bacterial LPS binding abilities similar to other cationic antimicrobial peptides (307, 310).

In addition to the gastrointestinal pathogens, histones have also shown bacteriostatic activity against skin pathogens, namely, *S.aureus*. According to Lee *at al*., histone H4 from sebocytes also exhibited antibacterial activity against the skin pathogen, *S.aureus* by a synergistic activity with the free fatty acids present in the sebaceous glands (311).

Since the present study also showed histone H4 sequences in the fractions, it is likely that this histone product can also be produced by gastrointestinal epithelial cells and is active against *S.aureus* (308).

Similar to the constitutive appearance of histones in other studies, our study also showed histone sequences without a bacterial stimulus.

Other sequences acquired from the samples are ubiquitin carrier protein, cystatin C and atrial natriuretic peptide complexed with its receptor.

Ubiquitin has shown antibacterial activity against a wide range of bacteria. They have been detected in the supernatants of stimulated adrenal medullary chromaffin glands. Studies have shown that the activity of ubiquitin can be seen against *Bacillus megaterium* at a concentration of 60 µM (312). It is highly unlikely that a peptide of this concentration may have been secreted in the CM of Caco-2 cells. Also as the MW of ubiquitin was 8.5 kDa it is highly unlikely that it contributed to the antibacterial observed in the samples.

Fractions analysed by Proteomics international also contained proteins like mammalian thioredoxin and insulin like growth factor matched against a human database. These have not shown any known antimicrobial activity.

Some amino acid sequences with potential antimicrobial activity have been listed below based on reports that suggests that amino acids such as valine, proline, phenyl alanine, cysteine, arginine, lysine and glycine.

Table 3.15: List of sequences with possible antibacterial activity

Accession number	Sequences	Mass
CAA24950.1	K.DGKSAAHR.K M.PEPAKSAPAPK.K	13606
CAI56716	Y.KGGTTPNLRLLCSITGGTS.P N.AATTSGYTPLHLSAREGHEDVAA.F	203420
CAA09865.1	T.VRPGTPPDF.I	28329
CAD38944.1	K.VVLRQVVVPRPGR.D K.VVLRQVVVPRPGR.D K.VVLRQVVVPRPGR.D K.VVLRQVVVPRPGR.D	55126
EAX03082.1 (Histone 1, H2bj)	M.PEPAKSAPAPK.K M.PEPAKSAPAP.K	8644
Q0VAS5 (Histone H4)	R.DNIQGITKPA.I	11307
CAB02542.1(Histone H2B)	M.PEPAKSAPAP.K R.STITSREIQT.A R.STITSREIQTA.V H.AVSEGTKAVTKYTSS.K	13928
B4DR52 (Histone H2B)	M.PDPAKSAPAPK.K	18030
BAJ83816.1 (cystatin C)	G.SSPGKPPR.L	3388
Q16881-2 (thioredoxin reductase)	R.MLSRLVLNSWAQAIIRPRPPK.V	59750

Several cationic antimicrobial peptides are formed by cleavage of whole or intact proteins that may or may not have antibacterial action themselves. An example is Lactoferricin B, which is a milk protein that is formed by the cleavage of bovine protein. Although bovine protein does exhibit antibacterial properties, lactoferricin B which is a 25 residue peptide shows an efficacy that is much higher to the intact protein (313, 314).

Sequences were also obtained from negative control, RKO cell line and HeLa MDR1 OFF. RKO cell line does not show P-gp expression and therefore hypothetically should not efflux the particular antibacterial factor. HeLa MDR 1 OFF is a cervical cancer cell line overexpressing P-gp, but it still should not express the antibacterial factor as it is not a gastrointestinal cell line. Some of the possible antimicrobial sequences such as Histones and most hypothetical sequences were not seen in both the cell lines. The HeLa MDR1 OFF cells showed the presence of sequences that did not match to the gut cell lines. Therefore, it could be suggested that the histone and some of the hypothetical sequences in the samples from Caco-2 and LS174T have contributed to the putative antimicrobial activity observed.

3.8 Limitations

As CM was used that contained a mixture of components, isolating one particular factor with antibacterial activity was not possible. Although many sequences were obtained from a high ultra-sensitive technique like protein sequencing, it may not be necessary that the sequences isolated from the samples may be attributed to the antibacterial activity in the samples at all. Studies have reported that histones could be a potential antibacterial agent, but no further validation, such as isolation of histone or measuring its concentration for antimicrobial activity was done. Also, as was shown in the known control sample (Amyloid beta-42), LC/MS/MS may not only be the mode of detection for protein sequences. As MALDI-TOF was not used for detection of samples from CM some potentially valuable peptides may have not been detected as LC/MS/MS was alone our choice of peptide sequence determination, especially since Amyloid beta-42 has been determined in other studies to be produced in Caco-2 cells (187).

3.9 Conclusions

Potential sequences with antibacterial activity have been observed in the CM of gastrointestinal cells that expresses P-gp, while these sequences were absent in cells without P-gp expression. Nevertheless, the sequences may not be related to the antibacterial activity in these cells. Hence such an association remains as such until more evidence is delivered, such as direct bidirectional transport, which will be scoped in later chapters of the thesis. It remains to be seen whether histones are responsible for antibacterial activity and a prospective substrate for P-gp, by various other experiments. In addition, many proteins may have not been identified due to limitations in the detection methods used in the study, therefore a single peptide could not be designated here as the primary factor for antibacterial activity shown in this project and related projects in this laboratory.

Chapter 4 : Investigation and determination of defined antimicrobial peptides in gastrointestinal cell lines

4.1 Rationale

The previous chapter has demonstrated various unknown protein/peptide sequences with potential antimicrobial activity in the conditioned medium (CM) of gastrointestinal cell lines. This chapter illustrates the presence of known antimicrobial peptides (AMP) in the cells and CM, when the gastrointestinal cells have been induced by lipopolysaccharide (LPS) which is a bacterial product to fully understand what transpires during the antimicrobial activity observed in the previous chapter.

4.2 Introduction

The gastrointestinal cell lines were induced with LPS and an array of AMPs were detected in their cell lysates and CM. Non-induced samples were also subjected to the same methods of detection and compared to the induced samples to demonstrate the causal effects of stimulation with LPS.

Humans are continually exposed to microbes and the most common sites of initial encounter with microbes are the epithelial surfaces including the moist surfaces of eyes, nose, airways, digestive tract, urinary and reproductive system (315). The intestinal tract is colonised by a variety of microorganisms some of which perform useful functions related to the biological activity of the body. The intestine is also extremely prone to both the enteropathogenic and enteroinvasive microorganisms that cause harm to the body by invading the epithelial barrier. AMPs in the intestine protect the epithelial surface, thereby providing a balanced microflora and assisting better absorption of nutrients through the intestine (315, 316).

Microorganisms are phagocytosed by the granules present on the surface of the epithelium (109). During the process, the granules either attach to the vacuoles that can ingest microbes or are secreted into the extracellular milieu, and consequently act as either bacteriostatic or bactericidal agents. These granules contain a large number of AMPs (109, 317, 318).

4.2.1 Structure of antimicrobial peptides

Antimicrobial peptides are positively charged, cationic peptides ranging between 10-40 amino acids although sometimes exhibiting negative charge at pH 7 (319). They are stored

as pro-peptides or mature C-terminal peptides. AMPs can be distinguished based on their size, amino acid assembly and conformational structure. They can also be divided into various classes depending on their composition and three dimensional structures. Group 1 constitutes linear, α-helical peptides, without cysteines such as LL-37/hCAP. Cecropins and magainins that are mainly found in animals such as pig and frog respectively are also examples of this group (320). Group 2 comprised of peptides with cysteine residues linked by disulphide bridges, such as α and β defensins while group 3 contains AMPs with short chain amino acids, and an increasing proportion of one or more of the amino acids e.g., PR-39 (that contains higher amounts of proline and arginine) (289).

4.2.2 Spectrum of the antimicrobial activity

AMPs are active against both gram positive and gram negative organisms. The difference in activity is attributed to their tertiary structure (109). Activity of AMPs may be, synergistic or an independent process. Synergism occurs when the AMPs are attached to the larger molecules such as polypeptides with enzymatic activity thereby providing a stimulus for increased antimicrobial activity. AMPs may also combine with certain bacterial macromolecules, like bactericidal permeability inducing protein-BPI (109, 321).

AMPs are effector molecules that are part of the innate immunity system present within the gut and thus contribute to the body's first line of defence. They upregulate the potential of the intestinal epithelial cell to diminish bacterial attachment (162). This criterion is also important for maintaining homeostasis within the intestinal system. Although some of the AMPs are endogenously secreted in the extracellular fluid, the presence of microorganisms or microbial products also stimulate their secretion. The toll like receptors (TLRs) and NOD like receptors (NLRs) which are both pattern recognition receptors (PRR) are also activated. Some of the AMPs produced by the gut are cathelicidins, defensins, phospholipase A2, lysozyme and lactoferrins. Antimicrobial peptides are made of different constituents and hence attach to the microorganisms diversely (157).

Secreted endogenous AMPs also form a physical and biochemical barrier by accumulating in the mucus layer, without disseminating into the gut lumen, assuring significantly reduced interchange with the microflora (315).

Antimicrobial factors are also proposed to alternate with complement factors, thereby regulating the expression of both. This relationship demands further enquiry in the intestine (322).

4.2.3 Relationship between antimicrobial peptides and neuropeptides

Neuropeptides (NP) may also display anti-infective activity, owing to their similarity with AMPs in terms of size, amino acid composition, amphipathic design and cationic charge. The conventional roles of NPs have been signal transmission and integration in the nervous system. Owing to their release from nerve fibres of innervated organs they also have the ability to act as an antimicrobial and immunomodulatory agent in the surrounding tissue (323). Lundy and Linden., have reported that neuropeptides such as vasoactive intestinal peptide (VIP), calcitonin gene-related peptide (CGRP) and Substance P (SP) have shown activity against microorganisms like *Escherichia coli (E.coli)* and *Pseudomonas aeruginosa (P.aeruginosa)*, with a similar potency to that of α-defensins (324). Hansen *et al.*, have also discussed the possibility of antimicrobial activity amongst the neuropeptides, wherein the nervous system may employ such neuropeptides to be active precisely in specifically innervated areas such as oral tissues (325, 326). Their method of action maybe similar to that of other antimicrobial peptides, wherein a combination of positively charged amino acids and hydrophobic residues is responsible for their activity, e.g., NPY, AM, α-MSH (327-329). Despite having a direct effect on microorganisms, their presence in the tissues is not sustainable and may not reach high enough concentrations for such activity. This may be due to the shorter half-life of NPs *in-vivo* that causes a delay in restoring these NPs at the nerve endings (330). Hence, the anti-infective activity maybe emphasised by their more potent immunomodulatory activity.

4.2.4 Effect of lipopolysaccharides and other bacterial products on microbial activity

Lipopolysacharide (LPS) is an endotoxin present on the outer membrane of gram negative organisms such as *E.coli* and *Klebsiella sp*. They can be released when the cells undergo cell division or apoptosis. LPS is divided into three segments, an outer O antigen segment,

core oligosaccharide and a lipid A layer (331). A number of AMPs are known to interact with the negatively charged LPS layer of an organism (331).

AMPs produce strong electrostatic interactions with LPS, thus causing them to attach to lipophilic micelles thereby neutralising the lipid A layer. Many factors influence the binding capacity of AMPs with the LPS. Hydrophobicity of AMPs helps in the attachment to the LPS micelles, while their positive charge helps in attenuating the negative charge of LPS with electrostatic interactions. The distance between the positively charged amino acids in AMPs also has an influence in its binding with the LPS. A distance of 12-15 Å is usually considered to be optimal for attachment (332).

In addition to its endotoxin characteristic, release of LPS in the blood stream also stimulates an array of inflammatory cytokines, namely, TNF-α, IL-6 and IL-8. AMPs neutralise these inflammatory cytokines to produce an anti-inflammatory reaction. Hence, AMPs have been known to have both anti-microbial and anti-inflammatory properties, deeming them to have a broader spectrum than originally envisaged (333).

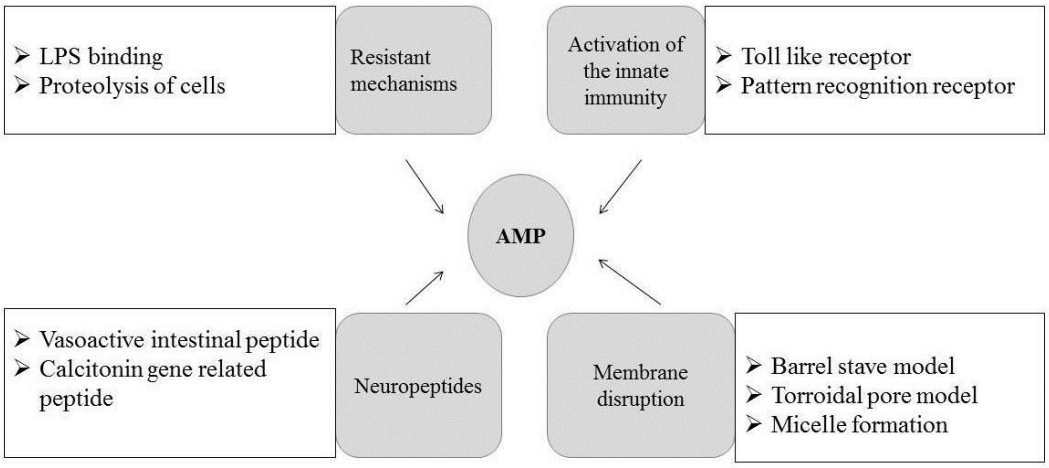

Figure 4.1: Schematic diagram of different properties of antimicrobial peptides (AMP). Diverse characteristics of AMPs include maintenance of haemostasis and as an immunomodulatory agent. Many neuropeptides may also be involved in antimicrobial resistance in addition to its other roles.

4.3 Aim

The aims of the chapter include:

- mRNA analysis of defined gastrointestinal AMPs in LPS stimulated and unstimulated cells cultured along with P-gp inducer-rifampicin and P-gp blocker-PSC-833 in Caco-2 and LS174T cells lines using RT-PCR.
- Protein analysis of defined gastrointestinal AMPs in LPS stimulated and unstimulated cell lysates and conditioned medium cultured along with P-gp inducer-rifampicin and P-gp blocker PSC-833 in Caco-2 and LS174T cells lines using Western blot.

4.4 Materials

Caco-2 cell line (P-80 to P-90), LS174T cell line (P-65-75), P-gp inducer rifampicin-5µM (Sigma-Aldrich, Castle Hill, Australia), P-gp blocker PSC-833-4 µM (kindly provided by Novartis, Basel, Switzerland), DMEM with 10% FCS with antibiotics (pencillin/streptomycin), DMEM with 2% FCS and 1% ITS-X/without antibiotics (in accordance to 2.5), LPS (E.coli 0111:B4, 99% purity, Sigma-Aldrich, Castle hill, Australia). Primers were obtained from geneworks (SA, Australia), RT-PCR was done using 2X Sensifast (Lo-Rox 2X, Bioline, NSW, Australia), primary antibodies against specific antimicrobial peptides were procured from Santa Cruz Biotechnology, Inc.(CA,USA) -Anti-human MMP-7, mouse monoclonal, (sc-101566), Anti-human α – defensin-5, mouse monoclonal, (sc-53997), Anti-human β – defensin-2 (sc-134314), mouse monoclonal, Mouse anti-human Monoclonal β-actin was obtained from Sigma (Castle Hill, Australia) and Anti-human β – defensin-1, rabbit monoclonal (ab170962) from Abcam Biotehnology (PA, USA). Secondary antibodies, HRP conjugated goat anti-mouse and HRP-conjugated goat anti-rabbit-Secondary antibody were obtained from Jackson Antibodies (PA, USA). PVDF membranes were obtained from immobilon 0.45 µM (Merck Millipore, Victoria, Australia). Chemiluminescent reagents (Clarity) was obtained from Bio-Rad Laboratories (CA, USA).

4.5 Methods

4.5.1 Cell culture maintenance

Caco-2 and LS174T cell lines were cultured in accordance with section 2.5.1. After the initial 4 days of culturing in growth medium with 10% FCS, the cells were replaced in growth medium with 2% FCS, along with 1% ITS-X. Conditioned medium from Caco-2 cells was collected between days 25-28 and between day 7 to day 10 for LS174T with and without addition of P-gp inducer (5 µM rifampicin) and repressor (4µM PSC-833). After collection, the samples were lyophilised overnight and reconstituted in 500 µL nanopure H_2O. Protein quantification was done using Modified Lowry test (section 2.5.3). The samples were stored at -20º C.

4.5.1.1 Stimulation with lipopolysaccharides

Cells were cultured in accordance with section 2.5.1. On day 27 Caco-2 cells were stimulated with LPS reconstituted in phosphate buffer saline (PBS). Stimulation for LS174T cells was done on day 9. 100 ng/mL were added to the cells and incubated for 24 hours. CM with LPS was collected and the cells were lysed in accordance to section 2.5.2. Alternatively, the cells were monitored over the 24-hour time period for observation of morphological changes that may be detected due to the addition of LPS. CM was lyophilised overnight and reconstituted in 500 µL nanopure H_2O. Protein quantification was done with Modified Lowry test (section 2.5.3). The samples were stored in -20º C.

4.5.2 mRNA analysis by RT-PCR

RNA was isolated in accordance with section 2.5.4 and cDNA was synthesised thereafter in accordance with section 2.5.5.

RT-PCR was performed in accordance with section 2.5.7. Briefly, the samples were prepared using 1 µL of both forward and reverse primer sequence (refer table 4.1 for primer sequence) from an aliquot of 5 µM, 1.5 µL of synthesised cDNA that is equivalent to 1.5 µg/mL RNA and was diluted with nuclease free water to make a total of 25 µL.

Table 4.1: List of primers used for gene expression of various AMPs

Gene	Primer sequences 5'-3'	T_A (ºC)	Amplicon size (bp)
MMP-7	AGA TGT GGA GTG CCA GAT GT- **F** TAG ACT GCT ACC ATC CGT CC- **R**	55	357
α-defensin-5	CCC AGC CAT GAG GAC CAT CG- **F** TCT ATC TAG GAA GCT CAG CG- **R**	56	306
β-defensin-1	CTC TCC CCA GTT CCT GAA AT- **F** GCG TCA TTT CTT CTG GTC AC- **R**	56	278
β-defensin-2	ATC AGC CAT GAG GGT CTT GT- **F** GAG ACC ACA GGT GCC AAT TT- **R**	54	172
LL-37	AGG ATT GTG ACT TCA AGA AGG ACG- **F** GTT TAT TTC TCA GAG CCC AGA AGC- **R**	58	276
GAPDH	ACC ACA GTC CAT GCC ATC AC- **F** TCC ACC ACC CTG TTG CTG TA- **R**	60	365

The gel was run in accordance with section 2.5.8, in 1.5% agarose with TAE buffer at 65 V for 90 mins.

4.5.3 Protein analysis by Western blot

Protein was quantified by Modified Lowry test (section 2.5.3.). Western blot was performed in accordance with section 2.5.4.

40 µg of the samples were loaded onto 4-12% Bis-tris gels using 1X MES running buffer at 150 V for 50 mins. The gel was transferred onto PVDF membranes. Transfer was done at 34 V for 45 mins. The blot was blocked in 2% casein in TBS overnight at 4º C for MMP-7, α-defensin-5 and β-defensin-2 (HBD-2) and 3% BSA in TBS overnight at 4ºC for β-defensin-1 (HBD-1) and 5% skim milk in TBS for 2 hours at RT for Amyloid beta (Aβ). Primary antibody (refer table 4.2) was added as per requirement in 1% casein for MMP-7, α-

defensin-5 and HBD-2 and 1% BSA in TBS for HBD-1 and incubated at RT for 2 hours and 0.5% skim milk in TBST for Aβ overnight at 4ºC. The blots were washed in TBST four times. Further, they were incubated with the following HRP conjugated secondary antibodies: HRP conjugated goat anti-mouse for MMP-7, α-defensin-5, HBD-2 and Aβ-42 and HRP conjugated goat-anti-rabbit for HBD-1 at RT for 2 hours, washed in TBST four times and developed thereafter using a 1:1 ratio of clarity incubated for 2 mins. Blots were scanned and imaged using the ChemiDoc ™ MP system (Bio-Rad).

Table 4.2: **List of primary and secondary antibodies for various AMPs**

Primary Antibody	**Dilution**
Anti-human MMP-7, mouse monoclonal (MM0022-4C21)	1:220
Anti-human α – defensin-5, mouse monoclonal (8c8)	1:220
Anti-human β–defensin-1, rabbit monoclonal (EPR69642)	1:2500
Anti-human β – defensin-2, mouse monoclonal (2-RY8)	1:220
Mouse anti-human Monoclonal β-actin (Endogenous control)	1:7500
Amyloid beta (Wo-2)	1:2500
Secondary antibody	**Dilution**
HRP conjugated Goat anti-mouse- secondary antibody	1:7500
HRP-conjugated Goat anti-rabbit-secondary antibody	1:7500

4.5.4 Statistical analysis

All data are reported as a mean parameter determined ± standard deviation (S.D.) unless otherwise stated. The difference between the groups was determined using Student's t-test. A P-value ≤ 0.05 was considered as statistically significant.

4.6 Results

4.6.1 Morphological analysis of cells

Both Caco-2 and LS174T cell lines were stimulated with 100 ng/mL LPS. Caco-2 was incubated with LPS on day 27, while LS174T cells were incubated on day 9 of cell growth. The cells were observed for a period of 24 hours for any change in the morphology of the cells. The effect of LPS in Caco-2 cells was minimal irrespective of the FCS content in the medium, while LS174T cells showed a significant effect by LPS when cultured in both 10% and 2% FCS. Although LPS was not broadly toxic to the cells as identified by trypan blue staining, the cells showed differences in growth pattern for a period of 24 hours. The cell lines appeared to show visible signs of cell structure alterations. A decrease in their proliferation was also noticed (Figure 4.2 and 4.3). The mosaic pattern of Caco-2 cells was less evident suggesting a possibility of reduced polarisation. Although LS174T cells usually displayed separated colonies, in the presence of LPS stimulation they were scattered further, with each colony showing fewer cells than the non-stimulated group.

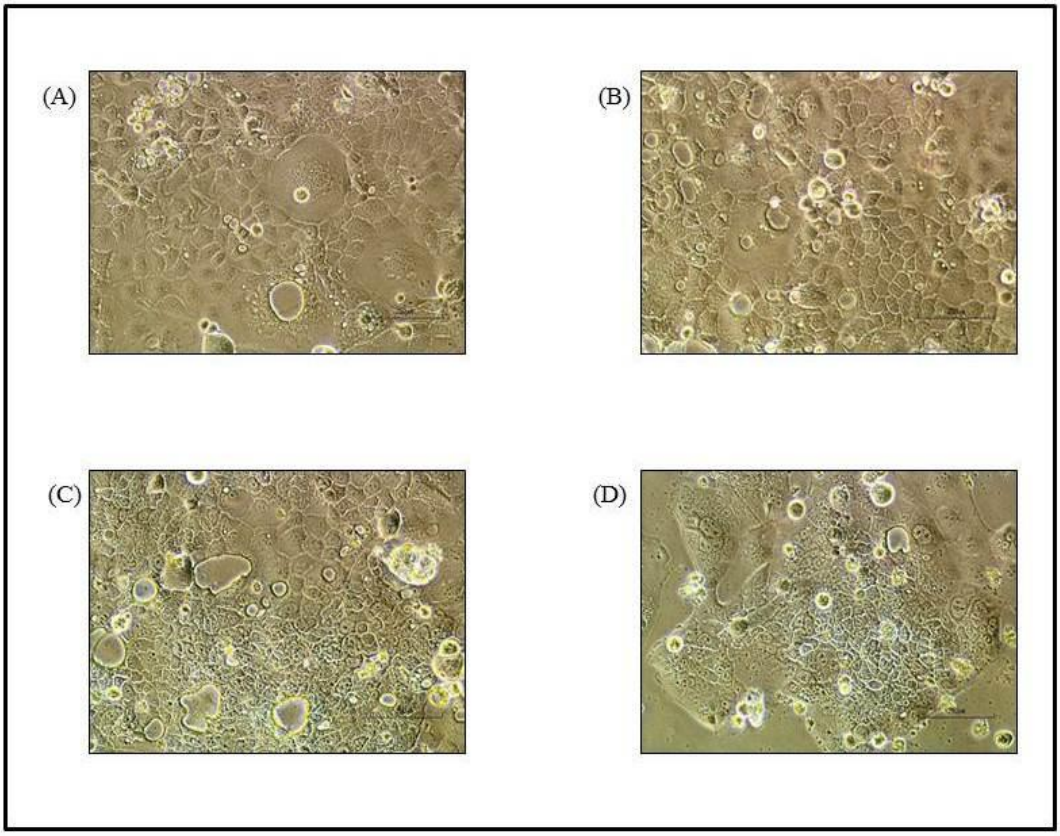

Figure 4.2: Phase contrast microscopic images of Caco-2 cells. Caco-2 cells were cultured in DMEM with 10% FCS or 2% FCS along with 1% vitamin, glutamine, non-essential amino acids appropriately in 6 well plates at $1.5x10^6$ cells. On day 27 the cells were incubated with 100 ng/mL lipopolysaccharides (LPS) and incubated in 37ºC, 5% CO_2 for 24 hours. **(A)** 10% FCS without LPS stimulation. **(B)** 10% FCS with LPS stimulation. **(C)** 2% FCS without LPS stimulation. **(D)** 2% FCS with LPS stimulation. Images were captured using inverted Carl Zeiss phase contrast microscope (Scale bar 250 µm).

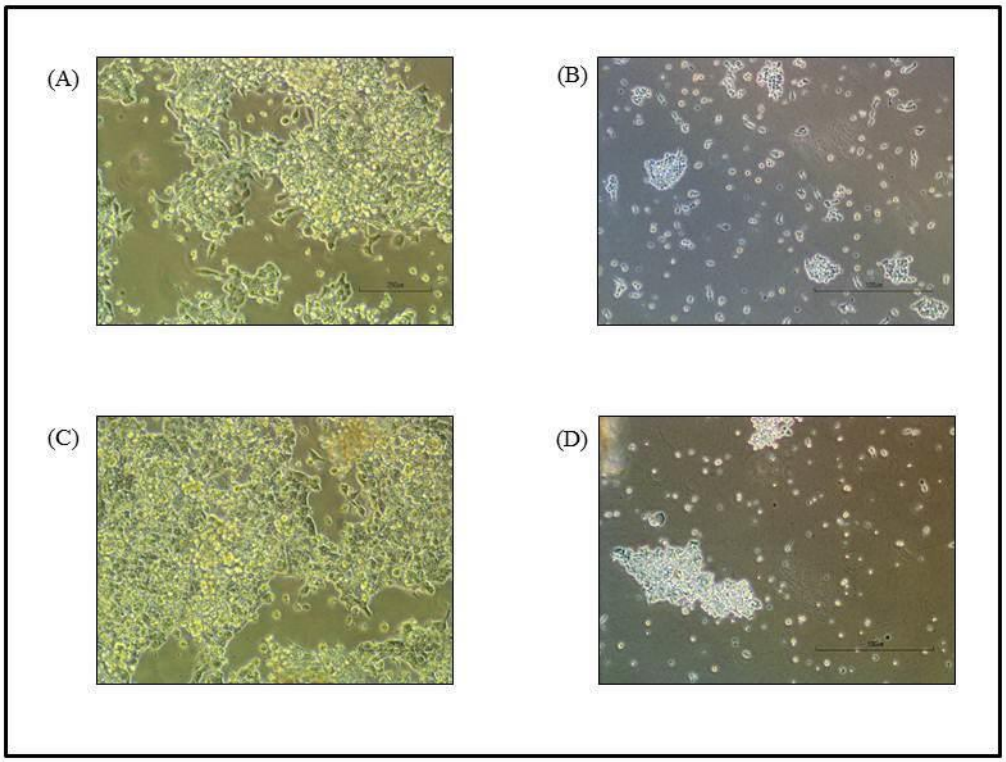

Figure 4.3- Phase contrast images of cells: LS174T cells were cultured in DMEM with 10% FCS or 2% FCS along with 1% vitamin, glutamine, non-essential amino acids appropriately in 6 well plates at approximately $1.5x10^6$ cells. On day 9 the cells were incubated with 100 ng/mL lipopolysaccharides (LPS) and incubated in 37ºC, 5% CO_2 for 24 hours. **(A)** 10% FCS without LPS stimulation. **(B)** 10% FCS with LPS stimulation. **(C)** 2% FCS without LPS stimulation. **(D)** 2% FCS with LPS stimulation. Images were captured using inverted Carl Zeiss phase contrast microscope (Scale bar 250 µm).

4.6.2 Gene expression of MMP-7

Both cell lines were analysed for MMP-7 expression. Caco-2 cells cultured in 10% FCS showed the presence of MMP-7, while the cells in 2% FCS only expressed MMP-7 when stimulated with 100 ng/mL LPS. This indicates that a lack of FCS affects certain properties of cells including its ability to endogenously produce antimicrobial peptides. LS174T did not display a band for MMP-7 in cells cultured in 10% FCS or 2% FCS, demonstrating that these cells do not elicit a response for MMP-7 even in the presence of the bacterial trigger in the current lab (Figure 4.4A(i)).

4.6.3 Protein expression of MMP-7

Protein expression of MMP-7 was similar to mRNA profile. Caco-2 cells cultured in medium with 10% FCS expressed MMP-7, while cells cultured in 2% FCS only showed a band when stimulated with LPS. The protein profile also indicated that there were not changes in the translation from mRNA to protein and that reduced FCS still affected the endogenous production of cells. LS174T did not show a band for cells cultured even in the presence of the bacterial stimulus (Figure 4.4 A (ii)).

Densitometry analysis was also done on the protein analysis using Western blot data. A significant (approximately 3-fold) difference was seen between control sample (Caco-2 cells in growth medium with 10% FCS) and Caco-2 cells in growth medium with 2% FCS and ITS-X (Figure 4.4B)

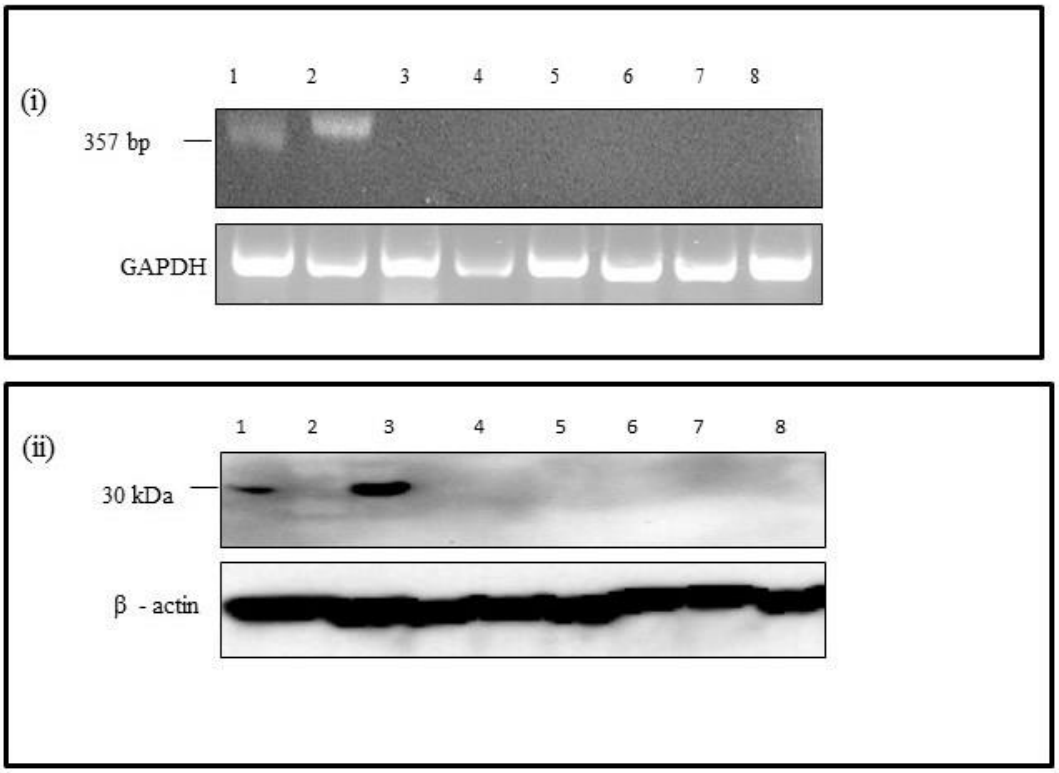

Figure 4.4A: Presence of MMP-7 in Caco-2 and LS174T cells. **(i)** mRNA expression in Caco-2 and LS174T cells. RT-PCR was done with appropriate forward and reverse primers of MMP-7 (357 bp). **1-4** represents Caco-2 cells cultured in growth medium with 10% FCS alone and 2% FCS with and without stimulation of 100 ng/mL LPS. **1**- 10% FCS. **2**- 2% FCS with LPS. **3**- 2% FCS without LPS. **4**- 2% FCS with 5 µM rifampicin. **5-8** represents LS174T cells cultured in growth medium with 10% FCS alone and 2% FCS with and without LPS. **5**- 10% FCS. **6**- 2% FCS, with LPS. **7**- 2% FCS without LPS. **8**- 2% FCS with 5 µM rifampicin. GAPDH was used as the endogenous control (365 bp). **(ii)** Protein expression (Western blot) using mouse monoclonal MMP-7 (MM0022-4C21) with HRP conjugated Goat anti-mouse used as secondary antibody in Caco-2 and LS174T cells. **1-4** represents Caco-2 cells cultured in growth medium with 10% FCS alone and 2% FCS with and without stimulation of 100 ng/mL LPS. **1**- 10% FCS without LPS. **2**- 2% FCS without LPS. **3**- 2% FCS with LPS. **4**- 2% FCS with 5 µM rifampicin. **5-8** represents LS174T cells cultured in growth medium with 10% and 2% FCS with and without LPS. **5**- 10% FCS without LPS. **6**- 2% FCS without LPS. **7**- 2% FCS with LPS. **8**- 2% FCS with 5 µM rifampicin. β-actin was used as the endogenous control at 42 kDa.

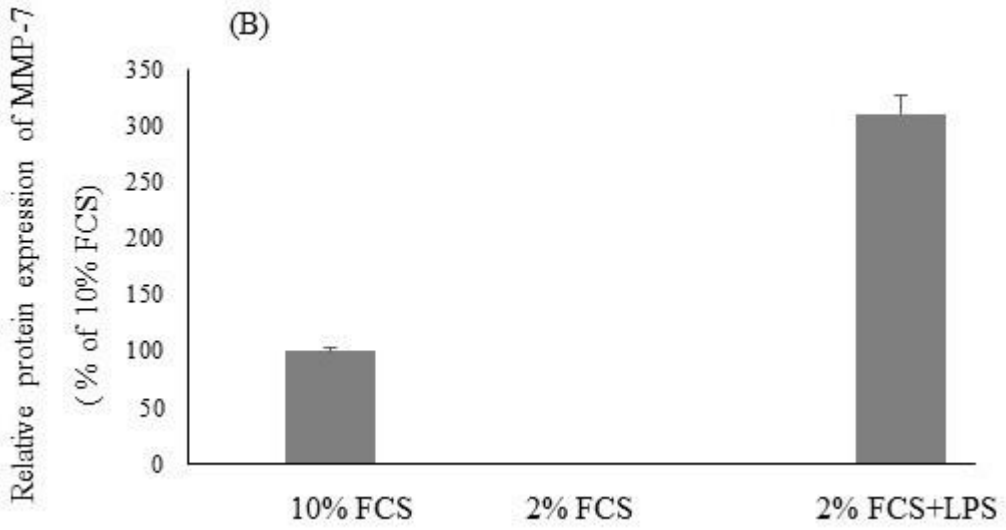

Figure 4.4B: Densitometry analysis of protein expression (MMP-7) of Caco-2 cells in different conditions of FCS and LPS. The values were compared to that of control values (β-actin). Data represents average protein expression ±SD (n=3).

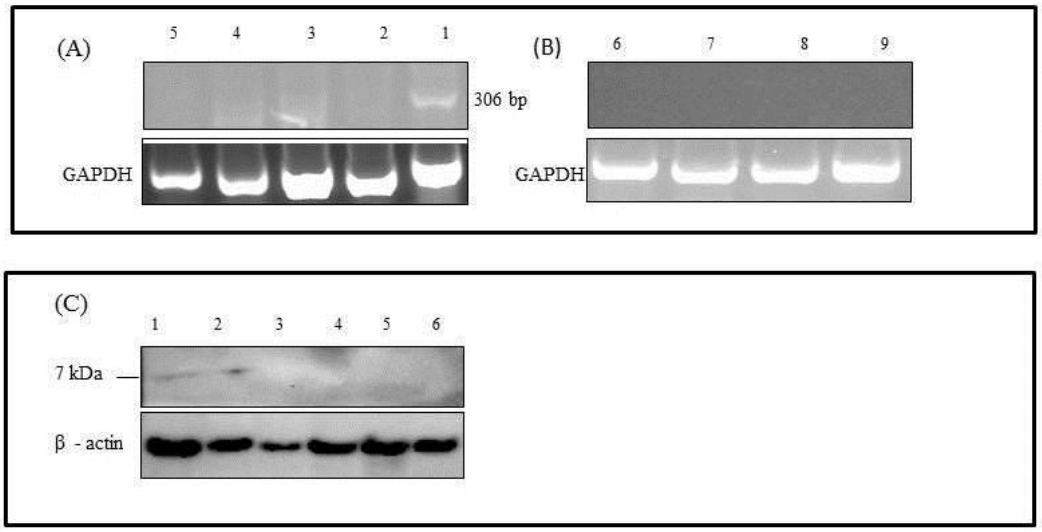

Figure 4.5: Presence of Human defensin -5 or α-defensin-5 (HD-5) in Caco-2 and LS174T cells. **(A)** mRNA expression in Caco-2 cells cultured in growth medium with 10% and 2% FCS with and without stimulation of 100 ng/mL LPS. **1-** 10% FCS stimulated with 1 µg/mL TNF-α (positive control). **2-** 10% FCS. **3-** 10% FCS with LPS. **4-** 2% FCS. **5-** 2% FCS with LPS. **(B)** mRNA expression in LS174T cells cultured in growth medium with 10% and 2% FCS with and without stimulation of 100 ng/mL LPS. **6-** 10% FCS. **7-** 10% FCS with LPS. **8-** 2% FCS. **9-** 2% FCS with LPS. RT-PCR was done with appropriate forward and reverse primers of HD-5 (306 bp). GAPDH was used as the endogenous control (365 bp) **(C)** Protein expression (Western blot) using α-defensin-5 mouse monoclonal (8c8) antibody with HRP conjugated Goat anti-mouse used as secondary antibody in Caco-2 and LS174T cells. **1-4** represents Caco-2 cells cultured in growth medium in 10% and 2% FCS, with and without 100 ng/mL LPS. **1-** 10% FCS stimulated with 1 µg/mL TNF-α. **2-** 2% FCS stimulated with 1 µg/mL TNF-α. **3-** 10% FCS without LPS. **4-** 10% FCS with LPS stimulation. **5 and 6** represent LS174T cells cultured in growth medium with 2% FCS with and without 100 ng/mL LPS. **5-** without LPS. **6-** with LPS. β-actin was used as the endogenous control at 42 kDa.

4.6.4 Gene expression of α-defensin-5

Cells stimulated with TNF-α increased α-defensin-5 mRNA synthesis. Bacterial stimulation with LPS did not activate a response for the AMP suggesting that the pattern of stimulation of the peptide was different to that of stimulation of MMP-7. Moreover, he presence of an attenuated microbial product may not induce α-defensin-5, the stimulation may occur only in the presence of live microorganisms (165) rather than the LPS product. The pattern was similar for both Caco-2 and LS174T. In this case, the cell lines respond to the stimulus similarly (Figure 4.5 (A)).

4.6.5 Protein expression of α-defensin-5

Protein expression did not differ from mRNA results. Caco-2 and LS174T did not show any evidence of α-defensin-5 using LPS in the laboratory (Figure 4.5 (B)).

4.6.6 Gene expression of Human beta defensin-1

Both Caco-2 and LS174T cell lines cultured in 10% and 2% FCS expressed Human beta defensin-1 HBD-1 (constitutive defensin). Caco-2 cells in 2% FCS stimulated with LPS displayed a thicker band indicating induction of HBD-1 in the presence of the bacterial stimulus in comparison to cells cultured in 2% FCS without LPS. LS174T did not show such induction by LPS under the same condition. In LS174T, HBD-1 displayed similar intensity when cultured in 10% FCS, 2% FCS and 2% FCS with LPS. However, cells when co-cultured with LPS and PSC-833, did not show the presence of any band in both Caco-2 and LS174T (2% FCS). Caco-2 cells when cultured with only PSC-833 showed similar band intensity to cells induced with rifampicin and increased intensity in comparison to cells without any stimulation. Here PSC-833 has an effect on the cells in the similar fashion as that of rifampicin. The effect of LPS does not allow for any other induction to take place, although LPS stimulation alone shows induction in Caco-2 cells. The effect of PSC-833 with and without LPS shows different results on Caco-2 cells. On the contrary, LS174T cells when cultured with only PSC-833 did not show an expression of HBD-1, indicating that both cell lines respond to P-gp blocker distinctly (Figure 4.6 (A) and (B)).

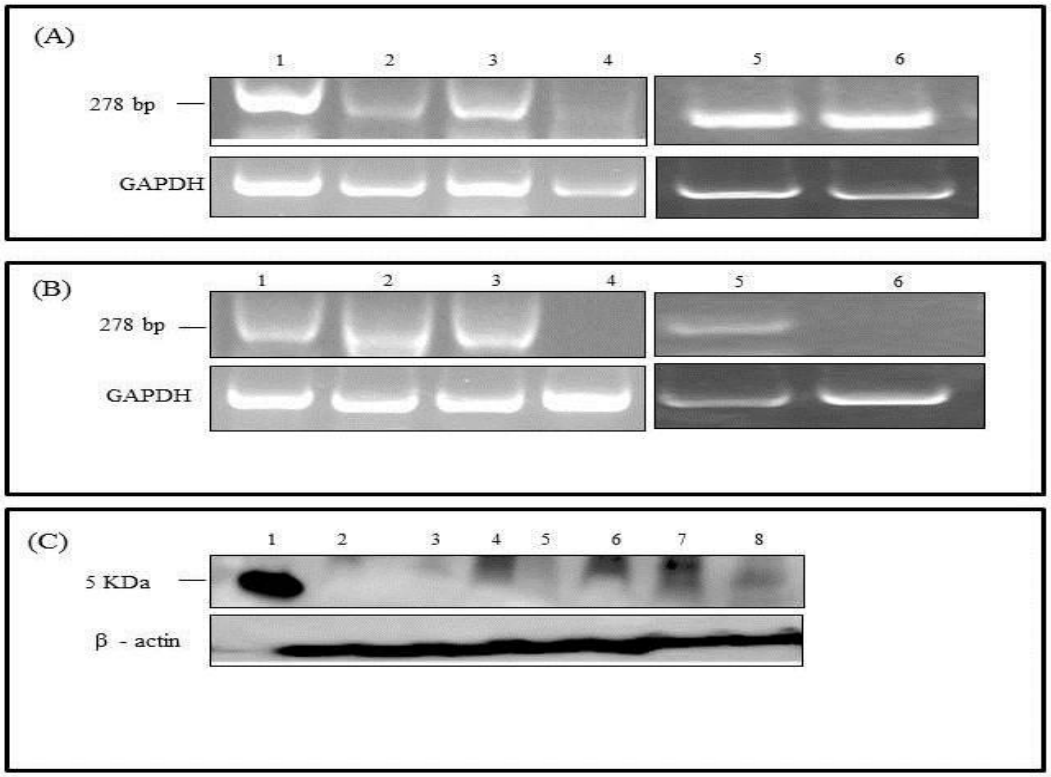

Figure 4.6: Presence of Human beta defensin-1 (HBD-1) in Caco-2 and LS174T cells. (A) mRNA expression in Caco-2 cells cultured in growth medium with 10% FCS alone or 2% FCS with and without 100 ng/mL LPS. **1**- 10% FCS. **2**- 2% FCS. **3**- 2% FCS with LPS. **4**- 2% FCS co-cultured with LPS and 4 µM PSC-833. **5**- 2% FCS stimulated with 5 µM rifampicin. **6**- 2% FCS stimulated with 4 µM PSC-833. **(B)** mRNA expression in LS174T cells cultured in growth medium with 10% FCS alone or 2% FCS with and without 100 ng/mL LPS. **1**- 10% FCS. **2**- 2% FCS. **3**- 2% FCS with LPS **4**- 2% FCS co-cultured with LPS and 4 µM PSC-833. **5**- 2% FCS stimulated with 5 µM rifampicin. **6**- 2% FCS stimulated with 4µM PSC-833. RT-PCR was done with appropriate forward and reverse primers for HBD-1 (278 bp). GAPDH was used as the endogenous control (365 bp). **(C)** Protein expression (Western blot) in Caco-2 and LS174T cells using anti-beta defensin-1 rabbit monoclonal antibody (EPR69642) with HRP-conjugated Goat anti-rabbit used as secondary. **1**- HBD-1 peptide at 10 µg/mL (positive control). **2-5** represents Caco-2 cells and CM cultured in growth medium with 10% FCS alone or 2% FCS with and without 100 ng /mL LPS. **2**- 10% FCS. **3**- 2% FCS. **4**- 2% FCS with LPS. **5**-CM in 2% FCS stimulated with LPS. **6-8** LS174T cells and CM cultured in growth medium with 2% FCS with and without 100 ng/mL LPS. **6**- without LPS. **7**- with LPS. **8**- CM stimulated with LPS. β-actin was used as the endogenous control at 42 kDa.

4.6.7 Protein expression of Human Beta Defensin-1

Protein expression in CM and cells of Caco-2 and LS174T of HBD-1 with various conditions as mentioned above could not be assessed. This can be attributed to lower protein levels that cannot be detected by Western blot. Only the control was detected in lane 1 (Figure 4.6 (C)).

4.6.8 Gene expression of Human beta defensin-2

In contrast to a study by Gacser *et al.,* who did not support the theory of induction of HBD-2 in Caco-2 cells by LPS (138), the current study showed that Caco-2 cells elicited a response for HBD-2 in the presence of the bacterial stimulus like LPS. The present study showed a band for the peptide when cultured in both conditions of 10% or 2% FCS along with LPS only in Caco-2 cells. This was supported by Hase *et al.*, suggesting that LPS stimulated HBD-2 in HCF-7 cells, a human colon epithelial cell line (334) and Witthoft *et al.*, who have shown that LPS induces HBD-2 in Caco-2 cells (335). This indicates that the percentage of FCS was not critical for mRNA induction, although HBD-2 was not produced endogenously. LS174T did not respond to bacterial stimulus for HBD-2 irrespective of the FCS content suggesting different methods of induction in different cell lines (Figure 4.7 (A, B)).

4.6.9 Protein expression of Human Beta Defensin-2

Protein expression of HBD-2 was different to the mRNA profile, especially in the Caco-2 cell line. Only Caco-2 cells stimulated with 1 µg/mL TNF-α, an inflammatory cytokine, showed visible response to HBD-2, indicating that HBD-2 is responsive to inflammation or is activated varyingly in different cell lines (Figure 4.7 (C)). While LPS activated the defensin in mRNA analysis, the effect was not translated to protein expression when studied in our laboratory.

4.6.10 Gene expression of LL-37

Gene expression of LL-37 was analysed in the study with samples of both Caco-2 and LS174T cells (Figure 4.8). LL-37 being a very important human AMP of the cathelicidin group was mandatory to analyse in the study. According to Hase *et al.,* (334) the expression of LL-37/ hCAP was constitutive in Caco-2 cells, with highest intensity when the cell line was spontaneously differentiated after 3 weeks of being in culture. Their *in-vivo* studies from normal human colon also suggested that the surface epithelium and adjacent upper crypts showed higher LL-37 expression in comparison to the proximal small intestine, indicating that highly differentiated colon epithelial cells showed maximal expression. The current study did not support this result as LL-37 did not appear in cells cultured regardless of the percentage of FCS present. The presence of LPS also did not induce LL-37 in both Caco-2 and LS174T cells.

4.6.11 Protein expression for Amyloid precursor protein (APP)

APP was monitored in different conditions in both Caco-2 and LS174T cell lines. Both cells cultured in regular conditions as 10% FCS showed the presence of APP, while cells cultured in 2% FCS did not express APP. When stimulated with LPS, cells cultured in 2% FCS expressed APP, although LS174T only elicited lesser response to LPS (lane 1 of LS174T (Figure 4.9C) in comparison to lane 2 of Caco-2 cells (Figure 4.9B). CM from these cells also revealed the presence of APP in a similar fashion. Caco-2 cells displayed a higher response to LPS (lane 4 (B)), while LS174T showed an increased response to rifampicin (P-gp inducer), rather than LPS (lane 3 (Figure 4.9C)).

APP expression was reduced, when both cell lines were co-cultured with LPS and PSC-833. This is illustrated in the comparison of lane 4 and lane 5 for Caco-2 cells (Figure 4.9B) and lane 3 and lane 5 for LS174T cells (Figure 4.9C).

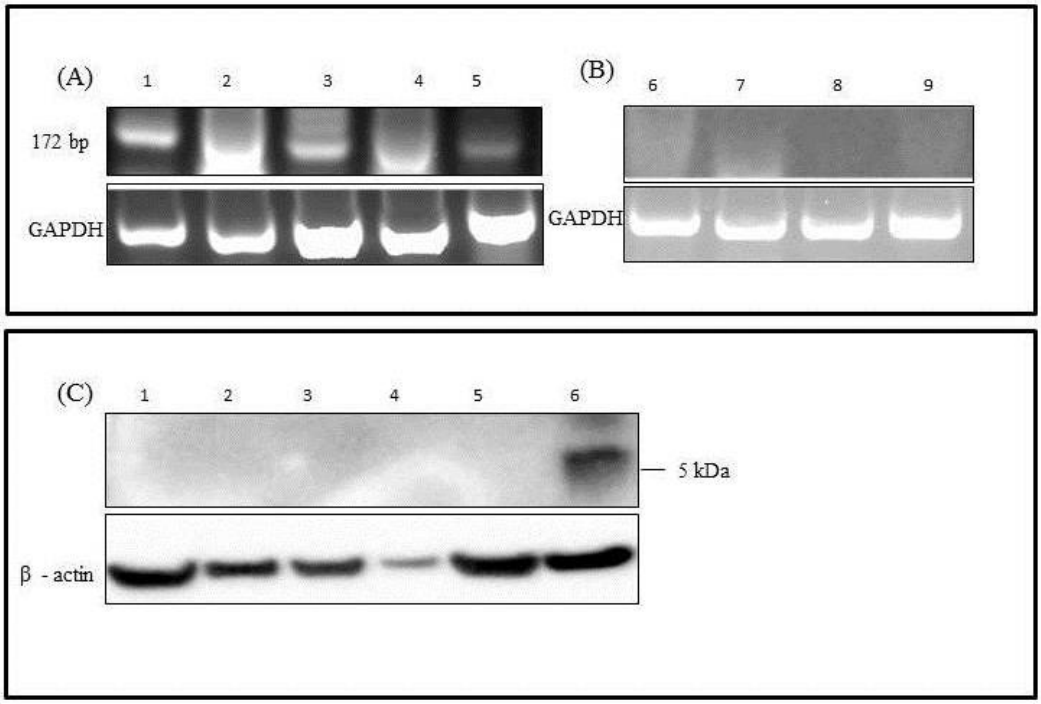

Figure 4.7: Presence of Human beta defensin-2 (HBD-2) in Caco-2 and LS174T cells. (A) mRNA expression in Caco-2 cells cultured in growth medium with FCS (10% or 2%) with and without stimulation of 100 ng /mL LPS. **1-** 10% FCS stimulated with 1 µg/ mL TNF-α (positive control). **2-** 10% FCS without LPS. **3-** 10% FCS with LPS. **4-** 2% FCS without LPS. **5-** 2% FCS with LPS. **(B)** mRNA expression in LS174T cells cultured in growth medium with 10% and 2% FCS with and without stimulation of 100 ng/mL LPS . **6-** 10% FCS without LPS. **7-** 10% FCS with LPS. **8-** 2% FCS without LPS. **9-** 2% FCS with LPS. RT-PCR was done with appropriate forward and reverse primers for HBD-2 (172 bp). GAPDH was used as the endogenous control (365 bp). **(C)** Protein expression (Western blot) using β–defensin-2 mouse monoclonal antibody (2-RY8) with HRP conjugated Goat anti-mouse used as secondary antibody in Caco-2 and LS174T cells. **1-3** represents Caco-2 cells cultured in growth medium with 10% and 2% FCS with and without 100 ng/mL LPS. **1-** 10% FCS with LPS. **2-** 2% FCS without LPS. **3-** 2% FCS with LPS. **4-5** represent LS174T cells cultured in growth medium with 2% FCS with and without 100 ng/mL LPS stimulation. **4-** without LPS. **5-** with LPS. **6-** Caco-2 cells cultured in growth medium with 10% FCS stimulated with 1 µg/mL TNF-α (positive control). β-actin was used as the endogenous control at 42 kDa.

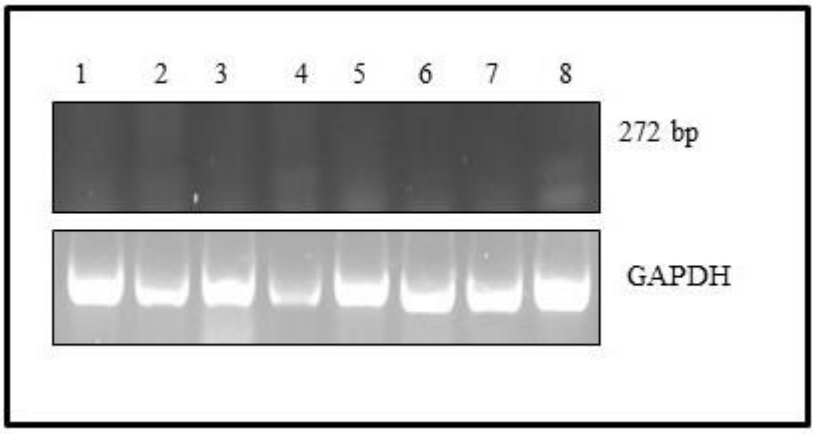

Figure 4.8: Presence of LL-37 in Caco-2 and LS174T cells. mRNA changes was observed by RT-PCR that was done with appropriate forward and reverse primers of LL-37 (276 bp). **1-4** represents Caco-2 cells cultured in growth medium with 10% FCS alone and 2% FCS with and without stimulation of 100 ng/mL LPS. **1**- 10% FCS. **2**- 2% FCS without LPS. **3**- 2% FCS with LPS. **4**- 2% FCS with 5 µM rifampicin. **5-8** represents LS174T cells cultured in growth medium with 10% FCS alone and 2% FCS with and without LPS. **5**- 10% FCS. **6**- 2% FCS, without LPS. **7**- 2% FCS with LPS. **8**- 2% FCS with 5 µM rifampicin. GAPDH was used as the endogenous control (365 bp)

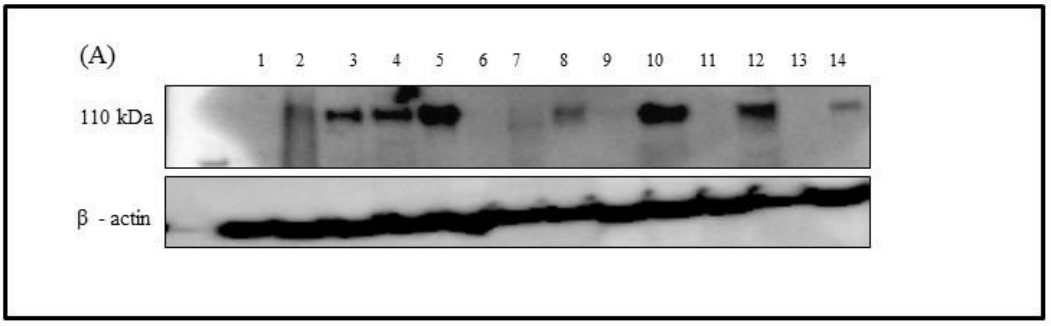

Figure 4.9(A): Presence of Amyloid precursor protein (APP) in Caco-2 and LS174T cells. **A** Protein expression (Western blot) using Wo-2 antibody in cell lysates and CM of Caco-2 and LS174T cell lines. **2-** Amyloid beta peptide at 10 µg/mL (positive control). **3-8**: LS174T cells in growth medium with 10% FCS alone or 2% FCS with and without stimulation with 100 ng/mL LPS. **3-** CM with 2% FCS with LPS and co-cultured with 4 µM PSC-833. **4-** CM with 2% FCS with LPS. **5-** CM with 5 µM rifampicin. **6-** Cells with 2% FCS without LPS. **7-** cells with 2% FCS with LPS. **8-** cells with 10% FCS. **9-14:** Caco-2 cells cultured in growth medium with 10% FCS alone or 2% FCS with and without 100 ng/mL LPS stimulation. **9-** CM with 2% FCS with LPS and co-cultured with 4 µM PSC-833. **10-** CM with 2% FCS with LPS. **11-** CM with 2% FCS. **12-** cells with 2% FCS with LPS. **13-** cells with 2% FCS. **14-** cells with 10% FCS. β-actin was used as the endogenous control at 42 kDa.

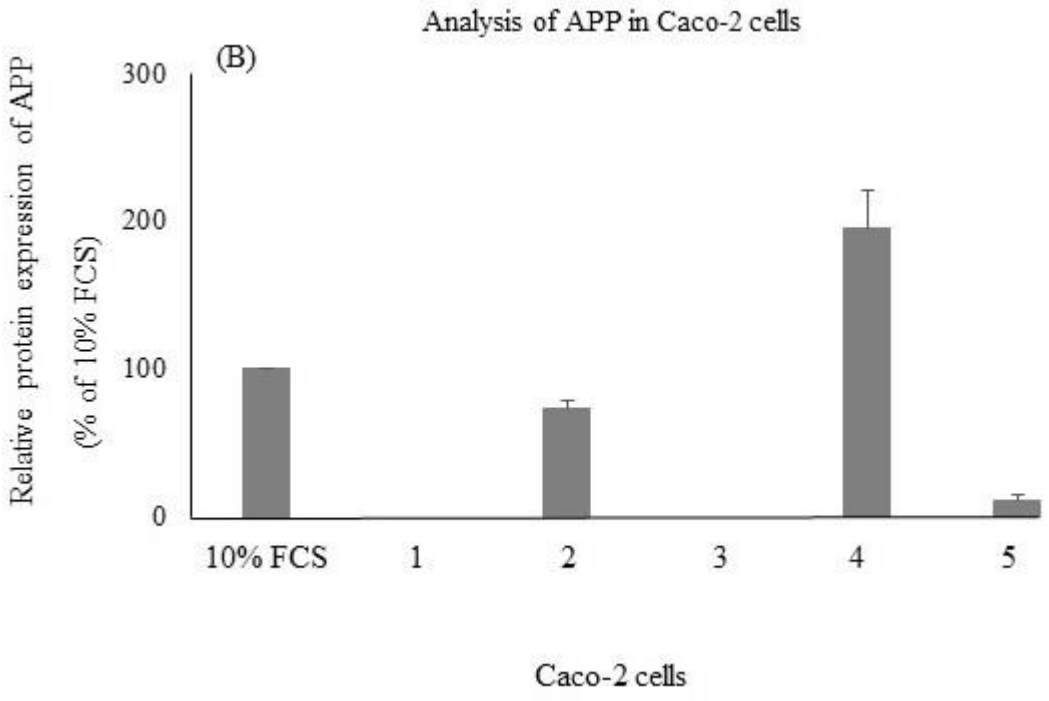

Figure 4.9 (B): Densitometry analysis of Amyloid precursor protein in Caco-2 cells normalised to β – actin. 10% FCS. **1**- 2% FCS. **2-** 2% FCS+100 ng/mL LPS. **3**- CM with 2% FCS without LPS. 4- CM with 2% FCS stimulated with 100 ng/mL LPS. **5**- CM with 2% FCS co-cultured with 100 ng/mL LPS and 4 µM PSC-833. The values have been compared to Caco-2 cultured in 10% FCS without LPS stimulation. Data represents average protein expression ±S.D (n=3).

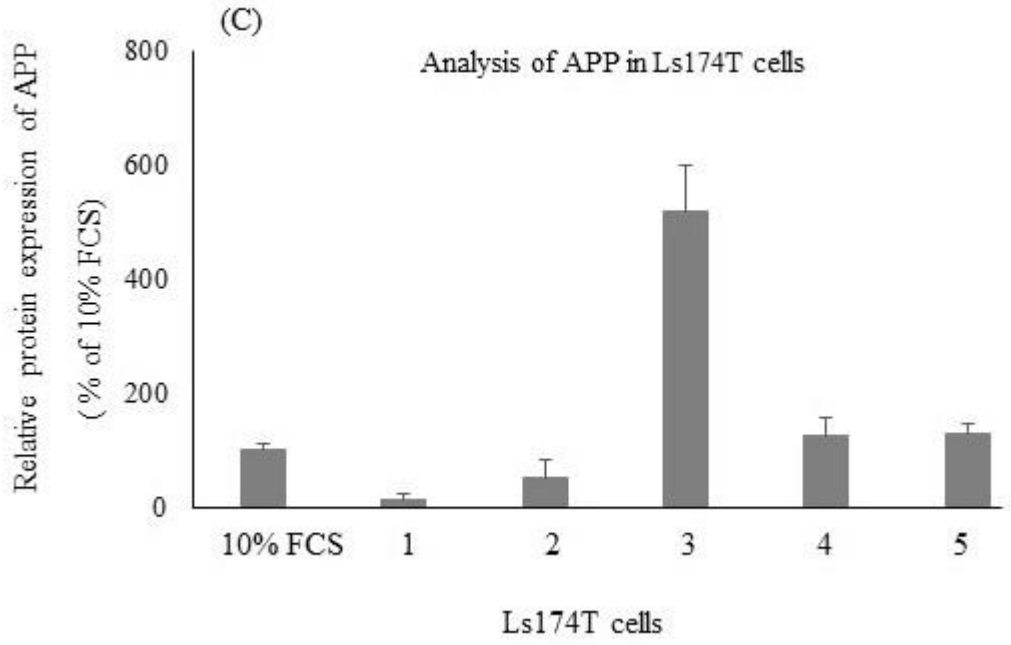

Figure 4.9 (C): Densitometry analysis of APP expression in LS174T cells. 10% FCS. **1**- 2% FCS **2**- 2% FCS stimulated with 100 ng/mL LPS. **3**- CM with 2% FCS stimulated with 5 µM rifampicin. **4**- CM with 2% FCS stimulated with 100 ng/mL LPS. **5**- CM with 2% FCS co-cultured with 100 ng/mL LPS and 4 µM PSC-833. The values have been compared to LS174T cultured in 10% FCS without LPS stimulation. Data represents average protein expression ±S.D (n=3).

4.7 Discussion

This chapter elucidates the presence of known antimicrobial peptides in the CM and cells of gastrointestinal cell lines, Caco-2 and LS174T, when stimulated with LPS. The previous chapter illustrated the presence of low molecular weight peptides produced without a bacterial stimulus in the CM, through mass spectrometry, while the present chapter demonstrates mRNA and protein analysis of defined AMPs with the use of RT-PCR and Western blot.

The cells were cultured in accordance with section 2.5.1 and were incubated with 100 ng/mL LPS for 24 hours on day 27 for Caco-2 and day 9 for LS174T cell lines. Cells were observed during the incubation period, to detect any change in morphology after stimulation with LPS. Caco-2 cells stimulated with LPS showed a minimal effect after 24 hours at the concentration tested, while LS174T cells appeared to be increasingly affected by LPS. A decrease in proliferation of the cells was observed along with decreased cell to cell adhesion (Figure 4.2 and 4.3).

For analysis of gene expression of the AMPs, RT-PCR was performed. cDNA was synthesised on day 28 for Caco-2 cells and on day 10 for LS174T cells. mRNA of MMP-7 was expressed in Caco-2 cells cultured in 10% FCS, but was absent in cells cultured in 2% FCS. Cells cultured in 2% FCS but stimulated with LPS showed the presence of MMP-7 and displayed an increased level of MMP-7 when compared to Caco-2 cells in 10% FCS (Figure 4.4A (i)).

The appearance of MMP-7 in the gastrointestinal cell lines here is significant as MMP-7 plays a role in the cleavage of the precursor defensins to mature peptides. Such peptides include HD-5 and 6 which are located in secretory granules present abundantly in human paneth cells *in-vivo* (336). They are stored as precursors in paneth cells, and are released upon stimulation with bacterial products like LPS. *In-vivo* studies have indicated that bacterial stimulus like LPS promotes secretion of 40% of paneth cell components. The absence of cleavage proteins such as MMP-7 have been shown to inadequately neutralise orogastrically administered gram negative microorganism such as *S.typhimurium* (165, 337).

Protein expression of MMP-7 was also detected and was similar to its mRNA profile. While Caco-2 cells cultured in the original medium of 10% FCS showed a band for MMP-7, only

LPS-induced Caco-2 cells cultured in 2% FCS expressed MMP-7 (Figure 4.4A(ii)). According to densitometry analysis, these cells showed an approximate 3 fold increase compared to cells cultured in 10% FCS without LPS induction (Figure 4.4B). LS174T cells did not express MMP-7 irrespective of the presence or absence of a bacterial stimulus, both for mRNA analysis and protein profile.

The cell lines were also analysed for expression of human alpha defensin-5 (HD-5). The presence of MMP-7 in Caco-2 cells suggested that HD-5 may also be present in the cells. HD-5 is an AMP of the paneth cells that is activated to the mature form by MMP-7 n mice (338). Earlier *in-vitro* and clinical studies have suggested that LPS does not stimulate HD-5 (336, 339). Therefore, TNF-α (a pro-inflammatory cytokine) was used for stimulation of HD-5 in both Caco-2 and LS174T cell line. Only TNF-α induced Caco-2 cells showed a presence of HD-5 (Figure 4.5, lane 1), while no band was detected in LPS stimulated samples. (338). Nevertheless, Wehkamp *et al.* stated that HD-5 was decreased in patients with colitis, in the presence of inflammation (340). Trend *et al.*, had reported that the concentration detected in preterm human breast milk samples were much lower than the minimal inhibitory concentration of the peptide, while the MIC itself was shown to be around 100 µg/mL (286). Although this could be attributed to HD-5 being an intestinal defensin, it may also give evidence suggesting the minimal possibility of the peptide in a complex mixture.

It could be concluded that irrespective of the presence of MMP-7, HD-5 was still not expressed or was below detection limits.

The cell lines were also analysed for beta defensins such as Human beta defensin-1 and 2 (HBD-1 and 2) as β-defensins are an important aspect of immunity in the gastrointestinal system (341).

Caco-2 cells constitutively produce HBD-1 in the cells and secrete it into CM (342, 343). In the present study, mRNA analysis of HBD-1 in both Caco-2 and LS174T cells was done. The cells were also incubated with LPS to show a difference in upregulation of HBD-1. Although, according to the study by O'neil *et al.*, it was suggested that there was no distinct difference in the upregulation of HBD-1 in the presence of LPS (343), in the present study, there was an increase in the mRNA of HBD-1 when the cell lines were incubated with LPS

(Figure 4.6. lane 3 in comparison to lane 2 (A and B)). As the hypothesis was to identify a P-gp substrate with antimicrobial properties, both the cells lines were incubated with rifampicin (P-gp inducer) to identify an increase in the expression of HBD-1, through PXR regulation. Although higher expression was seen in Caco-2 cells incubated with rifampicin (Figure 4.6 lane 5 (A)) an increase in density was not seen in LS174T cells that were incubated with rifampicin (Figure 4.6 lane 5 (B)). Ideally, greater relative efflux would be expected in rifampicin induced LS174T cells, compared to Caco-2 cells as they increase P-gp expression to a higher degree when exposed to such PXR activating agents (202). Nevertheless, previous evidence of P-gp induction was based on the presence of the proteins themselves rather than mRNA. As the current study started with mRNA analysis this may explain the discrepancy. When both cell lines were co-incubated with both PSC-833 (P-gp blocker) and LPS, a faint band was seen in Caco-2 cells (Figure 4.6 lane 4 (A)) and no band was seen in LS174T cells (Figure 4.6 lane 4(B)).

However, when cells were only incubated with PSC-833, HBD-1 intensity was much higher than cells cultured in 2% FCS without any LPS induction and only minimal difference in band intensity was seen in Caco-2 cells in comparison to Caco-2 cultured in 10% FCS (Figure 4.6 lane 6 in comparison to lane 1 (A)). While, LS174T cells when incubated with only PSC-833 showed an absence in HBD-1 band, similar to when co-incubated with LPS (Figure 4.6 lane 6 (B)). From the study we could infer that during a bacterial trigger like LPS, the mechanism of PSC-833 is different in comparison to when being incubated by itself suggesting that LPS does not allow any other mechanisms of induction to take place. The effect of P-gp blocker is to attenuate the efflux activity of P-gp thereby reducing the efflux of its substrates. The presence of the substrate itself should not be affected. Therefore it is suggested that the absence of HBD-1 when incubated with PSC-833 in LS174T cells cannot be completely credited to the repressor function of PSC-833.

The protein profile was also investigated for HBD-1 in stimulated and unstimulated cells and CM. Only the positive control (HBD-1 peptide) was visible showing the detection method was working, but none of the samples showed anything other than possibly very faint detection for HBD-1. Concentration of HBD-1 in the samples could be well below the level of detection by Western blot. A preliminary dot blot test to decide the antibody concentration was done with the samples. Dots were spotted at a low concentration of

1:3000, for unstimulated and stimulated cells and CM and no detection was evident in samples incubated with PSC-833, thus giving a brief overview of the protein profile of HBD-1 (refer to appendix for image). Since Western blot did not allow for detection of the peptide in samples it is difficult to make conclusions from the dot blot analysis alone. Reasons for poor Western blot detection can be due to the presence of different isoforms of HBD-1 that appears in Caco-2 cells and the possibility of cleavage of proteins during sample preparation (342, 344).

According to Ho *et al*., regulation of HBD-1 was related to peroxisome proliferator activated receptor gamma (PPAR-γ) in the Caco-2 cells (345). Mice deficient in PPAR-γ, also expressed an absence in HBD-1 expression and exhibited nonexisiting antimicrobial activity against *Candida albicans, Bacteroides fragilis, Enterococcus faecalis and E.coli* (345).

HBD-1 and HBD-2 show 39% homology, indicating they developed from the same ancestral gene. The products utilise different NF-κB sequence, thereby being differentiated into HBD-1, which is constitutive and HBD-2 which is inducible (343, 346).

To study mRNA expression of the inducible beta defensin HBD-2, RT-PCR was performed. HBD-2 was studied as they were known to show bacteriostatic properties rather than bactericidal (289). Cells were stimulated with both 1 µg/mL TNF-α and 100 ng/mL LPS. HBD-2 is an inducible defensin triggered by inflammatory stimuli. Induction of HBD-2 is mediated by NF-κB and AP-1 dependent pathway (347) and by the stimulation of TLR. TLR are activated in the intestinal epithelial cells by the presence of Th1 cytokines in intestinal bowel disease or by infection with pathogens. Deletion of 938 nucleotide upstream of the transcription start site of the HBD-2 gene also facilitates in LPS dependant expression in SW480 cells (348).

Certain bacterial strains such as *E.coli* Nissle 1917 and the flagellin filament structural protein of *S.enteritidis* have also been identified to be potential activators (349, 350). While both the pellet and supernatant of the organism could induce HBD-2, supernatant was found to show better potency in eliciting HBD-2 induction (347). *H.pylori* also stimulates HBD-2 in the intestinal epithelia indicating that organisms that are not a part of the regular microflora of the gut stimulates HBD-2 (351). Gacser *et al* suggested that HBD-2 can modulate the yeast commensals of the gut, like *C.albicans(138)*.

In the present study, for detection of mRNA, cells incubated with 1 µg/mL TNF-α for 24 hours, showed a HBD-2 related band. HBD-2 was not endogenous though, as Caco-2 cells cultured in 10% FCS and 2% FCS without a bacterial trigger did not express HBD-2. On the contrary, Caco-2 cells cultured in the same conditions stimulated with 100 ng/mL LPS showed HBD-2 (Figure 4.7 (A)). Previous reports have suggested that stimulation with 1 µg/mL LPS for 1 hour, did not elicit a response for HBD-2 (343, 348). However, these conditions have been known to stimulate HBD-2 in mucosal tissues such as gingival and epidermal keratinocytes and respiratory epithelium (285, 352, 353). This could be attributed to the differences in regulations of expression of HBD-2 at different mucosal sites and the level of exposure to organisms.

In the present study, LPS was stimulated for a longer period of time, thus being a potential reason for eliciting a response to HBD-2. Alternatively, *in-vitro* studies may not always show translational simulation with *in-vivo* studies, suggesting that LPS responsiveness of cell lines and gastrointestinal epithelium may show differences.

Although the induction was seen at the mRNA level, HBD-2 was not detected at the protein level. Differences in post translational modification may be the reason for the absence of HBD-2 mRNA, as these molecules are known to be synthesised as inactive precursors (138).

While LPS induction showed a positive response in Caco-2 cells, LS174T cells did not show any bands corresponding to HBD-2 (Figure 4.7 (B)). This supports studies from Fahlgren *et al*.,(338) who examined the effect of LPS, IL-1β and live *S.typhimurium* in LS174T and HT-29 cells. Incubation for 1 hour with 10 µg/mL LPS did not have any effect in the induction of HBD-2. In contrast, incubation with *S.typhimurium* induced HBD-2 (338). Other microorganisms such as *E.coli* Nissle 1917, *Lactobacilli* and *Bifidobacterium* have also been shown to induce HBD-2 in LS174T cells (354).

LL-37 (the only human cathelicidin) was also examined in this study (Figure 4.8). In addition to its role as a potent antimicrobial peptide, they have also shown to stimulate inflammatory genes (141, 355). Studies from the lab of Hase and colleagues have shown that LL-37 is constitutively produced in fully differentiated colon epithelial cells, such as, Caco-2 (334). In addition, its appearance also is stimulated by the MEK-ERK signal transduction pathway. ERK has shown to activate LL-37 transcription through MEK

induction. Different differentiation inducing agents such as flavone and butyrate have shown an increase in the presence of LL-37 in colon cells such as HT-29 and SW620 (356). However in our study, Caco-2 cells that spontaneously differentiate did not show the presence of a visible LL-37 band. Moreover, LS174T that does not differentiate spontaneously also did not show any band for LL-37.

The present study also observed the presence of neural associated peptides as a possible antimicrobial factor in stimulated cells. Protein expression of Amyloid beta (Aβ) and Amyloid precursor protein (APP) was studied (Figure 4.9). In contrast to most AMPs, Aβ are anionic peptides with an ability to bind and disrupt anionic lipid bilayers and mitochondrial membranes.

In this study, protein analysis of Caco-2 cells showed APP was expressed in cells cultured in 10% FCS without LPS stimulation, but needed LPS added to 2% FCS cells to show it, thus 2% FCS and defined medium components were not able to replicate what was shown in 10% FCS. When co-cultured with PSC-833, these cells only showed a faint APP related band. Similarly CM from the cells also expressed APP only in the presence of LPS induced 2% FCS cells and in 10% FCS cells. APP related band was only faint in CM when co-cultured with LPS and PSC-833. This has large ramifications for the earlier work in this project, as it shows that a precursor to a possible AMP was not likely to be secreted in the 2% FCS that was used to collect potential AMPs, meaning that although not found APP and its product of Aβ was still a valid target to continue exploring in subsequent studies.

LS174T responded to LPS in a similar manner. Cells stimulated with LPS showed a higher band intensity in comparison to unstimulated cells. The difference in expression ensued from the fact that when LS174T cells were incubated in rifampicin, higher band intensity was seen in its CM in comparison to CM stimulated with LPS. This can be attributed to rifampicin inducing P-gp in LS174T at much higher levels than in Caco-2 (202). Co-incubation with LPS and PSC-833 gave different results for both cell lines. Caco-2 cells did not express APP, while LS174T showed a faint band for APP (Figure 4.9 (A) lane 9 and lane 3 respectively). While the activity of PSC-33 is low in LS174T, it remains very high in Caco-2 cells, suggesting the use of Caco-2 as a model cell line for transport experiments (190).

Other studies have also shown Aβ to be a possible antimicrobial agent. In the study by Soscia *et al.,* Aβ peptides were known to show inhibition against many gram negative and gram positive organisms including, *Streptococcus pneumonia*, *C.albicans*, *E.coli* and *S.aureus* (182). Moreover, when compared to another gastrointestinal peptide, LL-37, Aβ showed better resistance in *Enterococcus faecalis* cultures by bacterial proteases. The anionic nature of the peptide can be associated with resistance. Also, oligomerisation of Aβ permits the peptide to be bacterial resistant, making Aβ more potent than LL-37 (182).

Vijaya kumar and colleagues have also demonstrated the significance of Aβ as an antimicrobial peptide (357). Infection of APP-knockout mice showed high mortality rates and low Aβ expression. They have also indicated the adhesion and agglutination activity of Aβ. The reported physiological concentration of cell derived Aβ ranged from 1-5 ng/mL in cerebral cortex and increased to 10-50 ng/mL during leptomeninges. They have also established that cellular derived Aβ is a potent anti-candida agent. On exposure to Herpes simplex virus-1, HIV-1, spirochetes and chlamydia, the expression of Aβ increases in host cells. Hence, it could also be deduced that Aβ is an inducible AMP (357).

Other reports have also indicated that Aβ may have antimicrobial activity, and have attributed the activity to their ability to form channels in the membranes, thereby disrupting the membrane. The presence of antimicrobial β sheet structures in Aβ plays a pivotal role in pore formation through the membrane, resulting in degeneration of the membrane (181, 358, 359).

Puig *et al*., have demonstrated that Aβ can be stimulated by LPS in Caco-2 cells. In our study, it has been demonstrated that 100 ng/mL LPS stimulated APP, but Aβ could not be detected, possible reason being the level of detection through Western blot is far too low. Another reason could be attributed to cross linking of APP in Caco-2 cells that triggers an APP-dependant signalling response without being processed to Aβ (187) .

4.8 Limitations

The action of LPS is dependant on the presence of certain receptors in the cells. TLR-2 and TLR-4 are some of the receptors present in Caco-2 cells. A decrease in their presence associated with decreased serum may contribute to the decreased stimulation of antimicrobial peptides. LPS may not trigger in the reduced presence of FCS due to

decreased CD14 or lipopolysaccharide binding protein (LBP), as FCS is a source of CD14 and LBP, and this may be another limitation to showing increased antimicrobial stimulation in our cell lines (360). Concentration of LPS may also affect the phosphorylation of MAPK, JNK and nF-κB pathways. As nF-κB-MAPK is responsible for the induction of HBD-2, it could be one of the reasons for its absence in LS174T cells (361). Also, p65 the subunit of nF-κB is known to be phosphorylated by LPS in a concentration and dose dependant manner.

4.9 Conclusion

Data from this study revealed that selected known gastrointestinal antimicrobial peptides could not be detected in the CM of LPS stimulated Caco-2 and LS174T cells. Yet, increases in the levels of APP could be seen in CM of Caco-2 and LS174T cell lines when exposed to LPS. Moreover, on co-incubation with PSC-833, which is a known blocker of P-gp, CM did not express APP, indicating that PSC-833 can also be speculated to prevent efflux of APP. More investigation and techniques that can detect lower quantities of proteins are required for conclusive results relating amyloid beta to antimicrobial activity. Similarly, on co-incubation with LPS and PSC-833, HBD-1 was also absent in mRNA samples of both Caco-2 and LS174T cells. The exact mechanism cannot be elucidated as Western blot analysis of HBD-1 did not show any conclusive results. Also, being a P-gp blocker (PSC-833) should only reduce the efflux of P-gp substrates and not decrease HBD-1 expression in the mRNA of the cells. HBD-2 and HD-5 could not be seen in the cells and CM of Caco-2 and LS174T further confirming that an unknown factor is playing a role against the pathogenic organisms in gastrointestinal cells.

Use of antimicrobial peptides as therapeutic drugs may come into practice in the near future. This could be advantageous for improved health of human beings.

Chapter 5: Determination of amyloid beta as a substrate for P-glycoprotein

5.1 Rationale

The previous chapter described the identification of defined antimicrobial peptides in the gastrointestinal cells (Caco-2 and LS174T) and its conditioned medium (CM). Neural peptides such as amyloid-β (Aβ) have also demonstrated antimicrobial properties (325) and have been included in this study. To identify whether Aβ is a potential substrate of P-gp, the cells were co-incubated with a P-gp inhibitor or inducer (PSC-833 or rifampicin respectively) and a bacterial trigger. Some changes were seen that led to expectations of Aβ being a potential substrate for P-gp, supporting previous studies that had also shown that Aβ was a likely P-gp substrate (95, 362). For substantiating and providing evidence of Aβ as a substrate of P-gp or otherwise in the current study, transport experiments were performed.

5.2 Introduction

5.2.1 Structure and function of amyloid beta

Amyloid beta (Aβ) is derived from its precursor protein, the large 110 kDa amyloid precursor protein (APP) (180). The process of Aβ formation includes cleavage of APP by β and γ-secretase, which are membrane bound endoproteases. β-secretase, a membrane bound aspartyl protease cleaves the precursor protein to form sAPPβ (secretory form). Cleavage of APP by β-secretase only constitutes 10% of the total cellular APP with the remaining 90% being cleaved by α-secretase, resulting in sAPPα and CTFα (363-365). BACE1 and BACE 2 are the two major forms of the enzyme and are approximately 65% homologous to each other. BACE1 is found in the brain at high levels, but is also present in other organs at lower levels (365). γ-secretase converts CTFβ, which is membrane bound, to Aβ. Cleavage by the γ-secretase is variable resulting in multiple peptide production from the precursor (366). Aβ-40 (90% of the population) is more abundant than Aβ-42. The Aβ-42 is more hydrophobic and fibrillogenic and despite being the minority, is the major deposit of amyloid beta in the brain (367).

5.2.2 Mechanism of clearance of Amyloid beta

The formation of plaques in Alzheimer's disease (AD) is believed to be attributed to the presence of amyloid beta (180). Accumulation of Aβ in the brain occurs when it is not transported out of the brain, indicating that removal of the peptide may assist prognosis in AD. This rationale has resulted in numerous groups examining transport of amyloid beta to the outside of the blood brain barrier (BBB) (95, 368, 369). The exact mechanism of this bidirectional transport has not been explored completely, although there may be an imbalance in the transport that contributes to the accumulation of cerebral amyloid deposits. There is some evidence that both low density lipoprotein receptor related protein (LRP1) and the receptor for advanced glycation end products (RAGE) present on both sides of the BBB may be involved in efflux of amyloid beta from the brain (370). Another important efflux transporter, P-gp has also been examined for its potential role in the efflux of amyloid beta. This ABC transporter is present in the luminal surface of brain capillary endothelial cells and limits xenotoxins from traversing the BBB (371). Lam *et al.,* provided some evidence of amyloid beta efflux by P-gp (93). Researchers still continue publishing works that suggest this link (362, 369, 372-374). Kuhnke *et al.,* have also shown evidence of an inverse relationship between vascular P-gp and the quantity of Aβ-deposited plaques (375). They have also suggested a decrease in the efflux of rhodamine-123 (rho-123) a well-known P-gp substrate on exposure of the cells (MDR 1 transfected LLC cells) to the peptide suggesting competitive inhibition. *In-vivo* studies have also indicated that a lack of P-gp increases the deposition of Aβ in cerebral plaques, while plaque decrease can be attributed to induction of the ABC transporter (95, 362, 374).

5.2.3 Structural conformation of P-glycoprotein

The inward facing substrate binding site of P-glycoprotein (P-gp) is pivotal for efflux as it allows the transporter to screen the inner membrane for specific substrates (77, 376). X-ray images of the transporter showed binding of inhibitors to the substrate binding sites. Many small molecules have evolved as substrates for P-gp, including hydrophobic peptides used as immunosuppressant. Previously, separation between the two domains of P-gp was measured at approximately 15Å (77), though recent research on the structures of P-gp indicated that the space between the two domains was larger at 40 Å (377). This recent

evidence suggests that the transporter would allow larger substrates like amyloid beta to bind to the substrate binding site (375).

5.2.4 Usage of different chromatographic conditions for analysis of Aβ-42

Many different methods have been developed for the analysis of Aβ. Initially the peptide was detected by competitive ELISA. It was realised that quantification of ELISA may not be the best for measuring very low concentrations of the peptide from a complex sample such as blood or serum. The different components present in these samples may show cross reactivity with the antigenic epitope used in ELISA. To bypass these inaccuracies different methods using chromatography and mass spectrometry have been developed. Du *et al.,* have suggested that HPLC coupled to mass spectrometry could be ideal for detection of Aβ-40. A basic pH was used to allow for better retention and thereby recovery of the peptide (378).

Table 5.1: Different methods employed for detection of Aβ-42 by HPLC

Column	**Chromatographic conditions**	**Reference**
Waters-Xterra C8 (3.0*150)	Ammonium acetate: Nanopure H_2O	Du C et al., (378)
Agilent PLRP-S (4.6*250-300Å 5 µm)	Ammonium acetate: Acetonitrile	Warner et al.,(379)
C4 narrow bore column (4.6*250)	Nanopure H_2O: Acetonitrile	Mori et al., (380)
CN Capshell columns (4.6*250)	Nanopure H_2O:Acetonitrile	Walsh et al.,(381)
C8 Agilent column (50*4.6mm)	Nanopure H_2O: Acetonitrile	Clarke et al.,(382)

5.3 Aim

The aim of this chapter includes:

- Assessing the cytotoxicity level of Aβ-42 in Caco-2 cells
- Determining the substrate efficacy of Aβ-42 by transport experiments and its HPLC analysis on Caco-2 cells

5.4 Materials

Materials for cytotoxicity assay (MTT): 96 well plates from Thermofisher scientific, Scoresby, Australia (tissue culture treated), MTT (Sigma-Aldrich, Castle Hill, Australia), DMSO (Ajax Finechem, Taen point, Australia), Aβ-42 (kindly provided from the lab of Dr Guiesseppe Verdille, School of Biomedical sciences, Curtin University). Cell inserts for transport studies was procured from Millipore, Victoria, Australia. HPLC columns for chromatographic analysis were procured from the following: C18 (4.6x250mm) was obtained from Alltech, Grace discovery, QLD, Australia, C8 (4.6x150 mm, stable bond, Poroshell) was procured from Agilent, Victoria, Australia), CN column (4.6x150mm) was obtained from Alltima, Grace discovery, QLD, Australia. Analysis was done on an Agilent 1100 HPLC consisting of a low pressure quaternary pump with degasser and an inbuilt peltzeir temperature controlled 2 mL vial autosampler and a column oven (Agilent technology, Waldbronn, Germany). Organic solvents for mobile phase were obtained from VWR chemicals (QLD, Australia) and trifluoroacetic acid was procured from Sigma-Aldrich, Castle Hill, Australia. HBSS used for transport experiment was obtained from Sigma-Aldrich,Castle Hill, Australia, 10 mM HEPES (Sigma Aldrich, Castle Hill, Australia) and 25 mM glucose (Ajax Chemicals, NSW, Australia).

5.5 Methods

5.5.1 Sample preparation of amyloid beta-42

100 μM of Aβ-42 standard was prepared in 2% sterile DMSO and 98% sterile F-12 buffer (cold phenol red free). The peptide was vortexed for 15 s and then stored at 4º C for 24 hours. The peptide was ready for experiments after 24 hours. Standard ranging from 50 nM

to 5 µM were prepared in HBSS (with 25 mM glucose and 10 mM HEPES) at a pH of 7.4, from the stock of 100 µM.

5.5.2 Cytotoxicity assay

To determine the cytotoxicity of Aβ-42 in Caco-2 cells, an assay based on the principle of a coloured compound only generated by living cells was performed. 4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay was used in this assay to assess the level of cell viability at different concentrations of Aβ-42 by detecting a blue formazan product produced by Caco-2 cells over time.

The cells were cultured in 96 well plates in accordance to section 2.5.1. $1.5x10^4$ cells were seeded in each well. Cells were cultured in growth medium with 10% FCS. The experiment was conducted when the cells reached 80% confluency (approximately day 4), Concentrations of Aβ-42 varying from 50 nM to 5 µM were incubated with the cells for 6 hours or 24 hours in 200 µL of the medium (37° C, 5 % CO_2). 20 µL of the MTT stock solution (100 mg/mL) was added to each well to attain a final concentration of 0.45 mg/mL. It was then incubated for 4 hours at 37°C to form MTT formazan crystals. The reaction was terminated with the addition of 100 µL of DMSO after aspiration of medium from each well. The plate was placed on the shaker (Ratek instruments Pty Ltd, Boronia, Australia) for 10 mins at 50 rpm RT. The experiment was quantified by measuring the absorbance at 570 nm in the multimode plate reader (Enspire multi-mode Plate reader).

5.5.3 Protocol for transport study of Aβ-42

The transport study was based on the principle which suggests that molecules which are substrates for P-gp use the transcellular pathway, thus being effluxed out of the lumen of the gut. To assess if Aβ-42 was a substrate for P-gp in the current study, transport experiment was performed. Caco-2 cells were grown on 0.6 cm^2 inserts at 65,000 cells/cm^2 in growth medium with 10 % FCS. This cell line was used as Caco-2 has been known as an excellent *in-vitro* model for permeability studies (190). Medium was changed every third day. As has been shown before in section 2.4.5, (Figure 2.11), TEER values were shown to be the highest between days 21 and 24. The experiment was conducted on day 21. Cell inserts with

TEER values > 300 Ω cm^{-2} were used for the experiment. The inserts were placed in a fresh 24 well plate after measurement of their TEER values. The medium in the inserts and the wells were replaced with pre-warmed HBSS (with 25 mM glucose and 10 mM HEPES) buffer at pH 7.4 with Aβ-42 (200 nM). For understanding permeability related drug interactions, the cells were also co-incubated with PSC-833. Apical chamber had a volume of 300 µL and basolateral chamber had a volume of 600 µL to ensure equivalent volume level on both sides of the membrane. This ensured a constant pressure gradient on both sides. Thus a set of wells (n=3) that contained 200 nM Aβ-42 in both the apical and basolateral compartments and a set of wells (n=3) that contained both Aβ-42 (200 nM) and 6 µM PSC-833 in both the apical and basolateral compartments were prepared at the start of the experiment. At every time point (30, 60, 90, 120, 180 mins), an aliquot was collected from both chambers and replaced with same volume of HBSS and 200 nM Aβ-42 with or without PSC-833.

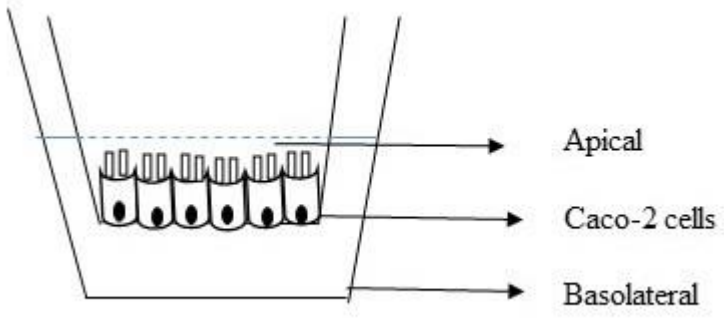

Figure 5.1: Pictorial representation of apical and basolateral chambers. The inserts were seeded with 65,000 cell cm^{-2}. A volume of 0.3 mL was present in the apical chamber, while the basolateral chamber held a volume of 0.6 mL.

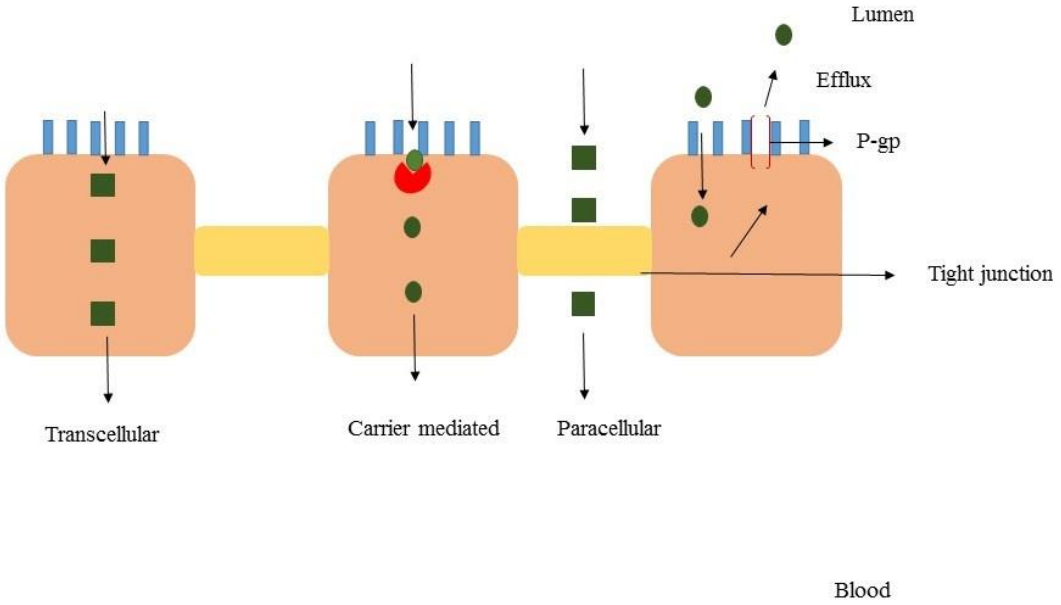

Figure 5.2: Illustration of different modes of transport in Caco-2 cells. Different forms of transport are demonstrated. Substrates of P-gp conform to the efflux form of transport.

5.5.4 Spectrophotometric determination of λ_{max} of Aβ-42

A stock volume of 1 µM Aβ-42 was prepared in HBSS (with glucose and HEPES) at a pH of 7.4 and scanned to determine the λ_{max} to be used for all experiments. The preliminary UV absorbance was determined using Hewlett Packard 8452A UV-VIS spectrophotometer (Hewlett Packard, Germany).

5.5.5 High performance liquid chromatography of Aβ-42

To estimate the changes that may have occurred during transport studies and to determine if the peptide was a substrate or inhibitor of P-gp, the samples were subjected to HPLC analysis. All the solvents used for HPLC were filtered through 0.45 µm filters.

5.5.6 Statistical analysis

All data are reported as a mean parameter determined ± standard deviation (S.D.) or standard error of mean (SEM). The difference between the groups was determined using Student's t-test. A P-value ≤ 0.05 was considered as statistically significant.

5.6 Results

5.6.1 Cytotoxicity assay

MTT assay was performed to observe the cytotoxicity of Aβ-42 on Caco-2 cells for 6 hours and 24 hours. Transport experiments were conducted for 3 hours, hence a six hour incubation of Aβ-42 for assessing cell toxicity was used as benchmark for relatively short term exposure. To follow any progression of observed toxicity, a full 24 hour incubation was conducted. At a concentration of 250 nM the peptide showed 72% cell viability, while still about 100% for 150 nM, therefore 200 nM was decided as an appropriate concentration for a 3 hour equilibrium transport study. A preliminary assessment of the level of detection of Aβ-42 by HPLC showed that a concentration of 200 nM was ideal for reproducible peak area measurement. Further increase in concentration of Aβ-42 resulted in gradually worse cell viability until there is less than 40% cell viability with 5 μM Aβ-42 (Figure 5.3).

5.6.2 Spectrophotometric analysis

The UV absorption maximum (λ_{max}) of Aβ-42 was determined at 212 nm at a concentration of 1 μM. This wavelength was used for analysis of Aβ-42 for standard curve analysis and in transport experiments.

5.6.3 HPLC-method development

Many different HPLC conditions were trialled to attempt to remove an interfering peak of PSC-833 which co-located with Aβ-42, especially when PSC-833 was used in the medium at 6 μM while the sample peptide was used at 200 nM. This proved to be difficult to overcome even with different columns, so only the data without PSC-833 added is shown in this section of the thesis. Flow rate was chosen according to the diameter of the column used. Initially a temperature of 40ºC was used in the column oven. As the elution times were quite short in these runs already, a lower temperature of 30ºC was considered optimal for the remainder of method development studies. TFA was used as the ionic agent to generate sharper peaks of the peptide. The peptide was shown to exhibit variable binding to the tubing and column when methanol was not used to wash the needle, as false peaks were

observed when injections were performed from multiple vials with different concentrations of Aβ-42. Hence methanol was used for washing, after which no carry over of peaks were seen in the subsequent samples.

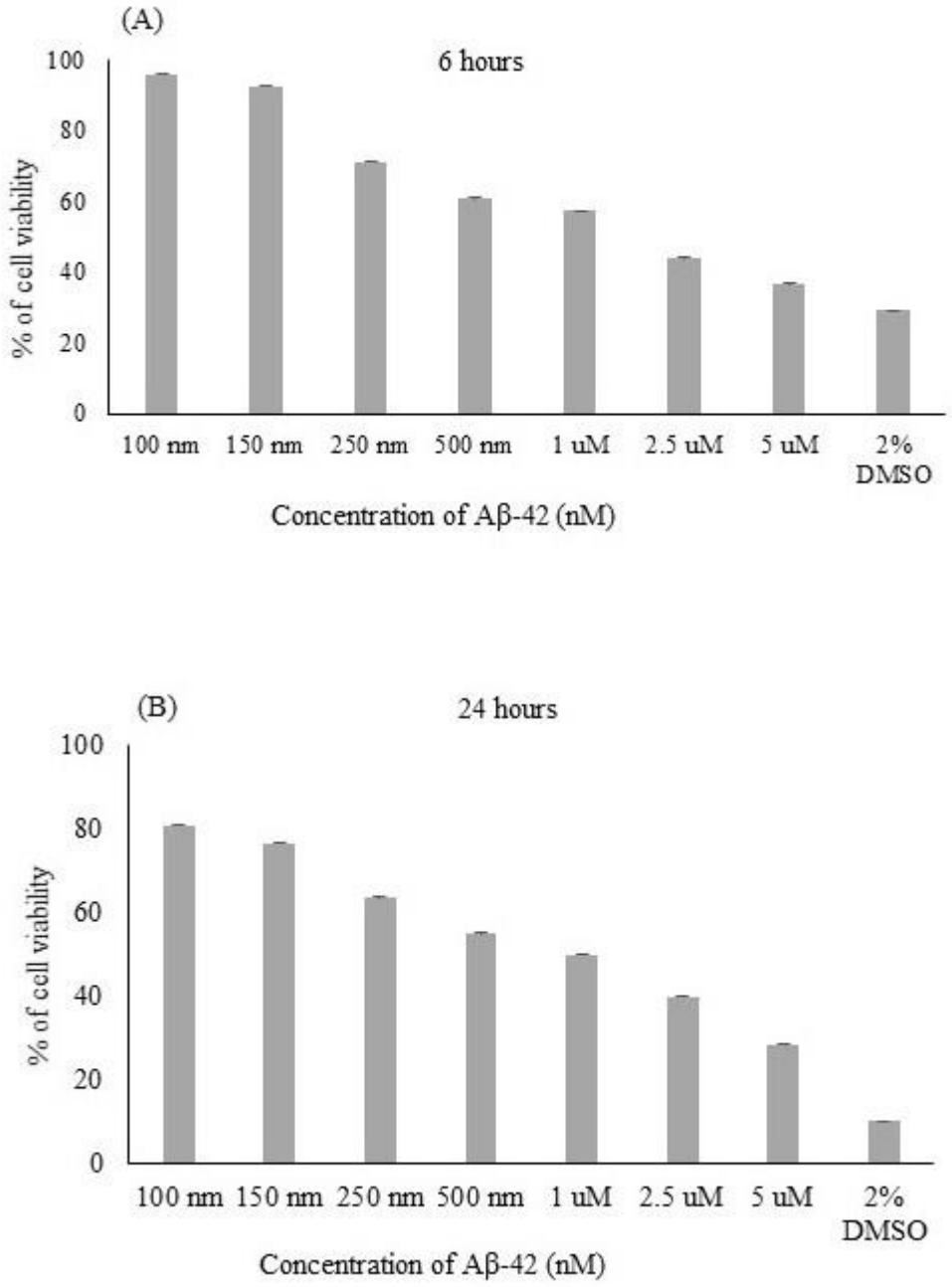

Figure 5.3: MTT assay performed on Caco-2 cells using Aβ-42 for (A) 6 hours and **(B)** 24 hours. The values were determined compared to the negative control 2% sterile DMSO. Data represents the average viability of Caco-2 cells ± SD (n=4).

Table 5.2: Results with different chromatographic conditions and columns.

Column	Mobile phase	Chromatographic conditions	Results
C18 (4.6*250mm)	Water/Acetonitrile with 0.1% TFA (pH 3.5)	Time:40 mins Temperature: 40°C Flow rate: 0.5 mL/min UV wavelength: 212 nm, 220 nm	Peak was seen at 9.5 mins
C8, stable bond (4.6* 150mm)	Phosphate buffer (25 mM)/ Acetonitrile with 0.1% TFA (pH 3.5 or 7.5)	Time:40 mins Temperature: 40°C Flow rate: 0.3 mL/min UV wavelength: 212 nm, 220 nm	Peak was seen at 7 mins
	Sodium acetate (25 mM)/ Acetonitrile with 0.1% TFA (pH 3.5, 5.8 or 7.5)	Time:40 mins Temperature: 40°C Flow rate: 0.3 mL/min UV wavelength: 212 nm, 220 nm	Peak was seen at 7 mins
	Ammonium acetate (25 mM) / Acetonitrile with 0.1% TFA (pH 6.0)	Time:40 mins Temperature: 30°C Flow rate: 0.3 mL/min UV wavelength: 212 nm, 220 nm	Peak was seen at 7 mins
CN column (4.6*150)	Sodium acetate (25 mM)/ Acetonitrile with 0.1% TFA (pH 3.5, 5.8 or7.5)	Time:40 mins Temperature: 30°C Flow rate: 0.5 mL/min UV wavelength: 212 nm, 220 nm	Peak was seen at 4 mins
	Phosphate buffer (25 mM)/ Acetonitrile with 0.1% TFA (pH 3.5 or7.5)	Time:40 mins Temperature: 30°C Flow rate: 0.5 mL/min UV wavelength: 212 nm, 220 nm	Peak was seen at 4 mins
	Ammonium acetate (25 mM)/ Acetonitrile with 0.1% TFA (pH 4.0 or 6.0)	Time: 40 mins Temperature: 30°C Flow rate: 0.5 mL/min UV wavelength: 212 nm, 220 nm	Peak was seen at 4 mins

The columns were used based on reports of their usage for Aβ and their ability to efficiently elute low molecular weight peptides (380, 381). Different pH and columns were used to observe if the peaks for Aβ-42 moved away from the solvent front. The pI value of the peptide was 5.5, hence efforts were made to use solvents with pH around the value.

Different solvents were used with pH around their appropriate pKa value to show effective utilisation of the buffer. Phosphate buffer showed effective elution at 3.5 and 7.5. Sodium acetate demonstrated good elution at pH 3.5, 5.5 and 7.5. Hence sodium acetate was used at all three pH values.

The difference in the peak area was shown to be best in C18 columns. As the transport experiment used the same concentration of peptide in both chambers, subtle differences from the basal concentration of 200 nM were observed. Understated differences were more readily seen in the C18 column. Therefore, a standard curve of the peptide from 50 nM to 250 nM was performed (Figure 5.4). The solutions for standard curve were prepared from a stock concentration of 100 μM Aβ-42. They were prepared in pre-warmed HBSS pH 7.4 in HPLC vials.

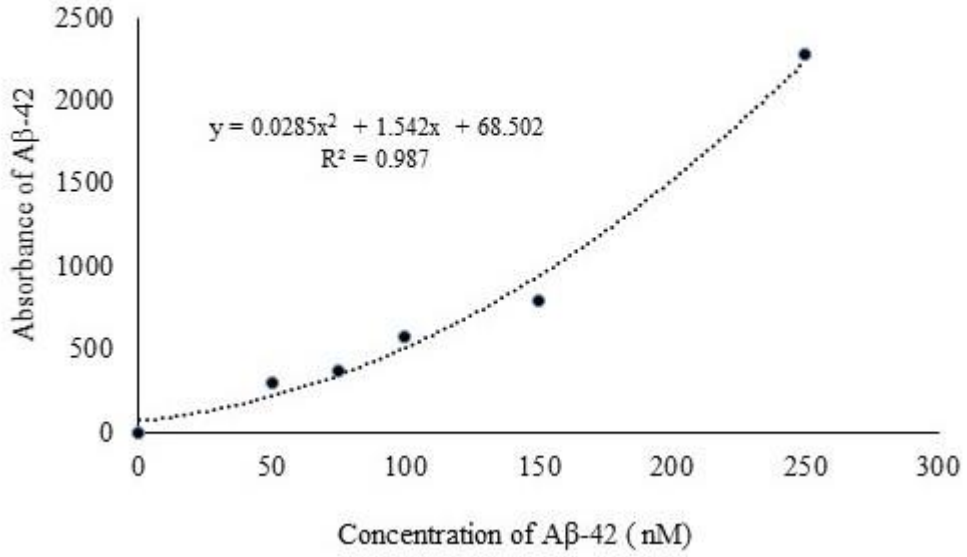

Figure 5.4: A standard curve for Aβ-42. Different peptide concentrations were prepared to quantitate Aβ-42 present as it moved through the apical and basolateral compartments in the equilibrium transport study of Caco-2 cells.

5.6.4 Equilibrium studies for efflux of Aβ-42 on Caco-2 cells

Equilibrium studies with the peptide was conducted on day 21. The method was developed by Dr Crowe and published earlier (383). Only inserts with cells showing above or equal to

300 $\Omega.cm^{-2}$ were used for the experiment with an average of 315 $\Omega.cm^{-2}$ measured. Initially the cells were washed with sterile PBS and conditioned with pre-warmed HBSS buffer at pH 7.4. After the TEER values were measured the inserts were transferred into a fresh 24 well plate. 200 nM of Aβ-42 was added to both apical and basolateral chambers. An aliquot was collected at various time points (30, 60, 90, 120, 180). They were immediately analysed by HPLC or stored at -20ºC for later analysis.

The peak area of the samples was analysed for net apical to basolateral (A-B) movement or efflux/basolateral to apical (B-A) movement. From the plot (Figure 5.5) it was suggested the A-B movement was higher yet only minimally than the B-A movement at all-time points implicating that there was no net flux of the peptide in the cell. This could be associated to oligomerisation, which is the characteristic feature of Aβ. A substrate of P-gp would show a higher B to A ratio than A to B. The transport of Aβ-42 from the apical to the basolateral compartment is shown to have a sharp increase in comparison to B to A from 30-60 mins. The rest of the experiment showed a slower increase in the transport of the peptide. However, the movement from B to A was constant with very slow increase in the peak area observed at different time points. Towards the end time points (120 and 180 mins) no difference in the movement from A to B and B to A was indicated. Degradation of the peptide was also observed as the peak area at 30 mins was below the initial concentration of 200 nM. The peptide may have been taken up by the Caco-2 cells and degraded by specific enzymes present in the epithelial cells such as Insulin degradation enzyme (IDE) and neprilysin. As the cell membrane, itself was not solubilised to observe the presence of Aβ-42, the possibility of uptake by the cells cannot be completely disregarded.

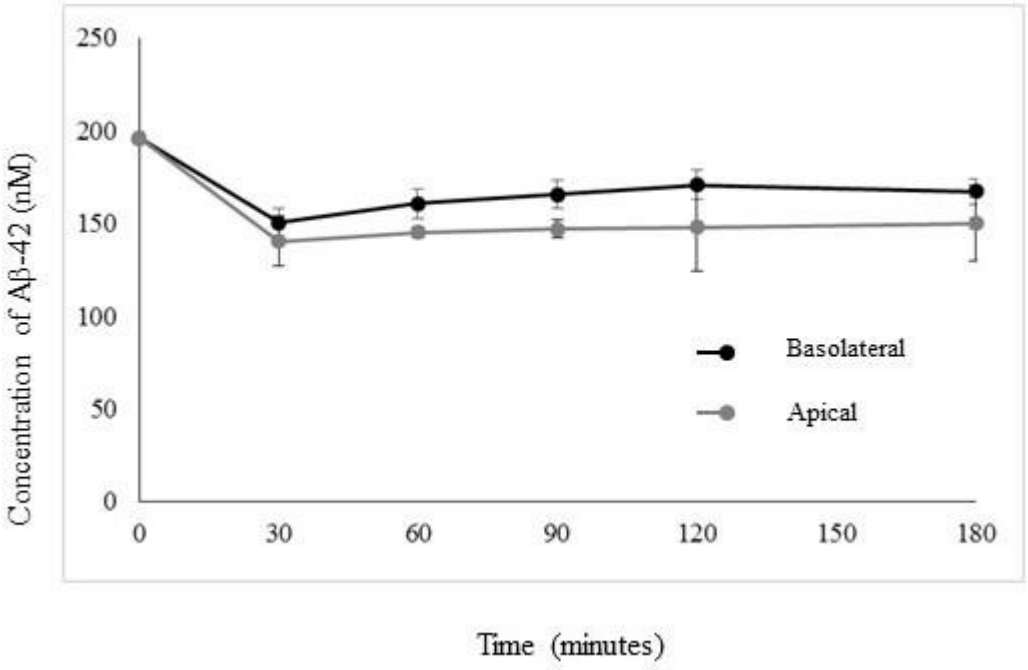

Figure 5.5: Transport studies using transwell inserts. The net movement of the peptide was observed for active efflux through Caco-2 cells. Concentration of Aβ-42 collected at each time point was depicted. Data represents the Aβ-42 remaining in each chamber in Caco-2 cells monolayers for each time point ± SEM (n=3).

5.7 Discussion

This chapter aimed to develop a method to understand the possibility of Aβ-42 being a P-gp substrate. The previous chapter showed that in the presence of a P-gp inhibitor (PSC-833), the presence of Amyloid precursor protein (APP) was reduced in CM of gastrointestinal cells, suggesting a possibility of APP and in turn Aβ acting via PXR regulation. This chapter has attempted to interpret the substrate efficacy of Aβ-42 through a modified bi-directional transport assay using Caco-2 cells.

Aβ-42 was used as the peptide of choice due to the fact that it is the predominant peptide found in amyloid plaques in the brain and the antibacterial potency of this peptide was higher than that of Aβ-40 (182). Puig *et al.*, have also reported that Aβ-42 was more abundant in Caco-2 cells than Aβ-40 (187). Since Caco-2 was the cell line used for *in-vitro* transport studies, Aβ-42 was deemed the ideal choice of peptide. The toxicity level of Aβ-42 was deduced using MTT cell viability assay. The peptide demonstrated toxicity even at a relatively low concentration of 250 nM (Figure 5.4). Toxicity was not linear though as 5 µM

concentration was not five times as lethal as 1 μM. This can be attributed to the aggregation and oligomerisation characteristic of the peptide which may limit its direct exposure to cell surfaces. The peptide is known to be toxic on interaction with the cell membrane (384). Also, as the cells were cultured in growth medium with 10% FCS, possibilities of non-specific binding of the peptide with albumin was high, which may explain why the toxicity over 24 hours was not vastly different in comparison to 6 hours (385).

To study efflux of the peptide on Caco-2 cells, equilibrium studies was conducted (Figure 5.5) (383). Apical and basolateral changes in concentrations of Aβ-42 transport were minimal. The amount in the apical chamber showed about 72% of the initial amount by 30 mins and increased to 76% at 3 hours, while the basolateral chamber reduced to 76% of the initial amount at 30 mins and increased up to 84% by 3 hours. The percent increase was more in the basolateral chamber than in the apical chamber. Overall the data was not significantly different between the apical and basolateral chambers. The decrease in the initial concentration may have come about through non-specific binding to the areas of the chambers and cells. This adsorption may have a saturation effect, representing the sticky characteristic of the peptide and as new peptide was added (at 200 nM) for every time point taken, this would have slowly brought each subsequent measure slowly back to the original concentration. Another possibility is the degradation of the peptide and certainly there may be some protease activity in the Caco-2 cells. In addition to the proteases present in the cells, Low density lipoprotein receptor related protein (LRP-4) has also been known to degrade Aβ (386). Nevertheless, if degradation was a real event, it would have been expected to continue to drop in concentration in this assay, so the conclusion that adsorption was a player in these results is suggested to be thus a greater factor than any possible degradation. Differences in change in the transport was seen best at 60 and 90 mins. As the time proceeded less movement was observed between the two chambers. Towards the end of the experiment no net flux between the two chambers was observed. This could indicate that the peptide did not conform to the characteristics of efflux movement which is mandatory for the substrates of transporters. Any drug related permeability reactions of the peptide could not be observed as cells on co-incubation with PSC-833 and Aβ-42 were not analysed by HPLC due to problems with PSC-833 creating a large peak area around the time frame of the Aβ-42 which was not able to be resolved after many changes to protocols. Regardless, PSC-833 would be unlikely to have added anything to these results given this

lack of separation of data and no upward trajectory of the apical data which would have suggested efflux into that chamber. This is in support with other studies suggesting that P-gp may not be responsible for Aβ efflux (386-388).

Ito *et al.,* in their *in-vivo* experiment observed that P-gp itself did not show any significant efflux mechanism of Aβ-42 and that the clearance of the peptide from the blood brain barrier was LRP-1 mediated (387). Nazer *et al.,* also concluded that an over expression of either LRP-1 or P-gp did not show any significant effect in the transport of Aβ-42 (386). They used transfected MDCK cells as their *in-vitro* model for transport of radiolabelled Aβ. Transfected MDCK-P-gp cells did not transcytose Aβ from the basolateral to apical side, while transfected MDCK-mLRP4 cells endocytose and degrade the peptide but do not transcytose the peptide. This suggested that neither of the two transporters were responsible for efflux of Aβ. Other researchers also reported that Aβ cannot be a substrate for P-gp (388). They also measured the efflux of pirarubicin (PIRA), a well-known P-gp substrate. Their findings suggested that the efflux of PIRA was not inhibited by Aβ-42. The effect of the peptide on a P-gp over-expressing leukemic cell line (K562/ADR) and its non P-gp expressing parent cell line (K562) remained the same. These reports suggested that Aβ-42 may not be a substrate for P-gp and the clearance mechanism of the peptide from the blood brain barrier may be attributed to other transporters. A substrate of P-gp can only be transported if it is present near the substrate binding site of P-gp. The substrate binding site is localised in the cytoplasmic leaflet of the membrane bilayer. The size of the peptide and its ability to aggregate in aqueous solution would deter the peptide from being transported through the membrane and effluxed out by P-gp (389). The binding site of P-gp can accommodate small molecules between 5 and 50 Å (33, 390), while the size of a single α-helix of the peptide is approximately 63 Å (391, 392). In addition, the substrates for P-gp ranged between 0.2 kDa and 1.9 kDa and were cationic at physiological pH. Aβ-42 is negatively charged at physiological pH (388). Thus, the current results shown here conforms with the non-substrate angle and that this is not the endogenous molecule we are looking for that contributes to antimicrobial action that is excreted by human gastrointestinal cells.

Yet, several lines of evidence suggest that P-gp is involved in Aβ efflux. Abuznait *et al.,* in their study concluded that on exposure to a P-gp inducer such as rifampicin, the human LS-180 cells gastrointestinal cells showed a decrease in intracellular Aβ-40 by about 16 %,

while the P-gp expression itself increased 6 fold (96). Wang *et al.,* in their *in-vivo* studies have shown that the accumulation of Aβ-40 was increased in the absence of MDR1a in Tg2576 APPsw mice that expresses high amounts of physiological APP (362). In their study, when crossed with MDR1-KO mice that do not express MDR1a gene, an increased amount of the peptide in supernatants of homogenised brain samples in comparison to MDR1-WT mice was observed (362). Do *et al.,* suggested that at 18 month of late plaque formation in transgenic mice used as AD model, an increase in the expression of ABCB1 and ABCG2 (by 1.7 fold) was seen that reduced the influx of blood to brain Aβ-40 (393). During that period although a decrease in LRP-1 and increase in RAGE was also seen that was counter balanced by the increase in ABCB1 and ABCG2. RAGE is thought to be responsible for influx of Aβ (393). This suggested that P-gp plays a pivotal role in the clearance of Aβ from the cells. Cirrito *et al*., also demonstrated the role of P-gp in clearance of Aβ in mouse models (95). In mdr1a/b–/– double-knockout mice the transport of Aβ-42 was shown to be vastly lower in comparison to wild type mice that expressed P-gp (6% compared to 16%). Nevertheless, there is a concurrent fall in LRP-1 expression in these mice (50% of wild type values) and this could have influenced the results. McCormick *et al.,* also showed that carboxyfluorescein attached Aβ-42 showed less accumulation in P-gp overexpressing cells (394). Another group used purified vesicle bound Mdr1 from hamsters and measured the binding affinity by flurophore quenching of carboxyfluorescein linked Aβ-42. The binding of Aβ-42 was at 6.7 ± 1.0 μm which was similar to many other substrates of P-gp (93).

Other studies have reported that the peptide can also inhibit P-gp expression *in-vivo* (91, 395). Hartz *et al.,* observed that the expression of P-gp and consequently its activity reduced in transgenic mice expressing high level of human APP (hAPP) (91). They also demonstrated that when mouse brain capillaries were incubated with fluorescein conjugated-Aβ-42, their luminal fluorescence was significantly reduced in the presence of P-gp inhibitors like PSC-833 and verapamil. Consequently the activity of P-gp was also reduced on consistent exposure to the peptide in hAPP mice. However, their levels could be restored on exposure to a PXR ligand inducer. Brenn *et al.,* also demonstrated that Aβ-42 and not Aβ-40 is responsible for the action of reducing expression of P-gp and other transporters like LRP-1 and RAGE in the BBB (395). This can also be attributed to the increased proportion of Aβ-42 in growing plaques in comparison to Aβ-40.

5.8 Limitations

The experiment was conducted to identify if Aβ-42 was a substrate of P-gp. The peptide only showed passive diffusion. To demonstrate degradation or uptake of the peptide by the cells, the cells should have been solubilised and observed to detect the percentage of Aβ-42 accumulated inside the cells, although it was detected in clean balanced salt solutions but would have been immensely difficult to accurately determine in the cytoplasm of the cells. As this was not done in the study, it cannot be concluded that the peptide was degraded or taken up by the cells. Additional experiments to show that P-gp may or may not be involved should use a P-gp inhibitor in the study that allows detection of the Aβ-42 without interference by successfully separating the peaks by HPLC, although lack of directional movement in the study without a P-gp inhibitor certainly suggests that the inhibitor would not have changed results. To rule out possibilities of a non-functional P-gp transporter, rhodamine-123 efflux should have been conducted on parallel cultures. Although, this was not done in this project, other projects underway in the lab with equivalent passage cells were done at similar times and continued to show efflux of Rho-123 (results not shown), so I am confident that P-gp was functioning adequately in this particular study. Although the HPLC methods in the current study identified the peaks for Amyloid-β, it could not be separated from the inhibitor used. Therefore based on other studies it could be suggested that use of other columns such as polymer laboratory reverse phase - styrenedivinylbenzene (PLRP-S) as a stationary phase can be used for more effective separation of the peptide from the inhibitor (379). Another approach could include the use of ion-exchange chromatography as the means for purification of Aβ peptide with urea as the buffer (381).

5.9 Conclusions

The toxicity of Aβ-42 in epithelial cells was observed at very low concentrations. The peptide did not show any validation of a possible efflux by P-gp in the current study by transport experiments. Moreover, no net transport of the peptide was observed between the two chambers. This indicated that the peptide is unlikely to be a P-gp substrate. Further the size of the peptide also may not allow it to be a substrate for the transporter, although there have been reports on possible efflux by P-gp (91, 95, 374). Although, transport studies with rho-123 was not shown in the current study, concurrent efflux studies by others in Dr Crowe's laboratory at similar times using the same batch of cells have shown efflux of rho-

123 in late passages (>P-80) in cells (232). Moreover, Western blot was also performed for all late passages from (P-75-P-90) and had demonstrated high level of P-gp expression. As it was previously shown that Aβ-42 was not detected by LC/MS/MS, therefore another technique like MALDI-TOF may prove to be necessary to detect any Aβ-42 directly secreted in growth medium.

Chapter 6 General Discussion and Conclusion

The thesis aimed to isolate putative low molecular weight fraction peptides that demonstrate antimicrobial properties. Studies conducted previously in the laboratory suggested possible antimicrobial activity in Caco-2 and LS174T cells linked to the presence of P-gp. Inhibition of P-gp with known P-gp blockers like PSC-833 or GF120918 increased bacterial attachment to the cells by 30% (219). These studies were repeated in the lab using conditioned medium (CM) collected from the confluent mature cultures of the same cell lines. The basis of the hypothesis was that although the previous study showed a direct relationship between functional P-gp expression on the surface of cells and attachment of bacteria to the cells expressing the P-gp, it was difficult to rationalise how P-gp could be preventing adhesion of the bacteria directly. A more acceptable hypothesis was that P-gp could secrete antimicrobial factors into the local environment thus preventing the adhesion of bacteria. Knowing that CM was able to have antibacterial properties further reinforced the notion that P-gp may have had this role. The purpose of the project was to confirm that P-gp was involved in the efflux of the factor detected in the CM, to try and isolate what this factor maybe and subsequently test its ability to be a substrate by conducting bidirectional transport studies with the purified factor. It was expected that the antimicrobial factor had to be of a low molecular weight (<3 kDa) as the largest known substrate of P-gp was only 1.9 kDa. However, most well defined and already characterised gastrointestinal antimicrobial peptides were of a larger molecular weight. Changes were observed in the appearance of known and unknown AMPs from CM of cells when P-gp was modulated. Hence CM was used for measurement of antibacterial activity against pathogenic microorganisms, namely, *E.coli*, *S.typhimurium* and *S.aureus* and simultaneously subjected to proteomic analysis for sequence determination.

To isolate these low molecular weight factors, it was necessary to culture cells in either a FCS free or very low FCS medium as albumin in FCS would readily bind to low molecular weight peptides making them difficult to be isolated by low MW filtration (259). Most cells in culture require serum for their growth. Depletion of serum from the growth medium would surely affect the properties of these epithelial cell lines. For complete assessment and evaluation of the cells cultured without FCS and for determining the expression of the important epithelial properties, a series of experiments were done. Western blot was used as the primary technique for evaluation of protein expression of ABC transporters in three different cell lines, Caco-2, LS174T and RKO. RKO was used as a negative control human

gastrointestinal cell line so as to evaluate low molecular weight peptides expressed from its CM when P-gp was not involved. Theoretically, any factor secreted from RKO cells should not produce any substrate for P-gp as it is a P-gp deficient cell line.

Initially, the cells were cultured in normal growth medium with 10% FCS for the first four days. This was required as the cells needed FCS for attachment and propagation (229). The growth medium for the cell lines were then replaced with growth medium containing 0% FCS or 2% FCS and ITS-X for both the conditions, for the remainder of the growth period (230).

Caco-2 cells were lysed on the 24th day of growth for detection of expression of three transporters, P-gp, BCRP and MRP2. Cells cultured in fully defined growth medium with 0% FCS had variable expression profiles which ranged from low to below detection limits in Western blots. Cells grown in 2% FCS with ITS-X had greater consistency in expression of P-gp than fully defined, but also less than when cultured in 10% FCS. The values were compared to the HeLa MDR 1 OFF cell line and to the Caco-2 cells cultured in 10% FCS (P-81). P-gp expression in LS174T did not differ greatly with different concentrations of FCS in the growth medium (Figure 2.5). High passage number in Caco-2 monolayers, e.g. from P-80 and above showed highest expression of P-gp in Caco-2 cells, which has been observed recently in our laboratory (232). BCRP, did not show significant difference between cells cultured in different concentrations of FCS in both cell lines. At all times the expression of BCRP in LS174T cells was lower than the expression of Caco-2 cells. Our results were further corroborated by a similar study by Gutman *et al.,* (236). MRP2 was only detected in Caco-2 cells in the current study. A significant difference in the expression of the transporter between the passages was observed with P-80 showing higher expression of MRP2. In repetition of expression of other transporters, MRP2 also showed minimal or no expression in cells cultured in 0% FCS. As expected, RKO cells did not show any expression of P-gp or MRP2 expression irrespective of the percentage of FCS used (204).

To evaluate the mRNA changes in the three cell lines, for ABCB1 (gene expression of P-gp) and ABCG2 (gene expression of BCRP), RT-PCR was performed. Similar to its protein expression profile, Caco-2 cell line showed a constant reduction of band thickness in cells cultured in 0% FCS, but increased in intensity in cells cultured in 2% FCS and 10% FCS relative to the GAPDH control (Figure 2.10) LS174T cells did not show a difference in

transporter expression irrespective of the percentage of FCS used. RKO cells lines did not express any P-gp in their cDNA samples.

Caco-2 are known to be an excellent *in-vitro* model for permeability and transport experiments (190). Cells cultured in 0% FCS unfortunately did not elevate TEER value any higher than 90 Ω cm^{-2}, while cells cultured in 10% FCS showed values up to 600 Ω cm^{-2} (Figure 2.11). This meant Caco-2 cells grown in fully defined medium could not be used in bidirectional transport studies. In addition, their P-gp expression appeared lower than normal as well. P-gp expression was only moderately lower than in cells cultured in 2% FCS plus ITS-X, but the TEER values was not a lot higher than in 0% FCS, showing that a complete growth medium with 10% FCS is required for tight junction formation. Hence it could be concluded that for transport experiments cells cultured in 10% FCS was a necessity. Expression of the tight junction protein occludin was also studied in cells cultured in 0%, 2% and 10% FCS on different days (Figure 2.12). Initially the expression was similar in cells cultured in different percentage of FCS. As the incubation days of cells increased, cells cultured in 2% FCS showed far less expression than the cells cultured in 10% FCS, while cells cultured in 0% FCS showed negligible expression. Quantitative analysis was not done in the study. This supports the finding that FCS is necessary for formation of tight junctions on cell monolayers grown in millicell inserts and also reaffirms the lack of tight junctions that were observed in the current study when FCS was reduced in growth medium (230).

For aspects of the project that did not require growth on inserts, 2% FCS with ITS-X was deemed appropriate as Western blot data did suggest that adequate P-gp expression was observed. Thus although the expression of occludin was affected, transporter expression in the later passages (>P-80) was not affected by reduced FCS. For stringent isolation of the factors that exhibited antimicrobial activity, 2% FCS appeared to be an ideal compromise between high P-gp expression and the ability to adequately isolate any low molecular weight secretions that were hoped to be quantitated and determined from any follow on studies.

Isolation of the antimicrobial factor from a complex sample such as CM required use of diverse techniques (257). Molecular weight cut off filtration (< 30 kDa and <3 kDa filters) and acetonitrile precipitation were used for decreasing the high MW protein load while

allowing low MW proteins to stay in solution and pass through the filters without blockage. Silver staining provided evidence that this combination of methods was functional at isolating LMW proteins from proteins such as albumin still present in the growth medium as 2% FCS was used in the studies. Size exclusion chromatography of the samples was performed for fractionating the sample further so that it becomes easier to identify the factor from a myriad of proteins present in the CM. AKTA purifier (P-900) was used for this purpose. PBS (50 mM pH 7.4) was used as the buffer for fractionation as it did not interact with the proteins expressed in the medium. Usage of other stronger eluents such as organic solvents used elsewhere would interact with the antimicrobial activity of the factor rendering in an ineffective way of its isolation. Once the samples were fractionated they were subjected to antibacterial sensitivity test and submitted to a local biotechnology company (Proteomics International) thereafter for sequence analysis. An antibacterial sensitivity test showed an increase in the activity of samples induced with rifampicin (a known P-gp inducer). Rifampicin was used as previous experiments had suggested P-gp induction would increase the intensity of antimicrobial effect (108). The pattern of inhibition between the three different organisms used was not uniform which is not surprising given both gram positive and gram negative organisms were used. Also as *S.typhimurium* and *E.coli* showed different levels of attachment to epithelial cells, the level of inhibition against both these gram negative organisms may not be in the same order of magnitude.

Proteomics International used LC/MS/MS as their prerogative technique for detecting sequences from fractions diluted in the buffer. From the many sequences that were detected in the growth medium after multiple days of exposure to the cell lines, histones were observed and based on the literature these do have some antibacterial activity (302, 306). It is not unusual to find histones in the medium as they are DNA binding proteins present in very high concentrations in the nucleus, which sometimes are exported instead of ingressed through the nuclear pore and are termed extracellular histones (396). Their antimicrobial activity has been shown to be similar to that of defensins (305). In this study they were observed in samples that were induced with rifampicin. Both Caco-2 and LS174T confirmed the presence of histones in the CM. (Table 3.2, 3.6, 3.7 and 3.9). CM from HeLa MDR1 OFF and RKO cells were also submitted for proteomic analysis by LC/MS/MS. Although histones may show antibacterial properties, their concentration of minimal inhibition was

high (3.8 µM) according to the study conducted by Tagai *et al* (305). It was unlikely that such a high concentration of histones was evident in the CM samples of the current study. Therefore the antibacterial effect of the samples cannot be fully attributed to the presence of histones identified in the samples. Many unidentified protein sequences were also detected in the study. The various amino acids present in these sequences rationalises them as potential antibacterial factors based on reports that suggest possible amino acids that contribute to antimicrobial activity in AMPs (299, 300). However, these sequences can also not be completely related to the antimicrobial activity observed in the samples. From these sequences, it is possible that antibacterial activity may not be recognised from a single factor rather a combination of proteins and peptides expressed in the cells in varying degrees.

However as only constitutively expressed endogenous peptides were targeted for detection, further peptides that may be induced by microorganisms or microbial products also needed to be investigated in the cells and CM of gastrointestinal cells. In addition, known peptides that may be stimulated during incubation with microorganisms may have been a possible mechanism of the observed antibacterial activity. To that end, the cell lines (Caco-2 and LS174T) were stimulated with LPS to simulate an environment that may induce various antibacterial peptides. After 24 hours of incubation, CM and lysed cells were observed for protein (using Western blotting techniques) and mRNA expression (using RT-PCR) of the various defined gastrointestinal peptides such as MMP-7, α-defensin-5, Human beta defensin-1, Human beta defensin-2 and Amyloid beta. MMP-7 was not produced endogenously in cells cultured in 2% FCS, but were observed in cells cultured in 10% FCS. As all the cellular secretion experiments were performed using cells cultured in 2% FCS, MMP-7 was not identified as the factor responsible for any antimicrobial activity. However on induction with LPS, MMP-7 was seen in both the protein and mRNA samples of cells cultured in 2% FCS. MMP-7 cleaves the precursor protein in paneth cells to produce active α-defensin-5 in the mouse small intestine (397) and by a paneth cell trypsin in human intestine (168). These defensins are only seen during Crohn's colitis and other types of inflammation of the intestinal tissue (339). α-defensin-5 was not seen in the samples endogenously or on induction with LPS and this has been supported by other studies (338). In contrast, α-defensin-5 has been shown to be induced in the presence of live microorganisms relatively faster (138, 165). Although LPS was used in this study, live

microorganisms were not used to encourage secretion in the cells due to the complications of long term bacterial/ mammalian cell co-growth in the laboratory. Hence the ability of the cells to express α-defensin-5 in such a condition was not observed. It could be suggested that an attenuated condition of microorganism or the microbial product was not adequate for expression of the defensin.

The appearance of β-defensins was also investigated. HBD-1 is constitutively produced by Caco-2 cells and is a gastrointestinal AMP (123, 341). The present study also showed a similar result, where HBD-1 was constitutively produced by both Caco-2 and LS174T cells by mRNA analysis. On induction with LPS a higher intensity in appropriate RT-PCR band was observed. In contrast, reports have suggested that LPS may not induce HBD-1 in Caco-2 cells (341, 343). Cellular exposure to the P-gp inhibitor, PSC-833 was also used to detect for any change in the mRNA expression of HBD-1. On co-incubation with LPS and PSC-833 no bands were observed for both Caco-2 and LS174T cell line. Inhibition of P-gp results in blocking the activity of P-gp thereby resulting in reduced efflux of P-gp substrates (although not immediately). As, HBD-1 was not detected in cells co-incubated with PSC-833 and LPS, this event seemed a likely reason to show that HBD-1 maybe a substrate for P-gp, however, both cell lines showed the presence of HBD-1 when incubated with PSC-833 alone. Moreover, the presence of the P-gp substrate itself in the mRNA samples should not be altered by PSC-833. Protein analysis of HBD-1 could not be achieved as the samples did not show clean bands on Western blot, although the control sample (HBD-1 peptide) transferred well. The plausible reasons could be due to the presence of the different isoforms expressed in Caco-2 cells and consequently LS174T cells (342, 344).

HBD-2 was also observed in Caco-2 and LS174T cell lines. In contrast to HBD-1, HBD-2 is an inducible defensin. Although reports have indicated that HBD-2 is not induced by LPS (341, 343), the current study showed greater HBD-2 expression in Caco-2 cells on induction with LPS in mRNA analysis. One reason for discrepancy may be that those earlier studies used only upto 4 hours of stimulation with LPS, while the current study used 24 hours. Protein analysis did not show any band for HBD-2. TNF-α was used as the positive control (pro-inflammatory cytokine) as reports had indicated that apart from α-defensin-5, HBD-2 also showed induction in the presence of TNF-α (335). The pathway for induction of HBD-2 is through Nf-κB and MAPK-1 in the presence of bacteria like *S. enterica serovar* Enteridis

flagellum, *Helicobacter pylori* and *E.coli* Nissle 1917 (347, 349, 350). However, other studies have reported that Nf-κB does not play a major role in the induction of HBD-2 (335, 398). So far none of the known gastrointestinal peptides tested against the samples indicated the possibility of being the antibacterial factor present in the CM samples. To further probe the source of the antimicrobial activity the samples were observed for amyloid beta within the cells and CM. Neural peptides have shown to exhibit antibacterial activity (325, 327). In addition to its characteristic ability to form plaques in the brain during Alzheimer's, Amyloid beta also has other diverse functions, including antimicrobial activity (182).

A LC/MS/MS was performed on samples containing only Aβ-42 and no sequences were matched with the peptide. This was also required as a positive control to demonstrate that the technique used for detection of sequences from CM was validated. The peptides did not seem to be detected even at a high concentration. This led to further analysis by a different method such as MALDI-TOF. The peptides could be identified only by MALDI-TOF, suggesting that certain peptides cannot be identified by the normal LC/MS/MS method.

Although Aβ-42 could not be detected in the study, Caco-2 cells showed an increase in presence of APP on LPS incubation. The expression reduced on co-incubation with LPS and PSC-833. Hence, the presence of APP could be speculated to be controlled by PXR activity. PXR regulates the three transporters P-gp, BCRP and MRP2 and reduction of PXR is directly proportional to the efflux activity of the transporter. In the study, PSC-833 was used as the repressor and the reduced expression of APP seen on incubation with PSC-833 can be associated with PXR activity of P-gp, thereby reducing the efflux of its substrate (Figure 4.9). LS174T indicated an increase of amyloid beta when incubated with P-gp inducer, rifampicin. From the expression of APP it could be speculated that amyloid beta may also act by the same mechanism. As the cells appeared to respond to amyloid beta in a PXR related mechanism and based on previous literature about amyloid beta being a substrate for P-gp (95, 375, 399) further investigation to understand whether amyloid beta is a substrate for P-gp was employed through transport experiments. Initially the MTT cell viability test using blue formazon precipitate formation was performed to measure the concentration of amyloid beta that manifests least toxicity on Caco-2 cells. A concentration of 200 nM showed cell viability of more than 80% after 6 hours of exposure and as the transport studies required only 3 hours, this was deemed an appropriate concentration that could also be

detected readily in our optimised HPLC protocols. Cells were cultured on millicell inserts and cultured for 21 days in growth medium with 10% FCS.

The movement of the peptide in either an apical or basolateral direction did not occur and thus did not conform to the characteristics of a substrate of P-gp which should have shown movement upward into the apical compartment of the suspended Caco-2 cell monolayers. Such lack of transport could suggest passive diffusion or a very limited transport that kept the protein in each chamber with little active transport (Figure 5.5). This latter possibility may have occurred due to oligomerisation and aggregation characteristic of Aβ-42 in an aqueous medium thus hindering its movement through the cell membrane, although the event maybe unlikely at this low a concentration (389). Another explanation for the cells not being able to show any uptake of the peptide is the size of the peptide. Degradation of the peptide may have been possible by specific enzymes in the epithelial cells. LRP which is present in Caco-2 cells is also responsible for degradation of the peptide (386) To study the transport in more detail, future work would need to overcome the detection limits of HPLC detection and use either radiolabelled or fluorescently labelled Amyloid-β-42 on only one side of the suspended cells and examine the apparent permeability across to the other side. As concentrations above 200 nM were deemed too toxic and as the experiment requires around 1% of the donor concentration to be detected on the receiver side of a bidirectional transport study, detection of 2 nM would be needed, which was much lower than our HPLC detection limits-hence the equilibrium change study was employed here instead.

The current study indicated the presence of various putative antibacterial factors such as histones and APP from the CM samples of gastrointestinal cells. Several lines of evidence of antimicrobial activity by Aβ-42 which is the cleaved product of APP, have been reported. As the study emphasised on determination of substrate efficacy of the factors with possible antibacterial activity, transport experiments were performed. Aβ-42 did not show any possible efflux in Caco-2 cells. This suggests that still further research on both endogenous peptides and peptides induced by microorganisms is necessary.

In addition to the various putative antimicrobial factors that maybe endogenously present in CM, the study also determined the mRNA expression of HBD-2 in the gastrointestinal cells on stimulation with LPS. This has not been observed in other studies, although their incubation times were much lesser than the current study. HBD-1 was also observed in the

study and have shown that in the presence of PSC-833 (P-gp blocker) and LPS, their mRNA expression was reduced. Although the other characteristics of HBD-1 such as their inability to remain stable in the presence of salt concentration as low as 20 mM did not fit them in the study as the antimicrobial factor, they could still be considered as potential substrates of P-gp. The study has also shown that although amyloid beta could be a possible antibacterial factor in the CM samples, they may not be P-gp substrates as observed by the transport experiments on Caco-2 cells performed in the study.

6.1 Future direction

- Different treatment options such as incubation with live microorganisms for a short period of time and detection of the samples with either microorganisms or LPS by LC/MS/MS or MALDI-TOF may have been performed. AMPs such as α-defensin-5 and beta defensins (1 and 2) have shown to be stimulated by the presence of live organisms. As the study itself used live organisms for assessing antimicrobial activity, it is imperative to understand the different peptides that could be present in the study and elucidate the mechanism of stimulation of α-defensin-5.

- Although histones have known to be representatives of antimicrobial activity, the percentage of their activity in the current study was not known. Hence concentration of histones and Aβ-42 need to be deduced in the conditioned medium of the samples by different methods that includes immunoprecipitation methods. This allows illustration of the proportion of antibacterial activity observed that has been caused by histones in conditioned medium.

- Although, Aβ-42 did not show substrate efficacy in the current study and this has been supported by other reports, consequently further analysis of the same is recommended. Different cell lines such as MDCK could be used for observing substrate efficacy of the peptide. Aβ-40 was not tested in the current study. This amyloid peptide also has shown antibacterial activity. As it does not form aggregates as Aβ-42, uptake and possible efflux by the cells may have been likely.

- Although, HBD-1 may not have played a part in the antibacterial activity in conditioned medium yet its absence when co-incubated with PSC-833 suggests that the inhibitor affects the expression of the peptide, thereby showing a PXR mediated mechanism. Further investigation to identify if they are substrates of P-gp is suggested. Usage of other inhibitors will help in the validation of the PXR mechanism. Western blot using antibodies identifying other isoforms in the conditioned medium is required.

- Identification of endogenous antibacterial substances would prove beneficial in the area of therapeutics and pave way for different avenues for defence against intestinal pathogens.

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Appendix

Appendix I: Mycoplasma Testing of Cell lines

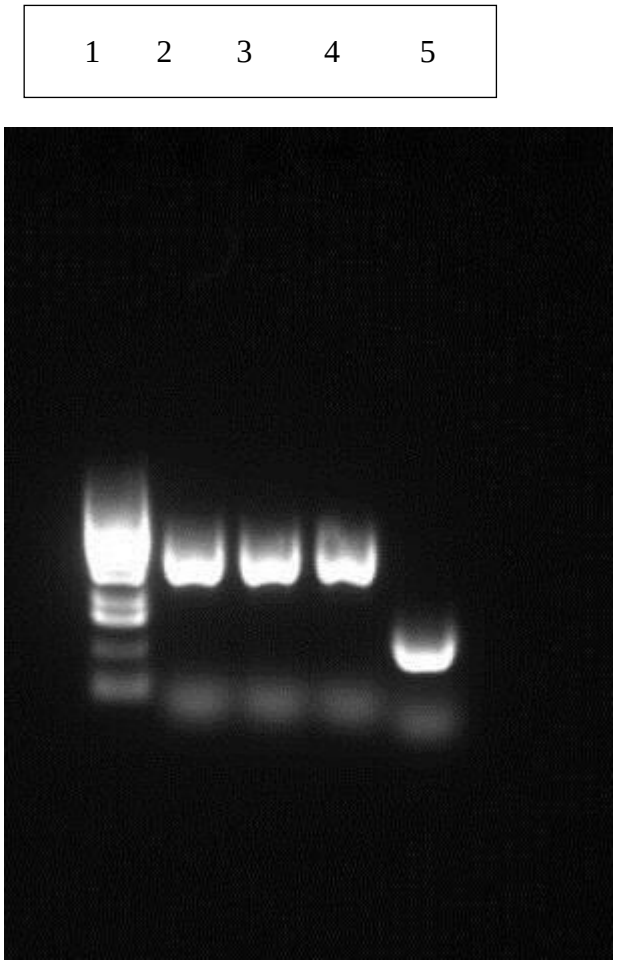

Figure 1: Mycoplasma test of the cell lines used in the study. (1) Ladder. (2) Caco-2 cell line. (3) Ls174T cell line. (4) Negative control. (5) Positive control (250 bp). The cell lines used in the study was tested negative against mycoplasma.

Appendix II: List of materials and volumes used in cDNA synthesis

Table 1: Volumes used in cDNA synthesis

Materials	**Volume**
10X RT Buffer	2 µL
25X dNTP	0.8µL
10 X RT random primer	2.0 µL
Multiscribe reverse transcriptase	1.0 µL
Template RNA	1µg/mL
Nuclease free water	Dilute the contents to 20 µL

Appendix III: List of materials and concentration used in Western blot

Table 2: Concentration of materials used in Western blot

Materials	Concentration
Running buffer	
MOPS buffer	20X diluted to 1X with nanopure H_2O
Transfer Buffer	
Methanol	20%
Transfer buffer	Diluted to 1X from 50X with 0.1% antioxidant with nanopure H_2O
Tris buffer saline (TBS)	
Tris HCL	3.14 g
Sodium Chloride	9 g
	Diluted in 1L of nanopure H_2O (at a pH of 7.4 using NaOH)
Tris buffer saline-Tween 20 (TBST)-Wash buffer	Same as above with addition of 0.5% Tween-20 after appropriate pH is reached
Blocking buffer	
Casein	2% diluted in TBS
Antibody Buffer	
Casein	1% diluted in TBS

Appendix IV: List of materials and concentration used in gel electrophoresis

Table 3: Concentration of materials used for gel electrophoresis

Materials	**Concentration**
<u>Preparation of TAE buffer</u> Tris base Glacial acetic acid Molecular grade EDTA	 40 mM 20 mM 0.5 M EDTA
Molecular grade gel	1.5% in TAE buffer
Loading dye	1:6 dilution

Appendix V: Sequences observed in the Conditioned medium samples

Based on reports of possible amino acids present in antimicrobial peptides a set of sequences have been identified from the various samples submitted to Proteomics International Pty Ltd. They have not shown a peptide match to any known protein or peptide, but may fit in the different parameters that assess the AMPs, such as presence of amino acids- lysine, cysteine, arginine, phenyl alanine, valine and proline.

Table 4: Sequences obtained from Proteomics International Pty Ltd

Mass	Sequence
721.67	AQESSEKPPEKPVKPERVK
422	SSSLEETIASLK
636.62	IDSEAHEKRPPILTSSK
461.92	SPGGASSITGFGSMK
731.35	MELMGCDLNSCSMPLGMESK
699.38	LWGTWVKAPLAR
633.35	FGSSASLISGLR
510.90	SRNPGSSCIGADPNR
616.78	SGGGGGGGLGSGGSIR
583.27	VLVASPKQAPR
560.31	TAAGWAGWGSR
791.33	VAHMMPDILLALTDDMGRTQK
725.67	QAPGQGLEWMGGIIPMFGTGK
791.41	AVGVKLSSFSVVGESLLYMLEK
735.34	ALCLLGADHADTGVSQDPRHK
765.34	PFDVDDAKVLLGLK
465.22	VGGGRAEAALLLAPR
765.34	APPSVPRLHCSTHK
455.11	KVWVPMKPYYTQVYQEIWVGVGLMS

Appendix VI: Dot-blot assay of HBD-1

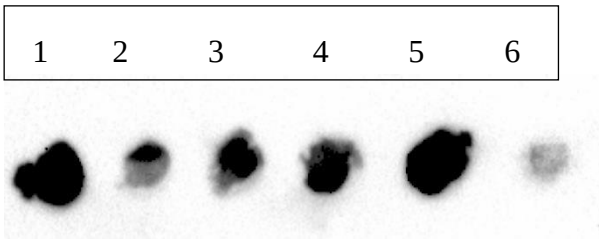

Figure 2: Dot bot preliminary screening of antibody used for HBD-1.

The samples were screened for determining the concentration used for HBD-1 antibody. 1- Caco-2 in growth medium with 10% FCS. 2- Conditioned medium of Caco-2 in growth medium with 10% FCS. 3- Caco-2 in growth medium with 2% FCS. 4- Conditioned medium of Caco-2 in growth medium with 2% FCS.5- Conditioned medium of Caco-2 incubated with LPS in 2% FCS. 6- Conditioned medium of Caco-2 incubated with PSC-833.

Appendix VII: Elaboration of Method used in HPLC for detection of Aβ-42

The gradient methods used for detection of Aβ-42 are as follows:

(A)

Time (in minutes)	Mobile phase	Percentage
0-5	Acetonitrile	5%
5-30	Acetonitrile	35%
30-35	Acetonitrile	5%
35-37	Acetonitrile	5%

(B)

Time (in minutes)	Mobile phase	Percentage
0-5	Acetonitrile	5%
5-25	Acetonitrile	40%
25-30	Acetonitrile	5%
30-32	Acetonitrile	5%

Appendix VIII: Preparation of MTT solution

MTT reagent was prepared according to manufacturer's protocol. The reagent was always protected from sunlight.

Briefly, the reconstituted MTT reagent was prepared in sterile PBS (pH 7.4) to make a stock concentration of 5 mg/mL. They were then filter sterilised using 0.2 µm syringe tip filter and stored in sterile containers wrapped in aluminium foil to protect against sunlight. They were stored in either 4ºC for short term storage or -20ºC for long term use.

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