The Association between *Fusobacterium nucleatum* Outer Membrane Vesicles and Colonic Cancer

A thesis submitted for the degree of

Doctor of Philosophy

By

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Declaration

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Dedication

To King Abdullah Bin Abdulaziz Al-Saud (may Allah have mercy on him),

To my father, Mohammed Munshi (may Allah have mercy on him),

To my brother, Rami Munshi (may Allah have mercy on him),

And to my beloved wife Mashael Khayyat for her support and patience.

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Special thanks to special people who helped me to complete this degree:

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I will say as Irish people say “Thanks a million” or “go raibh maith agat”!

Abstract

Fusobacterium nucleatum, a gram-negative spindle-shaped anaerobic bacterium, is a component of the human microbiome that primarily inhabits the oral cavity and is associated with many diseases, including sinusitis, tonsillitis, urinary tract infection, and inflammatory bowel disease. Recent research demonstrates an association between the presence of this bacterium and colorectal carcinoma.

This study aimed to investigate the ability of this bacterium and its outer membrane vesicles (OMV) to modulate colonic cell function. Like other gram-negative bacteria, *F. nucleatum* secretes OMV, but little is known about how they interact with host tissue. To investigate the potential role of these structures in the host-pathogen interaction the OMV were purified, proteomically characterised and their interaction with colonic cells investigated. In this regard, the mass spectrometry-based proteomic analysis revealed the presence of 366 proteins including several biologically active proteases which appeared to be selectively enriched in the OMV. In addition, many other proteins with documented or likely roles in *F. nucleatum*-mediated pathogenesis were identified.

The ability of the *F. nucleatum* OMV to modulate several colonic epithelial cellular behaviours was investigated and compared with the intact parental bacterium. The OMV induced a potent pro-inflammatory response in colonic epithelial cells, stimulating the expression and secretion of pro-inflammatory cytokines and chemokines. OMV also impaired the barrier function of model colonic epithelial monolayers, in part by degrading the tight junctional protein E-cadherin and down regulating the expression of *CDH-1*. This process was accompanied by cadherin switching and the induction of a mesenchymal phenotype in the colonic cells as judged prominent morphological changes, significantly increased cellular proliferation and migration and the appearance of several markers associated with EMT, including increased nuclear translocation of the pivotal EMT transcription factor, ZEB-1.

Taken together, these findings and the observation that the OMV enhanced colonic resistance to the chemotherapeutic agent 5-FU, support the hypothesis that these

nanostructures likely make a significant contribution to the pathogenesis of gastrointestinal disease in susceptible individuals.

Abbreviations

%	Percent
$\Delta\Delta C_q$	Relative Copy Numbers
5-Fu	5-Flourouracil
Akt	Protein Kinase B
ANOVA	Analysis of Variance
APC	Adenomatous Polyposis Coli
BHI	Brain Heart Infusion Broth
BMP	Bone Morphogenetic Protein
BSA	Bovine Serum Albumin
CAC	Colitis Associated Colon Cancer
CagA	Cytotoxin-Associated Gene A
CBA	Columbia Blood Agar
CCR	Chemokine Receptor
CD	Cluster of Designation
CDH-1	E-Cadherin
CDH-2	N-Cadherin
cDNA	Complementary DNA
CFU	Colony Forming Units
CIMP	Cpg Island Methylator Phenotype
COX-2	Cyclooxygenase-2
CRC	Colorectal Cancer
CXCL	Chemokine (C-X-C Motif) Ligand
DCU	Dublin City University
ddH_2O	Distilled Deionised Water
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic Acid
ECL	Enhanced Chemiluminescence
ECM	Extracellular Matrix
ELISA	Enzyme-Linked Immunosorbent Assay
EMEM	Eagle's Minimum Essential Medium
EMT	Epithelial Mesenchymal Transition
ERK	Extracellular Signal-Regulated Kinase
ETBF	Enterotoxigenic *B. Fragilis*
ETEC	Enterotoxigenic *E. Coli*
ExPEC	Extraintestinal *E. Coli*
Fad-I	*Fusobacterium*-Associated Defensin Inducer
FadA	*Fusobacterium* Adhesin A
FCS	Foetal Calf Serum
FN	Fibronectin

FomA	*Fusobacterium* Outer Membrane Protein A
FPLC	Fast Protein Liquid Chromatography
FSP1	Fibroblast-Specific Protein-1
g	Gram
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
GIT	Gastrointestinal Tract
GM-TriDAP	Diaminopimelate Tripeptide
GTPase	Guanosine Triphosphate Hydrolase Enzyme
HCS	High Content Screening
h	hour(s)
HUS	Haemolytic Uremic Syndrome
I-OMVs	Inner-Outer Membrane Vesicles
IBD	Inflammatory Bowel Disease
IEC	Ion-Exchange Chromatography
IFN	Interferon
IgA	Immunoglobulin A
IL-1	Interleukin-1
IL-1R	Interleukin-1 Receptor
IL-6	Interleukin-6
IL-8	Interleukins 8
ITGA-5	Integrin-A5
ITIM	Immunodominant Tyrosine-Based Inhibitory Motifs
KB cells	Hela Epithelial Cells
kDa	Kilo Dalton
KRAS or k-ras	Kirsten Rat Sarcoma
LC-MS	Liquid Chromatography–Mass Spectrometry
LEF	Lymphoid Enhancer Binding Factors
Lpp	Braun's Lipoprotein
LPS	Lipopolysaccharide
LT	Heat-Labile Enterotoxin
M	Mole
MAPK	Mitogen-Activated Protein Kinase
mg	Milligram
Min.	Minute(s)
MIP3-α or CCL-20	Macrophage Inflammatory Protein-3
ml	Millilitre
MLH-1	MutL α
mM	Millimole
MMP	Matrix Metalloproteinases
MMR	Mismatch Repair Protein
mRNA	Messenger RNA
MS	Mass Spectrometry

MSH	MutS Protein Homolog
MSI	Microsatellite Instability
MTS	Tetrazolium
NapA	Neutrophil-Activating Protein A
NF-κβ	Nuclear Factor-κβ
NK	Natural Killer Cell
NLR	NOD-Like Receptors
nm	Nanometre
NOD	Nucleotide-Binding Oligomerization Domain
NOD-like receptor	Nucleotide-Binding Oligomerization Domain-Like Receptors
O.D.	Optical Density
^{o}C	Degree Celsius
OmpA	Outer Membrane Protein A
OMV	Outer Membrane Vesicle
PADs	Peptidylarginine Deiminases
PAMPs	Pathogen-Associated Molecular Patterns
PBS	Phosphate-Buffered Saline
PFA	Paraformaldehyde
pH	Potential of Hydrogen
PI3K	Phosphoinositide 3-Kinase
pks	Polyketide-Peptide Genotoxin
PMS	Phenazine Methosulfate
PRRs	Pattern Recognition Receptors
pSORTb	Subcellular Localization Prediction Tool
PTGS-2	Prostaglandin-Endoperoxide Synthase-2
PVDF	Polyvinylidene Difluoride Membrane
RAC1 (gene)	Code for Ras-Related C3 Botulinum Toxin Substrate 1 Protein
RhoA	Ras Homolog Family Member A
RNA	Ribonucleic Acid
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
RPMI	Roswell Park Memorial Institute Medium
RT-PCR	Real Time Polymerase Chain Reaction
S	Seconds
SCFAs	Short Chain Fatty Acids
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SOCS-3	Suppressor of Cytokine Signalling-3
SPHK-1	Sphingosine Kinase-1
STAT	Signal Transduction and Activators of Transcription
STEC	Shiga Toxin Producing *E. Coli*
TCA	Trichloroacetic Acid

TEM	Transmission Electron Microscopy
TEMED	Tetramethylethylenediamine
TGF-β	Transforming Growth Factor Beta
TIGIT	T-Cell Immunoglobulin and ITIM Domain
TJ	Tight Junctions
TLRs	Toll-Like Receptors
TNF-α	Tumour Necrosis Factor Alpha
TP53	Tumour Protein P53
Tregs	T-Regulatory Lymphocytes
Tris	Trisaminomethane
U/ml	Unit Per Millilitre
UCC	University College Cork
v/v	Volume/Volume
w/v	Weight/Volume
WHO	World Health Organization
xg	Gravity Force Unit (The Standard Acceleration Due to Gravity)
ZEB-1	Zinc Finger E-Box Binding Homeobox 1
ZO	Zonula Occludens
β-catenin	β-Catenin
μg	Microgram

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CHAPTER ONE

Introduction and Background

1.1 Introduction

Microbial cells are found everywhere in the human body and human biology studies no longer concern themselves only with human cells (Gill et al., 2006). There are more than 1000 bacterial phylotypes in the human intestine that have been estimated at over 10^{14} microbes (Ley et al., 2006). Various factors contribute to the overall intestinal bacterial composition including environment, genetics, hygiene, and diet (Keku et al., 2015). The amount of bacteria and their diversity is commonly regulated by the location and the biology of different body habitats. The *Bacteroidetes, Firmicutes*, *Actinobacteria*, and *Proteobacteria* form the main phyla that are associated with the human body (Costello et al., 2009). There is a similar pattern in bacterial composition at individual sites, such as the intestine among individuals within a population than there are amongst sites within the same person. This suggests the existence of possible co-evolved beneficial functions among microorganisms in different body regions. Bacteria within the human body are malleable in nature, and their composition is highly affected by diet, hygiene practices (Pflughoeft and Versalovic, 2012), host genetics, early microbial exposure and antibiotic intake. Much of this diversity remains unexplained although ongoing studies clearly demonstrate an association between various human diseases and microbial dysbiosis.

1.2 Cancer and infection

In humans, cancer is considered as the leading cause of death worldwide with approximately 8.2 million deaths and 14.1 million new cases diagnosed in 2012 (Ferlay et al., 2015). Since the 1890s many studies have indicated the presence of bacteria in cancerous tissue (Russell, 1890). Yet, it remains unclear whether bacterial accumulation in tumours indicates a causal factor or whether they accumulate to exploit the high metabolic

activity and vascularization of cancer cells. The best example for bacterial association with inflammation-induced cancer is *Helicobacter pylori*, identified by the World Health Organization (WHO) as class I human carcinogen (Peek and Blaser, 2002). *H. pylori* infection is a potent risk factor for gastric adenocarcinoma (Polk and Peek, 2010). The chronic gastric inflammation caused by *H. pylori* leads to elevated β-catenin signalling in epithelial cells that increases the risk of malignant transformation (Franco et al., 2005, Bagheri et al., 2017). Although the previous example provides evidence for the association of pathogenic infection in the development of cancer, it has been found that some commensal bacteria could be involved in tumour-promoting inflammation (Tlaskalova-Hogenova et al., 2004, Goc et al., 2016). Other pathogens are associated with various cancers including human papillomavirus, a causative agent of cervical cancer (Crosbie et al., 2013). Hepatitis B and C also drive progression to liver cancer (Parkin, 2006). Trillions of bacteria colonize the human body, and they are essential for human physiology under homeostatic conditions (Huttenhower and Gevers, 2012) but are also associated with the inflammatory process in many pathological conditions (Gordon, 2012). Microbial dysbiosis is the term that refers to imbalances and perturbation in the microbiome, which has been linked to many diseases such as inflammatory bowel disease (IBD) (Hold et al., 2014), CRC (Keku et al., 2015, Dulal and Keku, 2014), and obesity (Brown et al., 2012). It is believed that infections are responsible for 18 % of cancers (Parkin, 2006). In spite of the fact that the precise mechanisms of carcinogenesis remain ambiguous, it is logical to minimise the risk of these cancers by identifying the pathogens.

Emerging data, largely due to next generation sequencing projects, have expanded our knowledge by generating enormous new data sets. These data sets of microbial genes and the pathways associated with them are revealing how they contribute to different tissue and organ function, which can be linked to many diseases and cancers (Pflughoeft and Versalovic, 2012), including CRC. However, it is not clear if these commensal bacteria contribute to tumour growth in the same manner as the inflammation induced by pathologic bacterial infection.

Many factors participate in cancer formation and development such as inflammation, cell proliferation, signalling, genomic destabilization, and induction of metastasis and invasion.

1.2.1 Inflammation promotes cancer formation

One of the earliest studies by Virchow (1881) made the connection between tumour growth and inflammation and now chronic inflammation is believed to be the key factor in cancer development (Medzhitov, 2008). For example, signal transducer and activator of transcription-3 (STAT-3) has been linked to tumorigenesis in many tissues and it is associated with inflammation in liver, gastric, colon (Grivennikov et al., 2009), pancreatic (Lesina et al., 2011) and lung cancers (Gao et al., 2007). In CRC for example, the production of IL-6 is essentially involved in the activation of STAT-3 (Waldner et al., 2012) which inhibits apoptosis and induces pre-malignant cell proliferation and cancer progression (Waldner et al., 2012, Bollrath et al., 2009). At the same time, IL-6 is also required for the activation of NF-κβ (Park et al., 2010a). In most tumours NF-κβ activation is induced by oncogenic mutations and inflammatory stimuli, which is associated with proliferation, pro-survival gene expression, and pro-inflammatory cytokines production (Ben-Neriah and Karin, 2011, Karin and Greten, 2005). This suggests a connection between inflammation and cancers and indicates the important role of the tissue microenvironment as a source of pro-inflammatory cytokines (Pikarsky et al., 2004).

Interestingly, RAS signalling activation through IL-1, IL-1R and MYD88 resulted in the activation of NF-κβ (Cataisson et al., 2012). This could possibly link NF-κβ to tumour development during inflammation and could provide support to the involvement of toll-like receptors-2 (TLR-2) in the activation of STAT-3 in gastric tumorigenesis (Tye et al., 2012). During the initiation of neoplasia, tissue damage elicits pro-inflammatory cytokine production that induce epithelial survival and proliferation (Sonnenberg et al., 2011). Intestinal tumorigenesis is associated with Wnt activation, which links to the elevated level of NF-κβ signalling and enhances Wnt-β-catenin activation that enhances tumour initiation (Schwitalla et al., 2013). Also, loss of APC anti-tumour activity induces GTPase RAC1 activation, which results in the elevation of ROS production and NF-κβ activation, which initiates CRC and drives intestinal stem cell proliferation (Myant et al., 2013).

1.2.2 Genomic destabilization

Increased genomic instability is an important driving force of cancer. During DNA replication, mismatched DNA base pairs are removed by DNA mismatch repair (MMR), and 15-20 % of sporadic CRC have a defect in the MMR system (Li and Martin, 2016). Inflammation could also be involved in initiating the neoplastic transformation by compromising DNA repair and increasing the DNA damage rate resulting in increased genomic instability (Mantovani et al., 2008). Somatic mutation could be initiated as a consequence of inflammation, which functions either directly or indirectly on epithelial cells. Intercellular reactive oxygen species (ROS) and reactive nitrogen species (RNS) accumulation in the presence of inflammatory cytokines or they release from neutrophils and macrophages (Wiseman and Halliwell, 1996) could cause DNA breaks or single base mutations that enhance the formation of somatic mutation (Mills et al., 2003). Inflammation has been linked to mutation formation in cancer-associated genes including TP53 and MYC thereby promoting oncogenesis (Endo et al., 2008, Komori et al., 2008). Also, inflammation can promote intestinal tumorigenesis by altering the composition of the intestinal microbiome and induce an expansion of genotoxic bacteria (Arthur et al., 2012). It has been found that accumulation of random genetic alterations can be associated with inflammation in which it could increase cellular genomic destabilization and increase susceptibility to mutagenesis (Colotta et al., 2009, Singh et al., 2007). As a consequence, cell cycle checkpoint disruption could occur with down-regulation of DNA repair resulting in random genomic alteration (Campregher et al., 2008).

In this regard, inflammation enhances microsatellite instability (MSI) and DNA replication errors via repressing MMR, which plays a crucial role in preventing genetic instability (Colotta et al., 2009). Many inflammatory mediators including IL-1 β, TNF-α, prostaglandin E2 and ROS participate in the induction of hypoxia-inducible factor 1α, which down-regulates MMR gene expression (MSH-2, and MSH-6) (Schetter et al., 2010).

1.2.3 Induction of metastasis and invasion

Epithelial mesenchymal transition (EMT) is a stage that precedes cancer formation, in which epithelial cells lose polarization and acquire mesenchymal cell characteristics such as increased invasiveness to basal membranes and migratory capacity (Kalluri and Weinberg, 2009). EMT suppresses E-cadherin expression via the activation of STAT-3 and NF-κβ via IL-6, and TNF-α (Sullivan et al., 2009, Wu et al., 2009b). Also, EMT is associated with an increase in the production and activity of matrix metalloproteinases (MMPs) (Radisky and Radisky, 2010). Inflammation stimulates this process by increasing MMP-mediated tissue remodelling and enhanced tissue vascular permeability by TNF-α induction (Nguyen et al., 2009). In addition, inflammation enhances cell survival and upregulation of adhesion molecules in response to pro-inflammatory cytokines (McDonald et al., 2009, Mantovani et al., 2008).

1.3 CRC

CRC is one of the fourth most common cancers resulting in over 600,000 deaths annually and 1.2 million new diagnoses. CRC is the fourth most common cancer worldwide (Castellarin et al., 2012). According to National Cancer Registry of Ireland, the most common cancers in Ireland are non-melanoma skin cancer, prostate cancer, breast cancer, CRC, and lung cancer [1]. Commonly, CRC arises from precancerous polyps, which can progress to cancer if left untreated over approximately a 10 year period (Konishi and Morson, 1982). A study conducted in Tallaght Hospital, UCC, DCU, and Newcastle University found that patients commonly decided not to take a part in screening tests either due to emotional burden or poor background knowledge about the cancer (Clarke et al., 2014, Clarke et al., 2016). Proteomic analysis identified differentially expressed proteins and pathways perturbed between non-tumour and tumour tissues in CRC (Sethi et al., 2015). This finding is consistent with upregulation of extracellular matrix glycoproteins and down-

[1] http://www.ncri.ie, accessed on 08.03.16.

regulation of adhesin proteins (cadherin) which is observed in CRC (Zhang et al., 2014a). There are many factors associated with CRC etiology including genetic factors, internal environment factors such as immunological derangement, intestinal environment, and the activation of tumour-related signalling pathways and external environmental factors such as smoking, dietary structure, drinking, and other unhealthy lifestyle. Furthermore, research links CRC with the intestinal microbiota (Zhu et al., 2013, Wu et al., 2013a, Wang et al., 2012).

Sequential accumulation of epigenetic alterations and somatic mutations contribute to CRC (Mundade et al., 2014). Also, tumorigenic transformation of normal colonic epithelium that results from inactivation of tumour suppressor genes like adenomatous polyposis coli (APC), p53 (TP53), and the activation of the oncogene K-ras (Mundade et al., 2014, Fearon, 2011). Additionally, alteration in bacterial diversity (microbial dysbiosis) is also associated with CRC development (Keku et al., 2015). However, it remains unclear how a particular bacterial species contributes to CRC development despite various longitudinal studies across different cancer stages (Nugent et al., 2014), and comparing microbial communities in the human intestine over time (Rodriguez et al., 2015).

1.3.1 Host cell immunity, microbiota, and CRC

Pattern recognition receptors (PRRs) are part of innate immune response of the host that includes TLRs, NOD-like receptors (NLR), helicase receptors and C-type lectin receptors (Davis et al., 2014). PRRs are responsible for the detection of tissue damage and the presence of bacteria and play an important role in microbial and host homeostasis (Davis et al., 2014). Also, it has been reported that NOD-2 is associated with increased risk of GI cancer and increase in NOD-1 and NOD-2 correlated with CRC (Liu et al., 2014b, Liu et al., 2015b). In contrast, deletion of NOD-1 or NOD-2 exacerbates colitis associated colon cancer (CAC) suggesting that they exert a protective function against carcinogenesis (Chen et al., 2008). The exact function of NOD-2 is unclear, but murine models ($\mathrm{NOD2}^{-/-}$) show an increase in CAC formation which is linked to dysbiosis (Couturier-Maillard et al., 2013).

It is becoming increasingly clear that the human gut microbiota contributes to the overall health of the host primarily via homeostatic means. The intestinal microbiota is now recognized as an important contributor to human diseases such as CRC, IBDs, and obesity. The mechanisms by which bacteria contribute to many disease states including CRC are thought to be complex and are poorly understood, with many of the current studies being observational rather than identifying the causal attributes and disease associations. Associations between bacterial metabolites (e.g. short chain fatty acids (SCFAs), bile acids) and various human diseases are also under investigation (Ohtani, 2015) as are the contributions of specific species of bacteria to cancer. **Table 1.1** summarizes much of this latter information.

Table 1.1: Pathogenic (tumour promoting) GI bacteria.

Bacterium	Disease association
Enterotoxigenic B. fragilis	CRC
Escherichia coli	CRC
Fusobacterium spp.	CRC
Clostridium concisus	IBD/Barrett's oesophagus/OAC
H. pylori	Gastric MALT lymphoma
H. pylori	Gastric cancer
Borrelia burgdorferi	Skin MALT lymphoma
Campylobacter jejuni	IPSID
Salmonella enterica	Gall bladder cancer
Enterococcus faecalis	CRC

Ultimately, Koch's postulates must be fulfilled to establish disease causality and from there the molecular Koch's postulates as identified by Falkow (1988). Indeed, the complex task of establishing the causal link between pathogen and disease in the case of intestinal disease will likely prove considerably more complex than, say, similar studies defining the causal relationship between *H. pylori* and gastric cancer, largely due to the vastly more complex microbiota, which inhabits the intestinal tract compared with the stomach. Yet, studies are now beginning to provide robust data indicating significant associations between specific species of bacteria and different disease states including cancer and to propose mechanisms whereby this may arise (Schwabe and Jobin, 2013) see **Figure 1.1.**

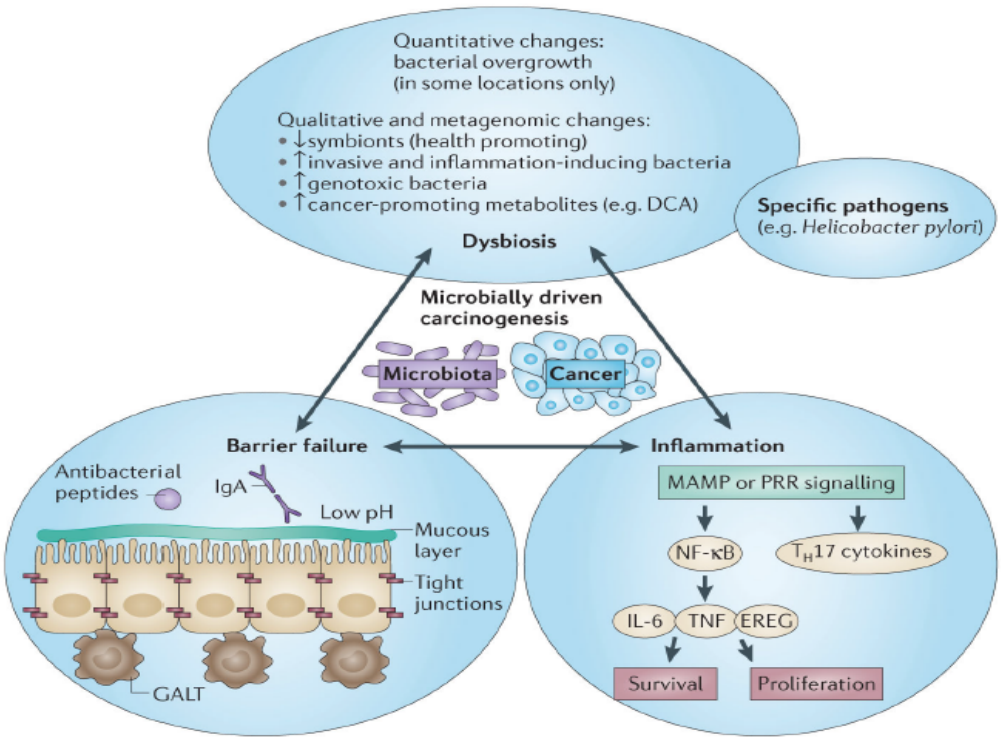

Figure 1.1: Mechanisms controlling host-microbiota interactions and associated failures implicated in cancer development. Microbial dysbiosis is now frequently found as a potential contributor to disorders of the GIT including IBD and CRC and suggestions or indicators for this contributing to disease progression include intestinal epithelial barrier failure, chronic inflammation and bacterial genotoxicity. From Schwabe and Jobin (2013).

1.3.2 Tumorigenesis, microbial dysbiosis and CRC

CRC starts as a genomic alteration (APC mutation) in which Wnt/β-catenin activation is required for the colorectal tumour maintenance (Scholer-Dahirel et al., 2011). Alteration in APC/Wnt signalling pathway causes uncontrolled cell proliferation that leads to adenoma formation and ultimately, invasive carcinoma that could result from additional driver mutations (Willett et al., 2013, Vogelstein et al., 2013). It was believed that the majority of CRC were sporadic in nature rather than being due to inheritance (Lichtenstein et al., 2000). For a long time, most research was focused on environmental risk factors. However, recently attention has been drawn toward microbes colonizing the gut as potential cancer-promoting factors. There are many bacterial species and tumour-promoting virulence factors that have been investigated and could be linked to CRC. The relationship between cancer and the microbiota could be bidirectional since cancer could influence

environmental changes that have a great impact on bacterial composition, which may further influence carcinogenesis. Using germ free model studies along with other cancer models including gut, breast, lung, and immune system have contributed to expanding research prospective concerning the role of microbiota in tumour development, allowing the study of the effect of single bacterial species and communities of species (Schwabe and Jobin, 2013).

Approximately 18 % of all human tumours are estimated to be linked to infection and many epidemiological studies support a link between bacterial infection and cancer incidence (Gagnaire et al., 2017). Yet, a causal relationship has not been clarified. Various bacteria with oncogenic properties have been found to be linked to CRC (Gagnaire et al., 2017) including *Fusobacterium nucleatum (F. nucleatum)* (Kostic et al., 2013b, Allen-Vercoe and Jobin, 2014), *E. coli* (Arthur et al., 2012, Allen-Vercoe and Jobin, 2014), *Bacteroides fragilis* (Ulger Toprak et al., 2006, Wei et al., 2016) and *E. faecalis* (Amarnani and Rapose, 2017), and *Streptococcus bovis* (Waisberg et al., 2002) (**Table 1.2**).

Table 1.2: Bacteria with oncogenic properties associated with CRC.

Bacteria	Associated cancer	Proposed mechanisms		Evidence	
		Direct	indirect	Experimental evidence	Epidemiology
F. nucleatum	CRC	β-Catenin-mediated activation of inflammatory and mitogenic transcriptional programmes.	Immunomodulation.	Promotes tumours in $APC^{min/+}$ mice, recruits in tumour-infiltrating myeloid-derived suppressor cells to tumours	Epidemiological studies
B. fragilis	CRC	β-Catenin activation, MAPK signalling activation	Induction of ROS and RNS, which cause DNA damage	Promotes tumours in $APC^{min/+}$ mice, reduces colon tumorigenesis in $APC^{min/+}$ mice after antibiotic treatment	Epidemiological studies

E. faecalis	CRC	Unknown	Production of ROS, bystander effect through activated macrophages	*IL-10*$^{-/-}$ mice exposed to *E. faecalis* are prone to tumours	Epidemiological studies
S. bovis	CRC	Unknown	Inflammation	None	Epidemiological studies
E. coli	CRC	Colibactin toxin causes DNA damage	Unknown	IL-10$^{-/-}$ mice treated with AOM and exposed to *E. coli* pks^{+} are prone to tumours	Epidemiological studies

Taken from Gagnaire et al. (2017) p. (2-3).

For many decades, *S. bovis* (Waisberg et al., 2002), and *Clostridium septicum* (Seder et al., 2009) were described as the most prominent bacteria associated with CRC. However, they are only seen in less than 1 % of cases. An increase in the exposure to *S. bovis* antigens correlates with the early stages of CRC even in the absence of bacterial infection (Tjalsma et al., 2006). Active immune system can repress the opportunistic infection, which give the advantage to other competing gut bacteria in the CRC microenvironment (Marchesi et al., 2011). Also, high abundance of *Enterobacteriaceae* has been observed in CRC patients and in CRC mouse model (Yang and Jobin, 2014). It has been suggested that specific bacterial candidates could be involved in CRC and include *F. nucleatum*, *enterotoxigenic B. fragilis* (ETBF), *E. coli* that have been connected to the carcinogenic processes at various stages including production of specific microbial toxins and genotoxins (Garrett, 2015, Arthur et al., 2012, Buc et al., 2013), suggesting that single bacterial species could link to neoplastic growth. Three studies demonstrated prevalence of the anaerobe *F. nucleatum* in colorectal carcinoma (Kostic et al., 2012, Castellarin et al., 2012, Strauss et al., 2011). The introduction of ETBF in $\mathrm{APC}^{\Delta 716}$ murine model stimulates mucosal T-regulatory lymphocytes (Tregs) that associated with the initiation of cancer through IL-17 α production (Geis et al., 2015). *F. nucleatum* in contrast to *E. coli* and ETBF is not considered as pro-inflammatory in mice (Kostic et al., 2013b). Nevertheless, this microorganism exerts a great impact on host immune responses, and plays an important role in carcinogenesis (Kostic et al., 2013b). *F. nucleatum* has been reported to able to interfere with immunity via the binding to human inhibitory receptor T-cell immunoglobulin and ITIM domain (TIGIT) through its surface

protein Fap2 that causes inhibition of T-cell activity and natural killer cell cytotoxicity (Gur et al., 2015). It is believed that *F. nucleatum* is capable of avoiding the immune response by reducing $CD3^+$ T-cells, one of the hallmarks of cancer (Mima et al., 2015, Hanahan and Weinberg, 2011).

One main characteristics of oncogenic bacteria is their ability to induce DNA damage and ability to manipulate host cellular pathways directly linked to cancer development and progression (Gagnaire et al., 2017). For example, *E. coli* is able to induce DNA damage in host cells via the secretion of colibactin toxin (Cuevas-Ramos et al., 2010) and cytolethal distending toxin (Cortes-Bratti et al., 2000). As a consequence, gene mutation and chromosome instability rates are increased and the infected cells are stimulated to release growth factors that induce cell proliferation and are involved in CRC metastasis and progression (Cougnoux et al., 2014). Also, *B. fragilis* produces genotoxins that can induce DNA damage in host cells (Goodwin et al., 2011) via the induction of chronic inflammation and ROS elevation. The induction of genomic errors participates in the formation of tumours and cytolethal distending toxin provokes immune responses and induces pro-inflammatory cytokine production, all of which enhance cell proliferation and contribute to tumour formation (Ge et al., 2007).

E. coli downregulates MMR in intestinal epithelial cells (Maddocks et al., 2013) via EspF effect and deregulate various cellular process including cellular junction integrity, mitochondrial function, and phagocytosis (Holmes et al., 2010). This results in the depletion of MMR proteins (MSH-2 and MLH-1) that enhance the formation of spontaneous mutations at the site of MSI, which is a characteristic of CRC (Maddocks et al., 2013). Of note, *H. pylori* also downregulates the expression of MMR proteins in gastric cancer cells (Machado et al., 2009). Similar to *E. coli*, *H. pylori* infection enhances the frequencies of MSI in gastric cancer cells. The exact mechanisms of this phenomena remain elusive, but it is believed to be independent of the CagA virulence factor (Machado et al., 2009).

MMR activates p53, which induces apoptosis and inhibition of MMR promotes cell survival by the bacteria, which have mutated DNA that contribute in cancer progression and development (Gagnaire et al., 2017). Various studies indicated a link between *F. nucleatum* overabundance in CRC tissues and MSI-high, CpG island methylator phenotype (CIMP-

high), and MLH-1 methylation (Mima et al., 2015, Tahara et al., 2014). Another study similarly showed that *F. nucleatum* overexpression in CRC is significantly connected to MSI-high, but not associated with cancer-specific survival (Nosho et al., 2016). However, the exact mechanisms that are involved *F. nucleatum*-induced colorectal carcinogenesis remains ambiguous (Nosho et al., 2016).

F. nucleatum FadA is capable of binding to E-cadherin that results in the phosphorylation and internalization of E-cadherin (Rubinstein et al., 2013). E-cadherin belongs to the cadherin superfamily which possesses a potent malignancy suppressing activity, and inactivation of E-cadherin is associated with tumour formation and development (Berx and van Roy, 2009). As a consequence, β-catenin is released and accumulates in the cytoplasm and then translocates into the nucleus and Wnt signalling is activated (Rubinstein et al., 2013). Increased FadA is associated with adenomas and adenocarcinomas correlates with elevated expression of inflammatory and oncogenic gene and may contribute to cancer development (Rubinstein et al., 2013, Repass et al., 2016). Whereas ETBF secretes a metalloproteinase toxin (*B. fragilis* toxin) that interacts with epithelial cells and cleaves E-cadherin that release β-catenin (Wu et al., 2007), which translocates into the nucleus to regulate transcription and activate oncogenic genes (Wu et al., 2003).

Additionally, oncogenic bacteria can indirectly contribute to cancer formation via the induction of inflammation and oxidative stress (Gagnaire et al., 2017). As in the case of *B. fragilis* infection that is associated with inflammation and plays an essential role in the pathophysiology of the microorganisms, *B. fragilis* is able to induce colitis and tumours that is associated with inflammatory infiltrates in APC-deficient mice (Wu et al., 2009a). Also, *B. fragilis* induces STAT-3 activation, which is associated with colitis formation and CRC (Wu et al., 2009a). *B. fragilis* toxin is involved in the activation of the RAS-p38 MAPK-AP-1 pathway, which results in the induction of IL-8 and monocyte chemotactic proteins-1 by colonic cells (Kim et al., 2005). Also, *B. fragilis* toxin inhibits apoptosis, which is also associated with cancer development and progression (Kim et al., 2008).

Moreover, bacteria can effect cancer development and induce genomic mutation via the induction of oxidative stress that modulates immune responses and generates chronic

inflammation (Gagnaire et al., 2017). *B. fragilis* and *H. pylori* are able to activate spermine oxidase in infected cells, which is involved in polyamine catabolism and triggers the production of hydrogen peroxide, which enhances the production of ROS that induce DNA damage (Chaturvedi et al., 2011, Goodwin et al., 2011). Also, *E. faecalis* is associated with the induction of extracellular superoxide and derivative oxygen species including hydroxyl radical or hydrogen peroxide (Wang and Huycke, 2007). This promotes chromosomal instability (Wang and Huycke, 2007) and plays an essential role in development of CRC (Wang et al., 2015b). *E. faecalis* induces DNA damage, chromosomal instability and also induces chronic inflammation associated with increased TNF-α and COX-2 (Yang et al., 2013). It has been found that *F. nucleatum* induces an increase in the production of inflammatory cytokines and reactive oxygen species (ROS) in CRC (Kostic et al., 2013b). ROS and inflammation can result in epigenetic silencing of the mismatch repair protein-1 (MLH-1) by reducing the enzymatic activity of MMR proteins, which is associated with MSI formation (Schetter et al., 2010, Maddocks et al., 2013).

1.4 *F. nucleatum*

F. nucleatum is an opportunistic pathogen, involved in many forms of periodontal lesions and diseases and external infections, as well as CRC (Allen-Vercoe et al., 2011). It is able to aggregate with other bacteria, and it induces tissue irritation (Roberts, 1999). *F. nucleatum* is the type species of the genus *Fusobacterium* that belongs to the *Bacteroidaceae* family. *F. nucleatum* is a gram-negative rod with pointed ends, is non-spore forming, and non-motile (absence of pili and flagella) (Bolstad et al., 1996). *F. nucleatum* is an anaerobe but can survive 6 % oxygen and is able to exhibit a high degree of aerotolerance. It ranges in size from 5 to 10 μm, and colonial morphology is not sufficient for species identification (Tunér et al., 1992). Furthermore, *Fusobacterium* species can be differentiated from other anaerobic, gram negative, non-spore forming rods by their lipid composition together with the ability to produce butyric acid (butanoic acids) as a major product of the fermentation of glucose and peptone (Bolstad et al., 1996). *F. nucleatum* can be divided into five subspecies, including *nucleatum*, *polymorphum*, *vincentii*, *fusiforme*, and *animalis*. Recently, *Fusobacterium* subspecies *vincentii* and *fusiform* have been classified into a single

subspecies, *F. nucleatum* subspecies *fusiforme/vincentii* (Nie et al., 2015). However, classification is difficult due to the heterogeneous nature of the strains, including differences in antigenic determinants and cellular/colonial morphology (Citron, 2002, Allen-Vercoe et al., 2011).

1.4.1 Pathogenesis

Many virulence factors are associated with *F. nucleatum* pathogenesis such as FadA. FadA is an adhesin protein, which is linked to its binding ability to mucosal KB cells. This adhesin protein is composed of 129 amino acids with an 18 amino acids signal peptide that corresponds to 12.6 kDa in the secreted form and 13.6 kDa in the intact form. Experimental deletion of FadA resulted in an 80 % reduction in the bacterial binding ability to KB cells, which suggests an important role for this adhesin in host binding (Han et al., 2005). FadA is anchored in the inner membrane and extends through the outer membrane of the microorganism (Xu et al., 2007). FadA also plays an essential role in host cells invasion via the interaction with epithelial E-cadherin (Rubinstein et al., 2013). FadA has been found to be elevated in periodontal inflammation (Liu et al., 2014c). RadD is an arginine inhibitable outer membrane protein in *F. nucleatum*, which is involved in inter species adherence and the maintenance of structured architecture of multispecies biofilms. Another outer membrane protein adhesin is FomA that has been shown to be associated with bacterial co-aggregation and biofilms formation (Liu et al., 2010b). Experimental studies using mice have shown that *F. nucleatum* outer membrane proteins are a source of potential vaccine candidates for the treatment of periodontal infections (Liu et al., 2010b).

Fap2 is another adhesin protein that enables *F. nucleatum* to interact with TIGT that results in the inhibition of NK cell cytotoxicity and T-cell activation (Coppenhagen-Glazer et al., 2015). Fap2 is also associated with *F. nucleatum's* ability to bind to adenocarcinoma cells in CRC (Abed et al., 2016) via tumour associated disaccharides. The adhesins FadA and Fap2 could be involved in the activation and induction of inflammatory responses in early tumorigenesis (Rubinstein et al., 2013, Gur et al., 2015). Fap2 and RadD are associated with the induction of apoptosis in human lymphocytes (Kaplan et al., 2010).

1.4.2 Human infection

F. nucleatum is an abundant species in the oral cavity, that can be detected in both healthy and diseases individuals. *F. nucleatum* is involved in many forms of periodontal disease including gingivitis and periodontitis (Signat et al., 2011). The spread of *F. nucleatum* infection correlates with the severity of disease and the progression of inflammation (Yang et al., 2014). *F. nucleatum* is able to provoke inflammation in the human oral cavity and gut mucosa (Strauss et al., 2011). In the oral cavity, biofilm associated bacteria have been divided into three groups that include early colonizer (gram-positive species), intermediate (gram-positive and gram-negative species) and late colonizer (gram-negative species). The early colonizer has the ability to adhere and mediate the co-aggregation to the tooth surface and the salivary pellicle (Palmer Jr et al., 2003). It is believed that *F. nucleatum* acts as a bridge, which mediates aggregation between early and late colonizers (Diaz et al., 2002, Signat et al., 2011). *F. nucleatum* can be found associated with almost all oral bacteria and co-aggregated with many species found in the biofilm (Kaplan et al., 2009). *F. nucleatum* invades oral cells via the interaction of FadA with E-cadherin in epithelial cells (Rubinstein et al., 2013). Internalization of *F. nucleatum* results in NF-κβ activation (Bui et al., 2016), which induces the activation of pro-inflammatory cytokines/chemokines (TNF-α, IL-1 β, IL-6, and IL-8). *F. nucleatum* induce cytokines production via TLR-4 dependent and TLR-4 independent manner (Liu et al., 2007). *F. nucleatum* is capable of invading deferent cells via it adherence ability to epithelial and endothelial cells (Han et al., 2000, Fardini et al., 2011). Also, *F. nucleatum* is able to elicit a potent inflammatory response when compared to other oral pathogens (Han et al., 2000). *F. nucleatum* stimulates tumorigenesis by direct interaction with pro-cancerous and cancerous oral epithelial cells via the activation of TLRs (Gallimidi et al., 2015), which activate oncogenic pathways such as Wnt pathways (Rubinstein et al., 2013). This suggests the involvement of *F. nucleatum* in the initiation and progression of oral tumours. It is worth noting that the presence of biofilm is usually linked to increased cell proliferation, IL-6 expression, decreased E-cadherin expression, and STAT-3 phosphorylation, which could be a possible mechanism that promotes tumorigenesis (Dejea et al., 2014). Also, microbial metabolites may be involved in tumorigenesis. For example, it has been shown that high levels of acetylated

polyamines could be associated with biofilm formation in CRC patients compared with normal tissues (Johnson et al., 2015).

Most research on *F. nucleatum* focused on its role as a periodontal pathogen and as the causative agent of periodontitis and gingivitis. Recently, this view has changed, *F. nucleatum* is associated with various oral infection and linked to many invasive infections throughout the body (Lewis et al., 2016). **Table 1.3** summarizes some sites and infections from which *Fusobacteria* have been isolated. *F. nucleatum* is a pro-inflammatory agent in human epithelial tissues with a high invasive capacity, which stimulates the secretion of pro-inflammatory cytokines such as IL-8 associated with many diseases (Han et al., 2000). Commonly *Fusobacterium* infection is found in mixed infection (aerobic and anaerobic poly-microbial infection) (Bennett and Eley, 1993). *Fusobacterium* species can cause serious infections and are able to extend through the bloodstream resulting in distant abscesses. They are also able to invade the amniotic membrane and cause stillbirth and preterm labour (Han et al., 2004).

Table 1.3: Spectrum of Fusobacterial infections.

Site	Type of infection	
Blood	Septicaemia	Post-angina septicaemia
Head and neck	Otitis media (acute and chronic)	
	Sinusitis	
	Mastoiditis	Vincent's angina
	Parotitis	Thyroiditis
	Recurrent tonsillitis	Peritonsillar abscess
	Cervical lymphadenopathy	
	Retropharyngeal abscess	
	Dental infections	Gingivostomatitis
	Eye infections	
	Orbital cellulitis	Maxillary sinusitis
Chest	Empyema	Lung abscess
	Aspiration pneumonia	
Central nervous system	Meningitis	Cerebral abscess
	Cerebritis	
Cardiovascular system	Thrombophlebitis	Endocarditis
	Pericarditis	
Abdomen	Liver abscess	Intra-abdominal abscess
	Appendicitis	Peritonitis
	Perirectal and perineal infections	

Genital tract	Amnionitis (and intra-amniotic infections) Prostatic abscess Female genital tract infections	
Bone and joint	Osteomyelitis	Arthritis
Miscellaneous	Soft tissue infection Tropical ulcers	

Taken from Bennett and Eley (1993) p. 251.

1.4.3 *F. nucleatum* is associated with IBDs

IBD is a complex inflammatory disease, which includes ulcerative colitis and Crohn's disease, relapsing and remitting chronic gut disorders with environmental and genetic risk factors (Conrad et al., 2014). It has been suggested that IBD could result from an inflammatory response to gut microorganisms, particularly in a genetically susceptible host. Therefore, gut microorganisms have been indicated as one of the etiological factors of gut inflammation (Couturier-Maillard et al., 2013). Genomics and genetics associated investigations focus on the association of host-microbe interactions in the progression and development of IBD primary to signalling/sensing and gut mucosal-triggered effector responses (Jostins et al., 2012). Microbiota in human gut has been reported as one of the environmental factors that contribute to IBD pathogenesis (Kostic et al., 2014, Gevers et al., 2014). Among various microorganisms living in the human gut, bacteria possess the ability to decrease mucosal inflammation and train mucosal response (Belkaid and Hand, 2014). One of the best examples is a commensal bacterium in which they severed as a regulator to gut mucosal immunity and is able to balance the intestinal homeostasis via stimulation of anti-inflammatory immune responses, stimulating immune cells, and eliciting antibody synthesis. As a consequence, a balance between immunosuppression to gut normal flora and immune-stimulation against pathogenic microorganisms is needed to maintain gut haemostasis (Cario, 2013). Such balance is lost in chronic auto-inflammatory disease such as IBD, which provides a microenvironment associated with the pathogenesis of CRC (Sansone and Bromberg, 2011).

Pro-inflammatory molecules released during colonic inflammation such as in IBD result in colonic cell damage and compromise epithelial barrier integrity. This could facilitate *F.*

nucleatum invasion to colonic epithelial cells and contribute to colorectal tumorigenesis. Colorectal adenomas and gut inflammation association with gut microbes, the environment, and the pathogenesis of CRC is a complicated interaction. It is required to investigate how microbes are associated with CRC and how this association with microbes induces inflammation to effect and drive colorectal tumorigenesis (Rubinstein et al., 2013, Jostins et al., 2012). McCoy et al. (2013) indicate a connection between *F. nucleatum* and intestinal inflammation by observing an increase in the expression of pro-inflammatory cytokines (TNF-α, IL-6, IL-17, and IL-12) in which they compared *F. nucleatum*-enriched colorectal adenoma to normal control tissues. This research was in agreement with other work by Kostic et al. (2013b), which showed an upregulation of NF-$\kappa\beta$ in *F. nucleatum* enriched colorectal adenomas. Dharmani et al. (2011) reported that *F. nucleatum,* isolated from IBD patient, induced upregulation in TNF-α and IL-1 β. Also, the microorganisms that were isolated from the inflamed area showed a greater ability to invade Caco-2 cells and trigger TNF-α and mucin-2 production when compared with those isolated from healthy subjects (Dharmani et al., 2011). Various studies analysing the metagenome and metatranscriptome to compare bacterial communities in tumour tissues relative to normal control tissue have now demonstrated *Fusobacterium* species enrichment at tumour sites (Castellarin et al., 2012, Kostic et al., 2012, Flanagan et al., 2014b). According to Strauss et al. (2011) *F. nucleatum* can invade and provoke an immune response in intestinal mucosa due to co-aggregation and its adhesive ability, which contributes to IBD and Crohn's diseases etiology. *F. nucleatum* is found as a part of the GIT (gastrointestinal tract), and it has been isolated from a broad range of abdominal infections.

1.4.4 *F. nucleatum* modulates T cells in colorectal tumours

Commensal bacteria considered as a part of the human gut flora as well as colorectal adenomas and commonly depend on glucose as a main source of energy. Interestingly, *F. nucleatum* does not depend on glucose as a main energy source (Vander Heiden et al., 2009). Instead, it relies on short chain fatty acids and peptides produced by tumour stroma, which also serve as chemo-attractants for myeloid derived suppressor cells (Kapatral et al., 2002). Kostic et al. (2013b) indicate an increase in tumour associated macrophage infiltration, M2, neutrophils, tumour-associated macrophages, and dendritic cells in *F.*

nucleatum infected colonic adenomas compared with control. Also, infiltration of myeloid cells has been reported (Minot et al., 2011) but no significant proliferation of T lymphocytes ($CD3^+$, $CD4^+$, and $CD8^+$ T lymphocytes) in the colorectal microenvironment. It has been reported that an increase in the T-helper cell (Th17 cells and $CD4^+$) infiltration is observed but they did not correlate with the enrichment of *F. nucleatum* in colorectal tumours (Minot et al., 2011, Kostic et al., 2013a). These observations indicate the immunosuppressive ability of *F. nucleatum*.

1.4.5 In CRC *F. nucleatum* relies on the inactivation of NK cells

NK cells are granular lymphocytes that activated by pathogen associated molecular patterns and cytokines including interferon (IFNs), IL-2, IL-12, IL-15, and IL-18 (Levin et al., 2012). There are two types of receptors expressed on NK cells include activating receptors and inhibitory receptors (Koch et al., 2013). In addition, NK cells express inhibitory receptors that do not recognize MHC class I proteins and ITIM (TIGIT domain). The TIGIT receptor on NK cells delivers an inhibitory signal when activated (Seidel et al., 2012). According to Gur et al. (2015) *F. nucleatum* Fap2 protein inhibits tumour cells lysis via the interaction with TIGIT receptors and results in the inhibition of the cytotoxic potential of NK cells. The expression of TIGIT receptors are not exclusive to NK cells and they can also be expressed on tumour infiltrating lymphocytes including T-cells. This partially explains how *F. nucleatum* evade immune cells in colorectal tumours. The development of monoclonal antibodies targeting Fap2 represent a potential therapeutic strategy and could enhance in vitro and in vivo studies to explore the importance of Fap2 (Bashir et al., 2016).

1.4.6 *F. nucleatum* associated with CRC

Growing evidence has shown a difference between intestinal microbial structures in the healthy individual compared with the CRC patient (Wang et al., 2012). This is characterised by a decrease in the number of some beneficial bacteria and an increase of opportunistic pathogens, such as *Fusobacterium*, *Enterococcus*, *E. coli* and *Streptococcus*, which is suggests the potential involvement of these bacteria in the progression of CRC (Wang et

al., 2012, Yu et al., 2015). Other bacteria also have been found to be enriched in CRC including *Porphyromonas*, *Peptostreptococcus*, *Lactobacillales*, and *Mogibacterium* (Chen et al., 2012). Also, a reduction of *Bifidobacterium*, *Blautia*, and *Faecalibacterium* in CRC (Chen et al., 2012). **Table 1.4** summarises some of the bacterial community structure that are expressed in CRC.

Table 1.4: Summary of the bacterial community structure in CRC.

Authors (year)	Study subjects (n)	Type of specimens	Overrepresentation	Underrepresentation
Sobhani et al. (2011)	CRC (60), colonoscopy control (119)	Stool	*Bacteroides/Prevotella*	
Ahn et al. (2013)	CRC (47), surgical control (94)	Stool	*Fusobacterium*, *Porphyromonas*	*Clostridia*
Wang et al. (2012)	CRC (46), healthy volunteers (56)	Stool	*Porphyromonas, Escherichia/Shigella, Enterococcus, Streptococcus, Peptostreptococcus*	*Bacteroides, Roseburia, Alistipes, Eubacterium, Parasutterella*
Wu et al. (2013a), and Wu et al. (2013b)	CRC (19), healthy volunteers (20)	Stool	*Bacteroides* species *Fusobacterium Campylobacter* species	*Faecalibacterium, Roseburia*
Weir et al. (2013)	CRC (11), healthy volunteers (10)	Stool	*Akkermansia muciniphila*	*Bacteroides, Prevotella, Ruminococcus*
Chen et al. (2012)	CRC (46), healthy volunteers (56)	Stool, rectal swab, cancer tissue, adjacent normal mucosa	In cancer patient: *Lactobacillales* (tumour) *Erysipelotrichaceae*, *Prevotellaceae, Coriobacteriaceae* (stool). In CRC patient: *Fusobacterium, Porphyromonas, Peptostreptococcus*, and *Mogibacterium*	*Faecalibacterium* (tumour), *Bifidobacterium*, and *Blautia*.
Mira-Pascual et al. (2015)	CRC (7), adenoma (11), healthy volunteer (10)	Tissue (tumour or rectal mucosa), stool	*Enterobacteriaceae* (cancer tissue)	
Geng et al. (2013)	CRC (8)	Paired tissue (cancer, normal)	*Roseburia*	*Microbacterium, Anoxybacillus*

Geng et al. (2014)	CRC (8), adenoma (10), healthy volunteer (10)	Normal and tumour tissue	*Streptococcus, Porphyromonas, Veillonella* (cancer vs control)	
Kostic et al. (2012)	CRC (95)	Paired tissue (cancer, normal)	*Fusobacterium*	*Bacteroides, Clostridia, Faecalibacterium*

Taken from Sun and Kato (2016) p. 132 with minor modification.

In order to determine the potential link between microorganisms and gastrointestinal cancer Castellarin et al. (2012) performed a RNA sequencing analysis of tumours and compared them to adjacent normal tissues. Quantitative real time polymerase chain reaction (RT-PCR) analyses of the genomic DNA isolated from 99 CRC samples and compared with adjacent control tissues confirmed significant *F. nucleatum* overabundance in tumour tissues. Various studies have confirmed the overabundance of *F. nucleatum* in tissues isolated from CRC tumour in comparison to control sample (Kostic et al., 2012, McCoy et al., 2013, Warren et al., 2013). Mira-Pascual et al. (2015) also recently found a significant high presentation of *F. nucleatum* in mucosal samples isolated from CRC patients when compared with healthy subjects. Importantly, *Fusobacterium* species are overrepresented in human colonic adenomas (McCoy et al., 2013, Kostic et al., 2013b, Li et al., 2016, Yusuf et al., 2016) suggesting that *F. nucleatum* has a role in the early stages of cancer development (Flanagan et al., 2014a).

In addition, Rubinstein et al. (2013) also indicated *F. nucleatum* high abundance in CRC patients via measuring the mRNA expression of *F. nucleatum* FadA relatively to healthy subject control, which showed a potential mechanism of *F. nucleatum* action during tumorigenesis. By using the $APC^{min/+}$ mouse model, Rubinstein et al. (2013) demonstrated that *F. nucleatum* resulted in enhancement of small intestinal and colonic tumorigenesis with noticeable increase in the pro-inflammatory NF-κβ and infiltration of specific myeloid cell subsets into tumours (Rubinstein et al., 2013). In contrast, other colitis murine models ($IL\text{-}10^{-/-}$, $T\text{-}bet^{-/-}$, and $xRag\text{-}2^{-/-}$) did not show any acceleration in tumorigenesis upon introduction of *F. nucleatum*. These findings suggest that *F. nucleatum* induces tumorigenesis following the loss of the tumour suppressor APC, which results in intestinal dysplasia observed in $APC^{min/+}$ mice (Kostic et al., 2013b). APC mutation is one of the earliest molecular alterations that occurs in the epithelium during the transformation into

adenoma (Cho and Vogelstein, 1992). Thus, it may be that somatic mutation precedes *Fusobacterium* enrichment, which is consistent with the finding of Rubinstein et al. (2013) in which proliferation only occurs in CRC cell lines, but not in non-neoplastic cell lines. Mutation could also be associated with the defect in the epithelial cell barrier that result in the lost of tight junctions, epithelial polarity, cell-to-cell contacts and mucus layer (Grivennikov et al., 2012). Any defect in the intestinal barrier at sites of dysplasia could promote the infiltration of bacteria including *F. nucleatum* that allow *F. nucleatum* to be a part of tumour environment. This could be an important stage in colorectal neoplasia in which promoting tumour progression by selectively recruiting tumour infiltrating myeloid cells could be the driving force for inflammation end epigenetic changes that could ultimately lead to cancer formation (Kostic et al., 2013b).

It has been reported that *F. nucleatum* could modulate the tumour-immune microenvironment and potentiate intestinal tumorigenesis (Kostic et al., 2013b) or *F. nucleatum* colonization in the intestine may promote the onset of colonic tumours in vivo (Yu et al., 2015). *F. nucleatum* invades colonic mucosa and induces local inflammation, which contributes to CRC development and accompanied by an increased in cytokines expression (TNF-α, IL-1 β, IL-6, IL-8), which leads to colorectal disease (Strauss et al., 2011, Castellarin et al., 2012, McCoy et al., 2013) and promotes tumorigenesis (Jauch et al., 2011, Buning et al., 2002). Further, Flanagan et al. (2014b) confirmed the finding of previous studies in which Flanagan's group analyses samples of Irish patients with colorectal adenoma. As indicated by their Kaplan-Meier analysis (**Figure 1.2**) *F. nucleatum* levels display a definite increase during colorectal neoplasia progression. High level of *F. nucleatum* is correlated with shorter overall survival time than patients with moderate and low levels of the bacterium. This supports the role of *F. nucleatum* in the induction of mucosal inflammation in colorectal disease and suggest the use of *F. nucleatum* abundance as a valuable prognostic indicator tool in combination with the eradication of *F. nucleatum* infection, which may be helpful for CRC patients.

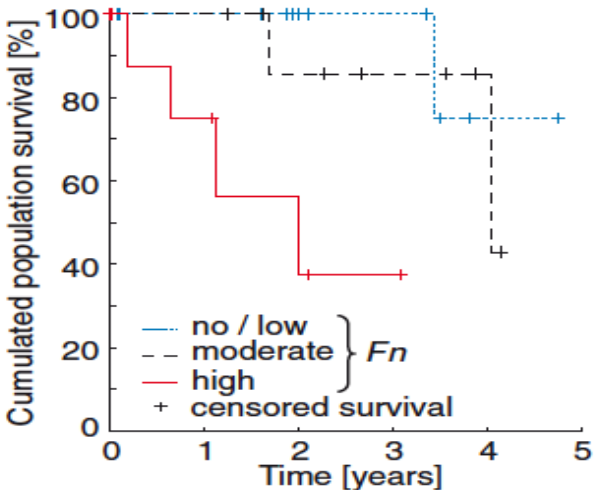

Figure 1.2: The overall survival of CRC. Kaplan–Meier curves showing the association between an increase in *Fusobacterium* quantification and the overall survival of CRC. Subjects were divided into groups of 'no/low' (dotted line), 'moderate' (dashed line) and 'high' (solid line) fold increase of *F. nucleatum*. Taken from Flanagan et al. (2014b).

During the colorectal carcinoma sequence, it is evident that *Fusobacterium* species tend to accumulate in the human intestine, this suggests that a fecal sample could be used as an early diagnostic detection method (McCoy et al., 2013, Yu et al., 2015).

There is now considerable interest in investigating the role of bacteria in CRC including *F. nucleatum* to determine whether or not they are a cause or a consequence of CRC (Ito et al., 2015). If a causative link is determined, antibiotics and/or vaccines could be potential therapies to treat and/or prevent CRC.

1.5 EMT

EMT can be defined as a biological process in which epithelial cells undergo multiple biochemical changes and lose their interaction and polarization with the basement membrane that enables it to acquire a mesenchymal cell phenotype. This change is characterized by an increase in migratory capacity, resistance to apoptosis, and increased invasiveness (Kalluri and Neilson, 2003). The acquisition of the EMT state is accompanied by degradation of the underlying basement membrane, these allowing the cells to migrate from the original cells. EMT engages various molecular processes such as expression of specific cell surface proteins, activation of transcription factors, production of ECM

degradation enzymes, changes in the expression of micro-RNAs, and the involvement of some biomarkers such as cadherin, vimentin, β-catenin, Snail, Slug, and Twist (Kalluri and Weinberg, 2009, Liu et al., 2015a).

1.5.1 The association of EMT with cancer progression and metastasis

Hallmarks of the initiation of the early stage of primary epithelial cancer are excessive cell proliferation and angiogenesis (Hanahan and Weinberg, 2000). EMT involved change in cells plasticity that include genetic and epigenetic changes as well as altering proteins expression, reduction in cell-cell adhesion, change in cytoskeleton, and cell polarization, which resulting in acquiring a highly motile mesenchymal phenotype (Ye and Weinberg, 2015). Followed by increase in invasiveness, which leads ultimately to metastatic dissemination and a life threating state. It has been proposed that EMT is a crucial mechanism that precedes the acquisition of a malignant phenotype of epithelial cancer cells (Thiery, 2002). Many studies reported that a mesenchymal state is acquired by cancer cells with the expression of mesenchymal markers such as β-catenin, vimentin, and fibroblast specific protein 1 (FSP1) (Yang and Weinberg, 2008). Also, it has been reported that in many carcinomas, EMT inducing signals are generated from tumour-associated stroma. TGF-β is one of the main players in the induction of transcription factors (Lamouille et al., 2014). The activation of these transcription factors is associated with various intercellular signalling networks including signal-transducing proteins, MAPK, ERK, PI3K, Akt, Smads, lymphoid enhancer binding factors (LEF), β-catenin, and Ras (Sánchez-Tilló et al., 2012).

The cell-cell adherence junction and cell-ECM adhesion also can be involved in the activation of EMT (Yang and Weinberg, 2008). It has been reported that TGF-β is a potent inducer of EMT in many cancers (Song, 2007). TGF-β plays a major role in epithelial proliferation suppression and is one of the primary tumorigenesis factors with a regulatory effect on tumour progression and metastasis (Oft et al., 1998). TGF-β can be induced by bacterial LPS and subsequent EMT (Zhao et al., 2011). Other studies also indicate the involvement of RhoA, p38, and MAPK with TGF-β in the induction of EMT using mouse

models (Bhowmick et al., 2001). Additionally, EMT is associated with a decrease in the expression of E-cadherin via ZEB-1 (containing the β-catenin binding site) and an increase in cell proliferation (Sanchez-Tillo et al., 2010).

Interestingly, recent studies have demonstrated that bacteria can also induce EMT in various tissue. For example, *P. gingivalis* (Sztukowska et al., 2016), *H. pylori* (Sougleri et al., 2016), *Klebsiella pneumoniae* (Leone et al., 2016), and *Mycobacterium tuberculosis* (Gupta et al., 2016). However, the implication of bacterial involvement in EMT are considered later in this thesis.

1.6 Bacterial outer membrane vesicles (OMV)

Microorganisms continuously send and receive signals from host cells and other microorganisms that allow them to initiate their own messages and communicate in various ways (Yoon, 2016). Commonly the secreted material, composed of soluble or insoluble compounds require specific mechanisms to be secreted (Kulp and Kuehn, 2010). The gram-negative envelope is composed of inner and outer membranes, a layer of peptidoglycan, and periplasmic space (Bos et al., 2007). The outer leaflet of the outer membrane is mostly composed of lipopolysaccharide (LPS), and the inner leaflet and the outer membrane consist mainly of phospholipids. The peptidoglycan layer is located in the periplasmic space and attached to the inner and outer membrane by anchored proteins (Braun's lipoprotein (Lpp) and OmpA) (Collins et al., 2007).

The production of OMVs from gram-negative bacteria was reported approximately 50 years ago (Bladen and Waters, 1963). Since then, OMV have been demonstrated to be released by many gram-negative bacteria (Kim et al., 2015, Veith et al., 2014). OMV are released in vitro (Schooling and Beveridge, 2006, Park et al., 2015) and in vivo (Lee et al., 2008, Ünal et al., 2011). OMVs are nanostructures that range in size from 20-250 nm (Schwechheimer and Kuehn, 2015). OMVs contain newly synthesized proteins and are synthetized without affecting the parent cells (McBroom et al., 2006). It has been reported that an increase in OMV production can be likened to bacterial stress responses (McBroom and Kuehn, 2007). Furthermore, OMVs serve as a delivery system that contain a wide variety of virulence

factors including toxins, LPS, DNA, adhesins, and enzymes. OMVs contain pathogen-associated molecular patterns (PAMPs) and are able to modulate host response to infection (Ellis and Kuehn, 2010). Microorganisms have unique packaging mechanisms for specific cargo within OMV (Haurat et al., 2011, Elhenawy et al., 2014). However, the exact mechanisms by which bacteria cargo enters OMVs remain unknown.

1.6.1 Characterization and composition of OMV

Various studies have been conducted to identify the proteomic composition of OMV recovered from bacteria, which are essential for the development of effective OMV-based vaccine and diagnostic tools (Lee et al., 2016). OMV are mainly composed of phospholipid, outer membrane proteins, and lipooligosaccharides in addition to content from periplasmic proteins and cell wall components that are trapped in the OMV lumen (Lee et al., 2008, Kulkarni and Jagannadham, 2014). Also, rising evidence indicates that various virulence factors are found in OMV including LPS, cytoplasmic proteins, and genetic material (DNA and RNA) (Kulp and Kuehn, 2010). Biochemical methods were used to identify OMV-associated proteins including SDS-PAGE with Coomassie or silver staining in addition to Western blotting and immunoblotting (Horstman and Kuehn, 2000, Kesty and Kuehn, 2004). Several virulence factors secreted by pathogenic bacteria including toxins, proteinases, adhesins, and other related enzymes have been identified in OMV (Kulkarni and Jagannadham, 2014, Kuehn and Kesty, 2005). Toxins are the main characterised features that could contribute to OMV-mediated pathogenesis. For example, a potentially toxic component in gram-negative bacteria is LPS, which is found in OMV and this can activate the immune response (Kuehn and Kesty, 2005). **Table 1.5** summarizes virulence factors that have been identified in OMV and potentially contribute to the pathogenesis of bacterial infections. Another important characteristic of OMV is their ability to adhere to and invade host cells through invasins and adhesins, which initiate vesicle-mediated pathogenesis and play a critical role in the interaction and invasion of host cells (Kesty and Kuehn, 2004, Kadurugamuwa and Beveridge, 1999).

Mass spectrometry-based proteomic analyses are responsible for the identification of hundreds of vesicular proteins (Lee et al., 2016). OMV act as carriers of active bacterial

toxins for *S. enterica, C. jejuni, Vibrio cholerae* (Guidi et al., 2013, Chatterjee and Chaudhuri, 2011, Lindmark et al., 2009). Vesicles from *H. pylori* contain several adhesins such as BabA and SabA in addition to proteases and urease (Olofsson et al., 2010). Also, it has been reported OMV recovered from enterotoxigenic *E. coli* (ETEC) are enriched with heat-labile enterotoxin (LT) (Horstman and Kuehn, 2000). Also, *Pseudomonas aeruginosa* OMV are enriched with aminopeptidases as well as B-ban LPS (Bauman and Kuehn, 2006). Furthermore, the presence of DNA within OMV was observed in *E. coli*, *H. influenzae*, *N. gonorrhoeae*, and *P. aeruginosa* (Mashburn-Warren and Whiteley, 2006) in which OMV serve as a protective shield for DNA against DNase (Kulp and Kuehn, 2010, Renelli et al., 2004). Also, RNA can be found in the OMV, but in lesser amount than DNA (Dorward et al., 1991).

Table 1.5: Virulence factors and activities associated with OMVs.

Species	Vesicle-associated factors	Virulence-associated activity
Actinobacillus actinomycetemcomitans	Leukotoxin, GroEL	Bone-resorbing activity, chicken embryo lethality, cytotoxic
Actinobacillus pleuropneumoniae	Apx toxin, proteases	Proteolytic.
B. fragilis	Hemagglutinin, alkaline phosphatase, esterase lipase, acid phosphatase, phosphohydrolase, α and β galactosidases, α-glucosidase, glucosaminidase, β-glucuronidase	Hemagglutinating and enzymatic activities
Bacteroides succinogenes	Cellulase, xylanase	Aryl-β-glucosidase, aryl-β-xylosidase, endoglucanase, xylanase activities
Bordetella pertussis	AC-Hly, FHA, pertussis toxin (Ptx)	ND
Borrelia burgdorferi	OspA, OspB, OspD	HUVEC adherence
Brucella melitensis	Omp25, Omp31	ND
Burkholderia cepacia	PLC-N, lipase, PSCP, 40-kDa protease	Enzyme activities
EHEC O111:H–	ClyA	Pore forming
ETEC	LT	Enterotoxic and vacuolating activities
ExPEC	Alpha-hemolysin, CDT, iron and hemin binding OMPs	Hemolytic, causing detachment of cells from monolayer .
STEC O157:H7	Shiga toxin 2	Cytotoxic
UPEC	CNF1	Cytotoxic

H. pylori	VacA, Lewis antigen LPS	Vacuolating activity (75), cytotoxic, stimulating proliferation, IL-8 secretion
Legionella pneumophila	Mip (lpg0791), IcmK/IcmX, flagellin, phospholipase C, LaiE/LaiF, phospholipase, chitinase, acid phosphatases, Hsp60, proteases, diphosphohydrolase	Inhibition of phagolysosome fusion, proteolytic and lipase activity
Moraxella catarrhalis	UspA1/UspA2	Binds C3 complement in serum
Neisseria meningitidis	PorA, NlpB, NarE (putative)	TNF-α, IL-6, activation of tissue factor (procoaggulant), profibrinolytic and antifibrinolytic factors.
Photorhabdus luminescens	Toxin AB, GroEL	Insecticidal
P. gingivalis (Bacteroides)	Arg- and Lys-gingipain cysteine proteinases	Cleavage and loss of CD14 from macrophage, cleavage of IgG, C3, IgM .
P. aeruginosa	Phospholipase C, hemolysin, alkaline phosphatase, Cif, PQS, quinolines, protease, β-lactamase	Decrease of apical CFTR expression, *in vitro* enzyme activities, bactericidal quinolines, IL-8 stimulation
Salmonella enterica serovar Typhimurium	Protective antigens	ND
Shigella dysenteriae serotype 1	Shiga toxin 1	Toxicity
Shigella flexneri	IpaB, IpaC, IpaD	Invasion
Treponema denticola	Dentilysin, adhesins, proteases	Chymotryptic activity, disruption of tight junctions
Vibrio anguillarum	Metalloprotease, hemolysin, phospholipase	Protease, metalloprotease, hemolytic activities
V. cholerae	RTX toxin	Cell rounding, depolymerizing actin
Xanthomonas campestris	Cellulase, β-glucosidase, xylosidase, avirulence proteins, type 3 secretion system proteins	ND
Xenorhabdus nematophilus	Bacteriocin, fimbrial adhesin, pore-forming protein, chitinase	Chitinase activity, insecticidal

Taken from (Ellis and Kuehn, 2010) p. 82. Abbreviations not defined in the text: AC-Hly, adenylate cyclase-hemolysin; FHA, filamentous hemagglutinin; CDT cytolethal distending toxin; OMP, outer membrane protein; PLC-N, phospholipase, nonhemolytic; UPEC, uropathogenic *E. coli*; CNF1, cytotoxic necrotizing factor 1; ND, not determined.

1.6.2 OMV functions

OMV serve many functions and are involved in both defence and offense (Olsen and Amano, 2015). OMV can have a predatory function due to their ability to fuse with the membrane of similar or other bacteria, which can provide protection against competing bacteria and can be involved in nutrition acquisition (Mashburn-Warren et al., 2008). OMV are serve as transport vehicles for virulence factors, DNA, and β-lactamase (Schaar et al., 2011), which provide that the contents of OMVs reach their destination at high concentration (Demuth et al., 2003). Since OMVs provide a protective shield for their contents from extracellular degradative enzymes and various protease (Horstman and Kuehn, 2000), they also serve as a means for long-distance transportation enabling them to reach sites distant from parental bacteria (Dorward et al., 1991). OMV contain specific virulence factors that are directed toward designated target cells, which can be used for mediating invasion (Bomberger et al., 2009). Likewise, membrane associated and embedded proteins, and other proteins lacking signal sequences for secretion can be exported by OMVs that do not rely on bacterial to host cell contact for delivery (Kuehn and Kesty, 2005).

1.6.3 Factors associated with OMV production

OMVs play a major role in bacterial pathogenesis, many factors are associated with OMV production. For example, exposure to antibiotics can induce an increase in the production of OMVs and toxins (Dutta et al., 2004) as in case of *Pseudomonas* (Kadurugamuwa and Beveridge, 1995). Furthermore, environmental changes could mediate OMV production e.g. temperature as in case of *E. coli* (McBroom and Kuehn, 2007) and *S. marcescens* (McMahon et al., 2012). The induction of OMV could play a primary role in bacterial survival in mixed bacterial infections with a limited source of nutrition. Besides, OMV can aid in the promotion of biofilm formation, which enhances colonization and antibiotic resistance (Murphy et al., 2014). OMVs are involved in aggregation and interaction with other bacterial strains (quorum sensing, communication) (Mashburn and Whiteley, 2005). Biochemical analysis revealed that OMVs can also provide a means for exchanging products

amongst bacteria including DNase-resistance and periplasmic antibiotics (Kadurugamuwa and Beveridge, 1999). This suggests the ability of the vesicles to fuse with other bacterial cells and the potential role of the OMV in the transformation and acquisition of antibiotic resistance during infection.

1.6.4 OMV biogenesis

OMV proteomic analyses indicate compositional diversity among bacterial species (Lee et al., 2016). Even when using identical bacteria, the OMV composition varies and is affected by the growth and isolation conditions. *S. typhimurium* OMVs were enriched with membrane proteins when grown under nutritional limiting conditions. In contrast, they were enriched with cytosolic proteins associated with cellular metabolism and translation when grown under nutrient-abundant conditions (Bai et al., 2014).

There are several models describing the formation of OMV. One model demonstrates that vesiculation is initiated when the cross-links which bridge peptidoglycan to the outer membrane are broken due to altering the balance between peptidoglycan synthesis and breakdown, with the result that a small portion of the outer membrane protrudes from the cell (Schwechheimer et al., 2015) and subsequently pinches off. In another model, OMV are produced due to environmental stress via the reduction in envelope integrity, which involves accumulation of aberrant envelop components and misfolded proteins. The abnormal accumulation and build-up of cellular components in the periplasm result in the extrusion of a portion of the outer membrane outward that is detached from the peptidoglycan layer and the inner membrane (Schwechheimer et al., 2014). Furthermore, vesiculation can be induced by altering biophysical characteristics of the outer membrane (Schertzer and Whiteley, 2012). Budding off and curvature formation due to the incorporation of phospholipids or LPS into the outer membrane resulted in alterations to membrane fluidity and flexibility (Yoon, 2016).

1.6.5 OMV can interact with epithelial cells

The OMV surface contains some of the parental cell surface proteins and adhesins, which enable them to interact with host cells and provoke an immune response (Chatterjee and Chaudhuri, 2013). For example, VacA enhances and facilitates *H. pylori* OMV uptake by gastric epithelial cells (Parker et al., 2010). Also, OMV derived from *H. pylori*, *P. aeruginosa*, and *N. meningitidis* bind to lipid rafts of epithelial cells and become internalized via endocytosis (Kaparakis et al., 2010). OMV peptidoglycan can bind to PRRs that activate NOD-1 and induce IL-8 production by the epithelial cell (Kaparakis et al., 2010). *L. pneumophilia* OMV attach to lung epithelial cells and modulate the cells response and induce pro-inflammatory cytokine production (IL-7, and IL-13), which are not induced by the binding of the parental bacterial cells (Galka et al., 2008). Furthermore, OMV attenuate the fusion of lysosomes of macrophages with phagosomes (Fernandez-Moreira et al., 2006).

It has been reported that *S. typhimurium* continually releases OMVs when interacting with macrophages and epithelial cells (Garcia-Del Portillo et al., 1997). OMV interact with epithelial cells and modulate various vital function within the cell including apoptosis (Mondal et al., 2016, Kunsmann et al., 2015) and proliferation (Li et al., 2015b). OMV from *Cronobacter sakazakii* are internalized by intestinal epithelial cells and induce cell proliferation and IL-8 production, which can be linked to bacterial persistence and could be also associated with infant enterocolitis and meningitis (Alzahrani et al., 2015). In addition, OMV contain bacterial RNA and can transfer it to host epithelial cells, which induce potent immune-stimulatory responses as in the case of *P. aeruginosa* (Koeppen et al., 2016), *E. coli* (Ghosal et al., 2015), *V. cholera* (Sjostrom et al., 2015), and *P. gingivalis* (Ho et al., 2015). OMV are considered as potent virulence factors that can induce caspase 9-mediated apoptosis and IL-8 production as in the case of *E. coli* (Kunsmann et al., 2015). Also, *H. pylori* release OMV that contain many virulence factors which interact with gastric tissue (Fiocca et al., 1999).

In the case of ETEC OMV, the vesicles mediate LT entry as well as other membrane components into the host through lipid rafts in a clathrin-independent manner and in this

way they mediate the toxicity of ETEC OMV (Kesty et al., 2004). Furthermore, *P. gingivalis* OMV induce platelet aggregation that cause systemic heart disease (Sharma et al., 2000). It is likely that the nature of the pathogen-host interaction has the main impact on the microorganism's ability to modulate OMV formation. This is effected by many factors such as the presence of antibiotics (Brodsky et al., 2005) or serum (Brandtzaeg et al., 1992), temperature and pH variations (Silverman et al., 2010), exposure to host tissues (Marsollier et al., 2007) that stimulate the release of OMV among gram-negative bacteria.

1.6.6 OMV modulate the innate immune response

The biological impacts of OMV are likely to be considerable since they act as a carrier for many compounds such as virulence factors and antigens. An innate immune response to infection is influenced by the detection of OMV via TLR and NOD signalling that induces cytokine secretion by macrophages and dendritic cells (Alaniz et al., 2007), which are connected to the pathogenesis of inflammatory infections (Brandtzaeg et al., 1992). *Acinetobacter baumannii* OMV can elicit potent innate immune responses and induce a pro-inflammatory response in vitro and in vivo, which contribute to immunopathology of the infected host (Jun et al., 2013). NOD-1 specifically recognises diaminopimelate tripeptide (GM-TriDAP) motifs that are exclusively found in bacterial peptidoglycans (Fritz et al., 2006). Peptidoglycan, the cytosolic ligand that provokes the innate immune responce and can be exported into OMV and initiate the NF-κβ dependent response (Kaparakis et al., 2010). OMV play an essential role evading complement-mediated killing of bacteria that facilitates pathogenic bacterial survival in the host. For example, *V. cholerae* OMV contain OmpU, which is involved in the evading the host immune system (Aung et al., 2016). *P. gingivalis* OMV contain peptidylarginine deiminases (PADs) enzyme, which inhibits complement factor C5a and enhances the bacteriaium's ability to evade the immune system (Bielecka et al., 2014).

OMV contain various PAMPs including LPS, lipoproteins, peptidoglycan, OmpA, DNA, and RNA that interact with host PRRs. PAMPs activate innate immune responses via NOD-1 (Kaparakis et al., 2010, Kulp and Kuehn, 2010, Ellis and Kuehn, 2010). Internalisation of OMVs into epithelial cells subsequently can result in the destruction of the OMV by NOD-1

dependent autophagy degradation, which induces an inflammatory response (Irving et al., 2014). *N. gonorrhoea* and *H. pylori* OMV are recognised by host cytoplasmic immune receptor NOD-1 and activate innate immune response (Kaparakis et al., 2010) and induce the production of cytokines/chemokines including IL-1 β, IL-6, and IL-8 (Jun et al., 2013). Similarly, *V. cholerae* and *A. actinomycetemcomitans* OMV are internalized by epithelial cells and able to activate NOD-1 and NOD-2 that induce inflammatory responses (Thay et al., 2014, Bielig et al., 2011). Furthermore, it has been reported that OMV can interact with TLRs to mediate inflammatory response in epithelial cells as in the case of *Yersinia pseudotuberculosis*, *Yersinia pestis*, and *S. typhimurium*, which mediate immune response through TLR-4 (Laughlin et al., 2015). Also, OMV mediate inflammatory response via NOD1 and NOD2 in addition to TLR-2, TLR-4, TLR-7, TLR-8 and TLR-9 (Cecil et al., 2016). One good example of the ability of OMV to provoke innate immune response via TLRs to regulate inflammation and pathogenesis is seen in *P. gingivalis* OMV, which provoke TNF-α production by monocytes in a TLR-4 dependent manner (Waller et al., 2016). Also, *B. fragilis* OMVs activate TLR-2 on T-cells via their polysaccharide capsule (Shen et al., 2012). *B. fragilis* OMV associated with IBD, which modulates T-cells, induces IL-10 expression, and involves in the inhibition of intestinal inflammation (Chu et al., 2016).

1.6.7 OMV modulate the adaptive immune response.

Interestingly, the OMVs released by gram-negative bacteria are nonviable but carry various antigens, which are recognized by the adaptive immune system (Alaniz et al., 2007). This suggests that these surface organelles could be potentially used as important source of antigen in vivo. OMV could serve as decoys in vivo to re-direct the antibody response and thereby attract and neutralize antibodies to prevent them from attacking the intact organisms (Vidakovics et al., 2010). As in the case of *M. catarrhalis* OMV can be utilized to avoid the interaction with B-cells by directing the adaptive humoral immune response toward the OMV as a decoy (Vidakovics et al., 2010). The content of OMV cargo and their bacterial origin mainly affect the ability of OMVs to modulate the adaptive immune response. For example, OMVs are able to inhibit $CD4^+$ T-cell activation and proliferation that induce an immunosuppressive effect in the infected area, as in the case of *N.*

meningitidis (Li et al., 2015b). Also, OMV could induce proliferation of $CD4^+$ T-cells (Jones et al., 2016). *H. pylori* OMV induce the production of pro-inflammatory cytokines (IL-6) and at the same time induce the production of anti-inflammatory cytokines (IL-10) from human mononuclear cells, which inhibit the proliferation of $CD4^+$ T-cell and stimulate T-cell apoptosis (Winter et al., 2014). Furthermore, OMVs are able to activate human B-cells too as in the case of *H. influenzae* and *M. catarrhalis* (Deknuydt et al., 2014, Vidakovics et al., 2010).

Additionally, OMVs are able to mimic the symptoms and condition induced by pathogenic bacteria. For example, Shiga toxin producing *E. coli* (STEC) infection can cause hemolytic uremic syndrome (HUS) that is characterized by acute renal failure. According to Kim et al. (2011) purified OMVs that were recovered from STEC were able to produce similar symptom of HUS when inoculated on a mouse model. Similarly, two other studies carried out by Park show that Intestinal *E. coli* caused sepsis characterized by systemic inflammation, the principal cause of death worldwide. Inoculation of purified OMV recovered from *E. coli* can induce host responses that resemble the clinical condition caused by whole bacterial cells (Park et al., 2010b). The other study shows that OMV recovered from *P. aeruginosa* can cause pulmonary inflammation without the involvement of the live bacteria in vivo (Park et al., 2013). Such findings could lead to a better understanding of the pathogenesis of disease caused by bacterial infections and could have important implications for the improvement of vaccines, diagnostic tools, and treatment of these diseases.

1.6.8 OMV as possible vaccine candidates

Conventional human vaccines are pharmaceutical products that stimulate the immune system to inhibit and destroy pathogens and thus prevent disease (Bachmann and Jennings, 2010). OMVs are believed to be an attractive vaccine candidate since OMVs are non-replicative mimics of parental cells, which are highly immunogenic and can modulate innate and adaptive immune responses when internalised into eukaryotic cells. (Kaparakis-Liaskos and Ferrero, 2015, Ellis and Kuehn, 2010). Genetic engineering of OMV can enhance and improve their use as vaccines as in case of the modification of lipopolysaccharide

reactogenicity to produce OMV vaccine that is effective and safe (van der Pol et al., 2015). Non-pathogenic bacteria have been used to isolate OMVs expressing bio-medically crucial foreign bacterial lipoproteins and glycan. For example, OspA has been isolated from *Borrelia burgdorferi* and expressed in *N. meningitidis* OMVs (Salverda et al., 2016). OMV isolated from *E. coli* that was genetically engineered to express influenza A virus proteins, which can provide vaccination against lethal doses of H1N1 influenza A virus (Rappazzo et al., 2016). Also surface glycans from *C. jejuni* and *S. pneumoniae* have been expressed on the surface of *E. coli* OMVs, which were capable of stimulating the immune system in similar way as induced by *Pneumococcal* vaccine (Price et al., 2016). In addition, gram-positive OMVs also induce a protective immune response in vivo. In mice, administration of *M. tuberculosis* OMV induced a protective immune response (Prados-Rosales et al., 2014). Similarly, using *Staphylococcus aureus* OMV as a vaccine produced protection against challenge with *S. aureus* (Choi et al., 2015a). Also, vaccines derived from *B. pertussis* OMV contain immunogens more similar to those found in causal agent than in the commercial vaccine. Additionally, the small size of OMV-based vaccines enable them to reach immune cells deeper in the tissue and induce a more potent immune response (Benne et al., 2016). Also, OMV vaccines are less toxic than whole bacterial vaccine and cheaper which makes them more attractive candidates than conventional vaccine (Hozbor, 2017). This suggests the broad possibility for the development and use of OMV in the future development of novel vaccine technology.

Based on a review of the literature, it is now appreciated that OMVs are constitutively released by all gram-negative bacteria and are recognized as major contributors to disease (Kaparakis-Liaskos and Ferrero, 2015, Ünal et al., 2011, Ellis and Kuehn, 2010). These vesicles appear to contain a unique combination of the various proteomic fractions within these bacteria, including cytoplasmic, inner membrane, periplasmic, outer membrane and extracellular material. OMVs are nanoparticles that are considered as important virulence factors, which contribute to the pathogenesis of many diseases. They can travel to distant locations within the host, permit bacterial survival, cause cellular destruction, and facilitate pathogenesis in the host. In addition, as OMVs can disseminate in the host they can communicate between and within bacterial communities.

H. pylori is classified by WHO as a class I human carcinogen (Peek and Blaser, 2002). It is involved in the development and progression of gastric cancer (Wu et al., 2013b, Peek and Blaser, 2002). Also, *H. pylori* OMV were shown to be genotoxic and rapidly internalised by gastric carcinoma cells (Parker et al., 2010). *H. pylori* OMV enhance the carcinogenic potential of *H. pylori* and induce cell proliferation and oxidative stress, which cause chromosomal instability, an essential step in carcinogenesis (Chitcholtan et al., 2008). Similarly, *E. coli* OMV induce a genotoxicity in colonic cells, DNA damage, mutagenic development, which is associated with CRC (Tyrer et al., 2014). This leads to the hypothesis that other bacteria could also be involved in the initiation of carcinogenesis (Lax and Thomas, 2002, Lax, 2005). Yet, there is a lack of knowledge about *F. nucleatum* OMV and their association with disease, which were demonstrated in one study only to contain a serine protease that could degrade extracellular matrix proteins and IgA (Bachrach et al., 2004). *F. nucleatum* OMV represent an understudied but potentially important mechanism of *F. nucleatum* virulence and thus are a major focus of this study. This work attempted to bridge this gap by investigating the association between *F. nucleatum* OMVs and CRC.

1.7 Aims of the study

The aim of this thesis was to characterise *F. nucleatum* OMV and investigate how these nanostructures could promote carcinogenesis and associate with CRC. The specific aims of the thesis were:

- To analyse the proteome composition of *F. nucleatum* OMV to determine whether or not they contain virulence factors (Chapter 3).
- To investigate if OMV from *F. nucleatum* interacts with colonic epithelial cells in vitro.
  - To evaluate the ability of the OMV to degrade host proteins and modulate barrier function (Chapter 3).
  - To examine the ability of *F. nucleatum* and OMV to induce pro-inflammatory cytokine expression in colonic epithelial cells (Chapter 4).
  - To examine the ability of *F. nucleatum* and OMV to induce EMT-like behaviour (Chapter 5).
    - Induction of cell proliferation.
    - Induction of morphological changes in colonic epithelial cells.
    - Induction of EMT markers.

CHAPTER TWO

Material and Methods

2.1 Cells culture

2.1.1 Bacterial strains and culture conditions

Fusobacterium nucleatum nucleatum (ATCC 25586), *Fusobacterium nucleatum polymorphum* (ATCC 10953), and *Fusobacterium nucleatum vincentii* (ATCC 49256) were purchased from the ATCC culture collection (ATCC, LGC, UK). All microorganisms were grown at 37^{o}C under anaerobic conditions using the atmosphere generating system (AneroGen, ThermoScientific). All Fusobacteria species were thawed from frozen stock and plated on Columbia blood agar (CBA) plates (Columbia agar base, Oxoid) supplemented with 7 % v/v defibrinated horse blood. For broth culture, Fusobacteria were grown in brain heart infusion broth (BHI) which was pre-reduced for at least 24 h under anaerobic conditions before use. Different preparations of the bacteria were routinely Gram stained (Sigma) and observed microscopically under an oil immersion lens to ensure purity and to check morphology and integrity when required. The number of *F. nucleatum* was quantified as described by Gendron et al. (2004). *F. nucleatum* were grown in BHI broth for 48 h. Before incubation with eukaryotic cells BHI broth was removed by low-speed centrifugation and replaced with suitable antibiotic-free medium.

2.1.2 Eukaryotic cell culture

The colon cancer epithelial cell lines T-84, SW-480, SW-620, Caco-2, and Lovo cells were grown at 37^{o}C and 5 % CO_2 in the appropriate medium as shown in **Table 2.1**. Eukaryotic cells lines and growth conditions.

Table 2.1: Eukaryotic cells lines and growth conditions.

Cell line	Description	Growth conditions
T-84	Human CRC adherent epithelial cells, derived from a metastatic site in the lung.	DMEM/Hams F12 medium supplemented with penicillin (100 U/ml) and streptomycin (100 µg/ml) and foetal calf serum (10 %, v/v)
SW-480	Human CRC adherent epithelial cells. Dukes stage B adenocarcinoma	RPMI medium supplemented as above
SW-620	Human CRC adherent epithelial cells. Dukes stage C adenocarcinoma	RPMI medium supplemented as above
Lovo	human colon adenocarcinoma	RPMI medium supplemented as above
Caco-2	human colon adenocarcinoma	EMEM medium supplemented as above with the addition of 2 mM L-glutamine.

2.2 Bacterial growth curve

Determination of the relationship between optical density (O.D) and colony forming units (CFU) is important for co-culture experiments with human cells that require usage of a specific ratio of bacteria to human cells. Typically, *F. nucleatum* was inoculated in a 25 cm^2 flask which contained 10 ml of BHI broth and incubated anaerobically as described above for 24 h at 37^{o}C. Then, if expansion of the culture was required, 150 µl of the bacterial suspension was subsequently inoculated into several flasks containing BHI to an OD_{600nm} of approximately 0.1. The $O.D_{600nm}$ was recorded at each time point (duplicate measurements) and 100 µl from each flask was serially diluted and spread on CBA plates followed by incubation at 37^{o}C for at least 72 h to allow time for individual colonies to become evident. The number of bacteria/ml was determined using the following formula:

$$\text{Number of bacteria/ml} = \frac{\text{number of CFU}}{\text{volume plated (ml) X } \left(\frac{1}{\text{total dilution used}}\right)}$$

2.3 Recovery of the protease activity from *F. nucleatum*

A crude extract of cell-associated protease activity was initially obtained from *F. nucleatum* whole cells grown in BHI broth (48 h). The bacteria were pelleted by centrifugation (2087 x g, 40 min, 4^{o}C) and resuspended in ice-cold 25 mM Tris (pH 7.2) and subjected to sonication on ice with a Dawe Soniprep 150 at 15-20 W for 30 s (x 3) with cooling intervals. After centrifugation (2087 x g, 10 min, 4^{o}C) to remove intact cells and cellular debris, the resulting supernatant was passed through a 0.45 µm pore size filter (Corning Incorporated, Germany).

2.4 Proteolytic activity obtained by subculture fraction

A crude extract of the protease activity was obtained from whole cells by using different salt solutions to recover cell-surface associated protease activity.

Weakly cell-surface associated or soluble protease activity was obtained by gently mixing and re-suspended *F. nucleatum* pellet in phosphate-buffered saline (PBS) prior to centrifugation (2087 x g, 40 min, 4^{o}C) to remove whole cells. The resulting supernatant was sterile filtered and assigned as S1 (PBS wash), and the resulting pellet was designated as P1.

Stronger cell surface-associated protease activity was obtained by gently mixing P1 cells in 25 mM Tris (pH 7.2) containing 0.3 M KCL to remove additional cell surface-associated protease activity prior to centrifugation (2087 x g, 40 min, 4^{o}C) to remove whole cells. The resulting supernatant was sterile filtered and assigned as S2 (KCl wash), and the resulting pellet designated as P2. **Figure 2.1** shows a Gram-stained image of P1, and P2 to confirm the presence of intact cells, indicating that the cells were not ruptured by the extraction procedures and that the recovered protease activities represent material recovered from the cell surface rather than from lysed cells.

An intracellular or membrane-anchored protease activity was obtained by suspending P2 in 25 mM Tris (pH 7.2) followed by sonication as described above. The suspension was

subjected to centrifugation (2087 x g, 40 min, 4°C) and the supernatant subjected to a further round of centrifugation (100,000 x g, 15 min, 4°C) to remove any small membrane fragments, vesicles or other cellular debris. The resulting supernatant (S3) was designated as the cytoplasmic protein fraction and the resulting pellet (P3) consisted largely of cell membranes. Finally, the membrane rich fraction (P3) was subjected to solubilisation by suspending P3 in 25 mM Tris containing 0.2 % Triton-X-100 and the resulting supernatant (S4) was considered to contain membrane bound proteins.

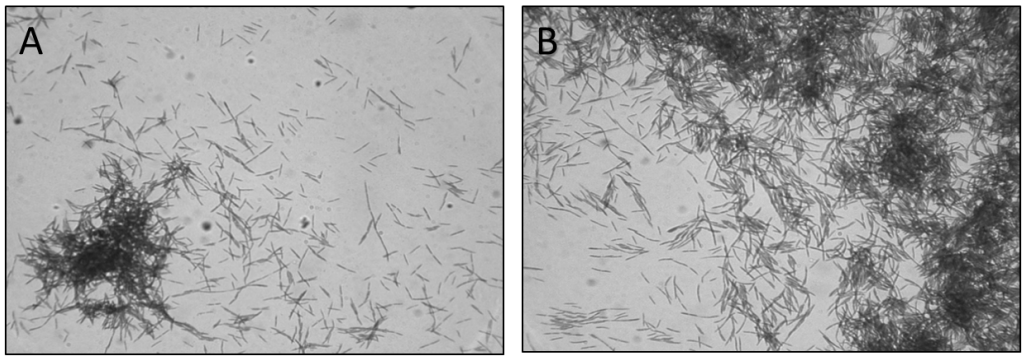

Figure 2.1: Gram stain of *F. nucleatum* recovered after extraction of protease activity by PBS and KCL wash. Panel A: *F. nucleatum* recovered after PBS wash, magnification (100 X). **Panel B:** *F. nucleatum* recovered after PBS containing 0.5 M KCl wash, magnification (100 X).

2.5 Protease activity assays

Proteolytic activity in crude extracts and partially purified fractions was assayed using the chromogenic substrates azocasein (casein conjugated to an azo-dye) or azoalbumin (Sulfanilic acid-azoalbumin) (Sigma, St. Louis, Mo.) essentially as described by Caldas et al. (2002) with minor modifications. 50 µl of sample were added to 50 µl of 2 % w/v azoalbumin in 25 mM Tris base (pH 7.2, supplemented with 0.2 M KCl, 5 mM $CaCl_2$, 1 mM $MgCl_2$ and 0.1 mM $ZnSO_4$). The mixture was then incubated at 37°C for 48-72 h. Non-digested azoalbumin was precipitated by the addition of 130 µl of 10 % v/v trichloroacetic acid (TCA), incubated on ice (4°C) for 30 min, and then centrifuged (1811 x g, 20 min). 100 µl of the resulting supernatant was transferred to 96-well plates containing 200 µl of 1 M NaOH and the absorbance was measured with a microplate reader (Victor) at 450 nm. Increased absorbance indicates the presence of proteolytic activity. Tris buffer (25 mM, pH

7.2) with and without KCl was treated exactly as a sample and was used as a control. Background absorbance was obtained by precipitating the substrate plus the sample in TCA without incubation.

2.6 Fractionation of the protease activities using fast protein liquid chromatography (FPLC)

Anion-exchange chromatography (Mono Q, HR 5/5) was carried out using a GE Healthcare AKTA FPLC apparatus. Bound proteins were eluted with a linear salt gradient (buffer A contained 25 mM Tris base (pH 7.2) and buffer B contained 25 mM Tris (pH 7.2) supplemented with 0.5 M KCl). Prior to FPLC, protease active material was dialysed (BioDesignDialysis tubing) against 25 mM Tris buffer (pH 7.2) using standard procedures to remove salt. Samples were loaded and eluted at a flow rate of 1 ml/min and 300 µl fractions were collected in 96-well plates.

2.7 *F. nucleatum* OMV recovery

F. nucleatum OMVs were recovered essentially, as described by Mullaney et al. (2009) with slight modification. *F. nucleatum* was grown in BHI medium under an anaerobic conditions at 37^{o}C in, typically, 10 x T-75 flasks, with each flask containing 50 ml of pre-reduced BHI. After 48 h of growth, the medium containing bacteria was transferred to 50 ml tubes and the bacteria were pelleted by centrifugation (2087 x g, 40 min, 4^{o}C). The resulting supernatant was filtered through a 0.45 µm filter. Following filtration, the supernatant was made 39 % with ammonium sulphate at room temperature and the mixture was incubated with stirring for 2 h at 4^{o}C prior to centrifugation (2087 x g, 40 min, 4^{o}C). The pellet containing the OMV was suspended in PBS and centrifuged (100,000 x g, 1 h, 4^{o}C) using a Sorvall Discovery SE100 ultracentrifuge. The resulting pellet was washed with PBS and centrifuged (100,000 x g, 30 min, 4^{o}C). Finally, the OMV pellet was re-suspended in 0.5 ml of PBS and stored in aliquots at -20^{o}C. Protein concentration was determined using either the BCA protein assay or a NanoDrop spectrophotometer (ND-8000).

2.8 Electron microscopy

OMV from *F. nucleatum* were subjected to electron microscopy analysis to determine purity and size heterogeneity. The OMVs were mounted onto carbon-colloidal-coated mesh grids and let settle for around 60 s. Residual non-adherent OMV were removed using filter paper wicks. The samples were left to air dry, and stained using 1 % v/v aqueous uranyl acetate prior examination by transmission electron microscopy on a Joel instrument.

2.9 Determination of protein concentration

Protein amounts were measured either by Nanodrop or by the DC or BCA protein assays (Bio-Rad), and a bovine serum albumin (BSA) was used as a protein standard where required. The standard curve was constructed by serially diluting a 1 mg/ml stock and all assays were performed in triplicate.

2.10 Proteins precipitation methods

2.10.1 Acetone precipitation method

Ice-cold acetone was used to dilute the sample (1:5), then it was mix thoroughly and incubated at -20^{o}C for at least 2 h. The sample then centrifuged at high speed for 5 min and the supernatant was then discarded. The remaining acetone was left to evaporate at room temperature. Then, the precipitated proteins, according to the need, were re-suspended by the reducing sample buffer (ordinary gel) or non-reducing sample buffer (zymography). The proteins were then boiled at 100^{o}C for 5 min.

2.10.2 Trichloroacetic acid precipitation (TCA) method

One volume of 100 % w/v of TCA was added to 4 volumes of the protein sample. This was then followed by 15 min incubation at 4^{o}C. The sample then centrifuged at high speed for

5 min and the supernatant was then discarded. The precipitated proteins were suspended in the reducing sample buffer (ordinary gel) or non-reducing sample buffer (zymography). In order to bring the solution into alkaline, 0.5 µl of saturated Tris base solution was added. The proteins were then boiled at 100^{o}C for 5 min.

2.11 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins were regularly fractionated and separated essentially as described by Laemmli (1970) using an Atto minigel apparatus (ATTO Corporation, Japan). Resolving and staking acrylamide gels prepared to the required as indicated in tables. Electrophoresis was carried out at 25 mA per gel for 1 h until the dye reached just above the gel base, at which stage electrophoresis was terminated. Two type of gels had been used throughout the experiments, both regular 12.5 % acrylamide gel (**Table 2.2**) or gradient gel (**Table 2.3**).

Table 2.2: Regular gel composition.

Component	Resolving gel (12.5 %)	Stacking gel (4 %)
ddH_2O	2.5 ml	3.0 ml
Acrylamide (30 %)	3.33 ml	0.67 ml
1.5 M Tris (pH 8.8)	2 ml	-
0.5 M Tris (pH 6.8)	-	1.25 ml
10 % SDS	80 µl	50 µl
10 % Ammonium persulphate	80 µl	50 µl
TEMED	8 µl	5 µl

Table 2.3: Gradient gel composition.

Component	20 %	15 %	5 %	4 %
Acrylamide (30 %)	6.66 ml	5 ml	1.665 ml	1.332 ml
1.5 M Tris (pH 8.8)	2.5 ml	2.5 ml	2.5 ml	2.5 ml
water	0.785 ml	2.445 ml	5.78 ml	6.168 ml
APS (10 %)	10 µl/3ml	10 µl/3ml	10 µl/3ml	10 µl/3ml
TEMED	1 µl/3ml	1 µl/3ml	1 µl/3ml	1 µl/3ml
Sucrose	1.5 g	1.5 g	-	-
Total volume	9.95	9.95	9.95	10

2.11.1 SDS-PAGE for MS analysis

Colloidal Coomassie G-250 was used to stain gel when required for MS analysis. The gel was incubated for overnight in fixer (50 % v/v methanol and 2 % v/v phosphoric acid) prior to a 1 h immersion in incubation solvent (34 % v/v methanol, 2 % v/v phosphoric acid and 17 % w/v ammonium sulphate). Coomassie blue G-250 (0.25 g in 20 ml methanol) was added to the incubation solvent and left to stain for 48-72 h with gentle agitation at room temperature.

2.11.2 Western blotting

Western blotting was carried out essentially as described by Towbin et al. (1979). Proteins (generally 25-50 μg/lane) were resolved typically by either by 12.5 % acrylamide or gradient acrylamide (4-15 %) SDS-PAGE gels. Proteins were transferred (1 mA/cm^2 for 1 h) to a polyvinylidene difluoride membrane (PVDF) using a semi-dry blotting apparatus (Atto). The blots were blocked with 5 % non-fat dry milk (Marvel) or 3 % BSA, in PBS, followed by probing of the membrane with the appropriate primary antibody at the recommended dilution, which then incubated for overnight at 4^oC. Blots were then washed several times with PBS containing 0.05 % v/v Tween-20, followed by incubation with the appropriate secondary antibody at the recommended dilution for 1 h at room temperature. After washing with PBS/Tween-20 antibody reactivity was visualised using enhanced chemiluminescence (ECL). Briefly, PVDF membranes were immersed for 1 min in 50 ml of Tris buffer (50 mM, pH 8.8) containing luminol (1.25 mM), iodophenol (400 μM), and hydrogen peroxide (0.01 %, v/v). Finally the blot was exposed to Kodak X-OMAT LS film for various periods of time to capture the image; the exposed film was developed and fixed using an automated AGFA developer.

In order to interpret western blotting results, loading control was used (Tubulin for cell extracts and PCNA for nuclear fractions). This is to compensate any different in proteins loaded in each lane. The size of each loading control band was measured by using image J software. The value of the largest band then divided by the value of other bands which

gave a factor (for each band). The resulted factors were multiplied by the size value of the corresponding sample bands.

2.11.3 Zymography

SDS-PAGE and Western blotting were performed using standard procedures. Zymography was performed essentially as described by Schmidt et al. (1988) with minor modifications. Gradient (4-20 %) acrylamide gels were copolymerized with 0.05-0.1 % w/v bovine gelatine. Non-reducing sample buffer was used to solubilise samples without heat denaturation. Gels were electrophoresed at constant current (25 mA/gel). Following electrophoresis, the gels were then washed for 1 h in Triton X-100 (2.5 %, v/v) with gentle shaking, to remove excess sodium dodecyl sulphate (SDS), followed by static incubation for approximately 24 h ($37^{o}C$) in 25 mM Tris buffer (pH 7.2), supplemented with 5 mM $CaCl_2$). Gels were then stained with Coomassie blue (R250) for at least 1 h followed by destaining in 30 % v/v methanol and 10 % v/v acetic acid. Zones of proteolysis were visualised as clear areas against a blue background.

2.12 Mass spectrometry analysis

Protein from SDS-PAGE gels were excised and transferred to sterile plastic tubes. These were then subjected to in-gel digestion, using a ProGest Investigator in-gel digestion robot (Genomic Solutions, Ann Arbor, MI) using standard protocols. The analysis was undertaken by Dr Catherine Botting (University of St Andrews, Scotland).

2.13 Transepithelial electrical resistance (TER)

Caco-2 or T-84 colonic epithelial cells were seeded on 0.4 µm filter inserts (uncoated polyethylene terephthalate plastic trace-etched membranes, Falcon, Beckton Dickinson, Oxford, United Kingdom) at a low density ($4X10^5$ cells/ml) in a 12-well plate (Falcon). The cells were fed both apically and basally every 24-48 h by changing the medium every 24-48 h. Cell confluence was assessed by phase contrast microscopy and measurement of the

TER, which was measured using an EVOM epithelial voltohmmeter apparatus. Typically fully polarised and differentiated monolayers took 10-14 days to form. TER measurements were taken at 24 h intervals. After the cells reached a fully polarised and differentiated state with functional tight junctions, as indicated by a stable TER reading, the monolayers were serum starved for 24 h prior to the addition of samples (bacteria cells, and OMVs). After treatment of the monolayers the incubation was continued and the TER monitored as appropriate, as indicated in the legend to figures. The TER of monolayers without any additions represented the control for each experiment. All treatments were made either in triplicate or quadruplicate, where indicated.

2.14 Biotinylation of *F. nucleatum* OMVs

50 µg of OMVs were biotinylated according to the manufacturer's protocol (EZ-Link Sulfo-NHS-Biotin #21217, Thermo Scientific). Briefly, the biotin reagent (10 mM) (freshly prepared) was added to the OMV suspension and incubated at room temperature for 30 min. PBS containing 100 mM glycine was then added to the suspension, which than centrifuged (21000 x g, 2 min) at room temperature; this was repeated 3 further times with re-suspension in PBS containing 100 mM glycine each time. A Western blot (probed with streptavidin-HRP) was undertaken to ensure the OMV were biotinylated.

2.15 Fluorescence labelling of *F. nucleatum* OMVs

Fluorescent labelling of *F. nucleatum* OMVs was performed according to the manufacturer's protocol (Alexa Fluor 488 Protein Labelling Kit, Invitrogen). Briefly, OMVs were suspended in PBS to make a total volume of 0.5 mL, 50 µL 1 M sodium bicarbonate was then added to the OMV suspension. This solution was transferred to the Alexa Fluor 488 reactive dye vial and incubated in the dark, with shaking for a period of 1 h. OMV were washed by ultracentrifugation to remove unreacted probe.

2.16 Immunofluorescence imaging.

Following treatment and incubation of cells as required, the medium was then removed by aspiration and the cells were washed with PBS (x 2) prior to fixing the cells with 4 % paraformaldehyde (PFA) in PBS for 15 min. This was then followed by two subsequent washes with PBS. Cells were then permeablized with 0.3 % v/v Triton X-100 in PBS for 5 min at room temperature, followed by blocking of non-specific protein binding sites with 3 % w/v bovine serum albumin (BSA) (Sigma) in PBS at room temperature for 1 h. Hoechst (nuclear stain, 1:2000) and phalloidin-TRITC (actin stain, 1:500) were added to the blocking buffer. The cells were again washed with PBS (x 2) and then incubated with primary antibody diluted in 3 % w/v BSA in PBS, using the manufacturer-specified dilution and then incubated overnight at 4^{o}C. The cells were washed then with PBS (x 3), followed by incubation with the appropriate secondary antibody (anti-mouse or anti-rabbit IgG) conjugated with Alexa 488 (Invitrogen) or Alexa 594 (Invitrogen) for 1 h at room temperature using the manufacturer-specified dilution. Cells were further washed by PBS and the cells were examined using an inverted light microscope (Nikon, Eclipse TE-300). Images were captured at different magnifications as indicated, where appropriate. Occasionally, analysis of immunofluorescence was undertaken by High Content Screening (HCS) or Confocal microscopy where indicated. HCS was performed using an InCell 1000 system. HCS images were obtained using a Nikon 10 X objective. Confocal microscopy was performed using Zeiss microscope using a 63 X objective.

2.17 Preparation of nuclear and cytoplasmic cellular extracts

Nuclear and cytoplasmic fractions of colonic epithelial cells (SW-480, SW-620, Lovo, and Caco-2) were prepared using Cytoplasmic Extraction reagent kit (Thermo Scientific, Meridian, USA). Extraction was performed according to the manufacturer's instructions. The nuclear and cytoplasmic extracts were stored at -20^{o}C until required. The protein concentration in each extract was determined by using NanoDrop spectrophotometer ND-8000.

2.18 STAT-3 phosphorylation

As described by Zhao and Dong (2016) with minor modifications, proteins were extracted from SW-480 using RIPA buffer (25 mM Tris-HCL pH 7.6, 150 mM NaCL, 1 % NP-40, 1 % sodium deoxycholate, 0.1 % SDS, and 1 % v/v of Phosphate inhibitor cocktail 3 (Sigma-aldrich)). This occurred, briefly, after the cells had been lysed and the contents were then centrifuged. The supernatant was collected for western blot. Then the proteins were denatured by loading buffer at 100^{o}C for 5 min and then subjected to 12 % SDS-PAGE. This followed by blotting the proteins into a PVDF membrane. The membrane was blocked by 5 % BSA in PBS at room temperature for 1 h. The primary antibodies were diluted in PBS (contains 5 % BSA, 0.1 % teen-20), and the membrane was incubated for overnight at 4^{o}C. The secondary antibodies were incubated on the following day for the same period of 1 h. The proteins bands were revealed by chemiluminescence detection system. All the antibodies were obtained from cell signalling technology, Ico. and were used at the recommended dilution.

2.19 RT-PCR

Different cell lines (SW-480, SW-620, T-84, and Caco-2) were seeded into a 24-plate until they reached around 80-90 % of confluence. The medium was replaced with serum, and penicillin and streptomycin free medium, which incubated for 2 h in 5 % CO_2 incubator at 37^{o} C. *F. nucleatum* (MOI of 500:1), and OMV (30 μg/ml) were added and co-incubated with the cells for time indicated (6, 24, 48, 72, and 96 h). the washed cells in PBS and the harvested cells were scraped with pastette, using PBS that transferred into four replicate wells. Cells were centrifuged and then stored at -70^{o}C. RNA extract using a total RNA isolation kit (NucleoSpin RNA, Macherey-Nagel) according to manufactory specification. The extracted RNA subsequently was subjected to reverse transcription using SensiFast Kit (Bioline) for making cDNA. Around 200-500 ng of RNA (as required) were added into 4 μl of 5x TransAmp buffer, in addition to 1 μl of reverse transcriptase. The total volume was then adjusted up to 20 μl with Dnase/Rnase free water. The mixture was then subjected to a thermal cycle using PTC-200 Peltier Thermal Cycler that performed under the following

condition: 25°C for 10 min (primer annealing), followed by 42°C for 30 min (reverse transcription), 85°C for 5 min (inactivation), and finally 4°C for 20 min (cooling step). The generated reaction product (cDNA) stored at -70°C. Subsequently, the generated cDNA from each specimen was subjected to RT-PCR. The RT-PCR was performed in PCR tube that contains 10 μl reaction mix (containing 1 μl of template cDNA, master SYBR (SensIfast, Bioline) mix, and each primer (0.4 μM)).

All used primers were obtained from Sigma (KicqStart) (**Table 2.4**) and were predesigned to amplify at least two exons in order to prevent the amplification of contaminating gDNA. Amplification was performed in a fluorescence thermocycler (Illumina Eco) under the following conditions: 15 min at 95°C (polymerase activation), followed by PCR (40 cycles), denaturation at 94°C for 1 min, annealing at 58°C for 1 min, and elongation at 72°C for 1 min. This followed by extension step for recording melt curve (default setting) at 95°C for 15 s, 55°C for 15 s, 95°C for 15 s. The specificity of the PCR product was verified by melting curve analysis. The housekeeping gene GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was amplified in parallel with the genes of interest. Relative copy numbers compared to GAPDH were calculated by $2^{-\Delta\Delta Cq}$ method (Livak and Schmittgen, 2001), using the following formula:

$\Delta\Delta C_q$ = A (C_q gene of interest – C_q internal control) – B (C_q gene of interest – C_q internal control).

RT-PCR was performed in triplicate for each RNA sample. For simplification, the RT-PCR data replicates of each experiment were analysed assuming that the additive effect of gene, concentration, and replicate can be adjusted, and the ΔCq can be obtained by subtracting the Cq value of the target gene from that of the reference. The ΔCq of the control and the sample can be analysed using the t-test that will yield the estimation of ΔΔCq (Yuan et al., 2006). Despite the fact that some published studies prefer not to present p-value and confidence intervals (Zenoni et al., 2004, Eleaume and Jabbouri, 2004).

Table 2.4: Primer sequences.

Primer	Forward sequence	Reverse sequence
GAPDH	ACAGTTGCCATGTAGACC	TTTTTGGTTGAGCACAGG
IL-8	GTTTTTGAAGAGGGCTGAG	TTTGCTTGAAGTTTCACTGG
CXCL-1	ATGCTGAACAGTGACAAATC	TCTTCTGTTCCTATAAGGGC
TNF-α	AGGCAGTCAGATCATCTTC	TTATCTCTCAGCTCCACG
IL-1 β	CTAAACAGATGAAGTGCTCC	GGTCATTCTCCTGGAAGG
IL-6	GCAGAAAAAGGCAAAGAATC	CTACATTTGCCGAAGAGC
CCL-20	TATATTGTGCGTCTCCTCAG	GCTATGTCCAATTCCATTCC
MMP-1	AAAGGGAATAAGTACTGGGC	CAGTGTTTTCCTCAGAAAGAG
MMP-2	GTGATCTTGACCAGAATACC	GCCAATGATCCTGTATGTG
MMP-9	AAGGATGGGAAGTACTGG	GCCCAGAGAAGAAGAAAAG
MMP-10	AGCGGACAAATACTGGAG	GTGATGATCCACTGAAGAAG
MMP-13	AGGCTACAACTTGTTTCTTG	AGGTGTAGATAGGAAACATGAG
BMP-1	GATGTGAAAAAGGACTATGGC	AATCTCAAAGGACTGGAATG
FN-1	CCATAGCTGAGAAGTGTTTTG	CAAGTACAATCTACCATCATCC
ITGA-5	AAGCTTGGATTCTTCAAACG	TCCTTTTCAGTAGAATGAGGG
CDH-1	CCGAGAGCTACACGTTC	TCTTCAAAATTCACTCTGCC
CDH-2	ACATATGTGATGACCGTAAC	TTTTTCTCGATCAAGTCCAG
Snal-1	CTCTAATCCAGAGTTTACCTTC	GACAGAGTCCCAGATGAG
Snal-2	CAGTGATTATTTCCCCGTATC	CCCCAAAGATGAGGAGTATC
Snal-3	TCCTTCCTGGTGAAAACG	CACCATTGATTTCTCTCTGC
ZEB-1	AAAGATGATGAATGCGAGTC	TCCATTTTCATCATGACCAC
TWIST-1	CTAGATGTCATTGTTTCCAGAG	CCCTGTTTCTTTGAATTTGG
Vimentin	GGAAACTAATCTGGATTCACTC	CATCTCTAGTTTCAACCGTC
NF-κβ 1	GACAACTATGAGGTCTCTGG	ATCACTTCAATTGCTTCGG
NF-κβ 2	TGAAGATTTCTCGAATGGAC	ACCTCAATGTCATCTTTCTG
SOCS-3	CCTATTACATCTACTCCGGG	ACTTTCTCATAGGAGTCCAG
SPHK-1	TTCCTTGAACCATTATGCTG	GATACTTCTCACTCTCTAGGTC
PTGS-2	AAGCAGGCTAATACTGATAGG	TGTTGAAAAGTAGTTCTGGG
Wnt-7 α	AAAGATCCTGGAGGAGAAC	TGATCTTCAGGAAGGTGG
Wnt-7 β	GCAGGAAGGTTCTAGAGG	GTTGTACTTCTCCTTCAGC
Wnt-9 α	GGTGTGAAGGTGATCAAG	TGCCGTCTCATACTTGTG
MYC	TGAGGAGGAACAAGAAGATG	ATCCAGACTCTGACCTTTTG
16S rRNA	AGAGTTTGATCCTGGCTCAG	GTCATCGTGCACACAGAATTGCTG
FadA	CACAAGCTGACGCTGCTAGA	TTACCAGCTCTTAAAGCTTG

2.20 Cytokine detection

Cytokines were detected by ELISA using Duo Set ELISA kits (R&D) according to the manufacturer's instructions. A summary of the antibodies used in immunofluorescence and Western blotting are found in the following (**Table 2.5**).

Table 2.5: Summary of antibodies used in immunofluorescence and Western blotting.

Antibody	M W (KDa)	Dilution used		Supplier	Code
		immunofluorescence	western blotting		
Phalloidin	-	1:500	-		
Hoechst	-	1:2000	-	Invitrogen, USA.	33258
Polyclonal Rabbit anti-ZEB1	124	1:200	1:500	Novus Biologicals.	NBP1-88845
Polyclonal Rabbit anti-ZEB1	-	1:200	1:2000	Novus Biologicals.	NBP1-05987
Goat anti-rabbit Alexa-green	-	1:500	-	Invitrogen, USA.	A11008
Monoclonal Mouse anti-vimentin	57	1:50	1:100	Darko, Denmark.	M0725
Monoclonal Mouse anti-vimentin	57	1:100	1:4000	BD Transduction laboratories, USA.	550513
Monoclonal Mouse anti-β-catenin	92	1:1000	1:1500	BD Transduction laboratories, USA.	610154
Mouse anti-E-cadherin	120	1:50	1:2000	BD Transduction laboratories, USA.	610182
Alexa-488 green anti-mouse IgG	-	1:500	-	Invitrogen, USA.	A11029
Alexa-568 red anti-mouse IgG	-	1:500	-	Invitrogen, USA.	A11031
Polyclonal swine anti rabbit IgG-HRP	-	-	1:2000	Darko, Denmark.	P0217
Rabbit anti mouse IgG-HRP	-	-	1:2000	Darko, Denmark.	P0260
Monoclonal Anti-PCA-HRP (nuclear loading control)	30	-	1:2000	Novus Biologicals.	NB500-106H
Polyclonal Rabbit anti-actin (loading control)	42	-	1:200	Sigma	A2066
Monoclonal Mouse anti-tubulin (loading control)	50	-	1:2000	Sigma	T6199-200UL
Monoclonal rabbit anti-STAT-3	86	-	1:1000	Cell Signalling	D3Z2G
Monoclonal rabbit anti-p-STAT-3	86	-	1:2000	Cell Signalling	D3A7

2.21 Lipopolysaccharide (LPS) isolation and lipid A from *F. nucleatum*

The method that was adapted is derived from Eugene and Hackett (2000) with some modifications, in which 50 ml of tube pellet with around 10 mg of whole *F. nucleatum* cells were suspended in a 1 ml of Tri-Reagent (Sigma) vortex which was used to achieve complete cell homogenisation. This was then followed with the addition of 200 μl of chloroform (20 μl/mg of cells) to create a phase separation. The mixture was then vigorously vortexed and the resulting mixture was centrifuged at 12000 rpm (18300 x g) for 10 min to separate the aqueous and organic phase. The aqueous phase was transferred into a new minifuge tube that was re-extracted by adding 200 μl DNAse and RNAse free water. This is followed by pooling the aqueous phases, which were dried by using speed vacd dry on medium heat. The left pellet at O/N and then the washed pellet dissolved in 0.5 ml 0.375 M $MgCl_2$ in 95 % ethanol, which was kept at -20^{o}C for 1 h. This was then followed by centrifugation 15 at 10000 rpm at 4^{o}C for 1 min and where white pellet become evident. The pellet was then washed with 95 % ethanol and subsequently with 100 % ethanol. The pellet was then dried on a heat block at 35^{o}C.

2.22 Incubation of SW-480 cells with 5-Flourouracil (5-Fu)

The procedure was performed essentially as described by Pereira et al. (2016) and Kong et al. (2015) with minor modifications. SW-480 cells were seeded in a 96-well plate and incubated in CO_2 for 24 h to allow cell attachment. Then the cells were exposed to 5-Fu (0.78, 1.56, 3.13, 6.25, 12.5, 25, and 50 μg/ml), *F. nucleatum* (MOI 200:1), and *F. nucleatum* OMV (50 μg/ml) which were incubated for 48 h. in vitro. Cell proliferation was assessed using the cellTiter 96-well non-radioactive cell proliferation assay (Promega, USA). The MTS metabolism was assayed by the addition of 20 μl of tetrazolium/phenazine methosulfate (MTS/PMS) reagent in to each well (19:1) and incubated for 1-4 h in 5 % Co2 incubator at 37^{o}C. Plate was shaken for 10 s and the florescence reading were taken at 590 nm. The statistical significant was calculated using the two-way ANOVA (Bonferroni multiple comparison).

2.23 In vitro wound-closure assay

Wound-closure experiment were performed as described by Matthay et al. (1993) with minor modification. T-84 cells were grown in 48-well plate until confluence. In each well a scratch was made using a sterile tip, and cellular debris was removed by washing with fresh medium. T-84 cell incubated with *F. nucleatum* OMV (10 and 20 μg/ml) for 48 and 72 h. The width of the scratch in each well was measured by using image J software at each time point.

2.24 Statistical analysis

Each experiment was carried out in triplicate or as indicated in each experiment. comparative analysis of the comparisons between the control and treatment groups using one-factor analysis of variance was carried out and the results were expressed as a mean ± SD. A student's t-test was used to determine the statistical significance using GraphPad Prism software (San Digo, CA, USA). Differences were considered significant with $P < 0.05$.

CHAPTER THREE

Proteomic and Biochemical Characterisation of *F. nucleatum* Outer Membrane Vesicles

3.1 Introduction

The study of the pathogenic potential of bacterial OMV is a recently emerging field concerned with how these microvesicles interact with human tissues and cells. Their importance as contributors to diseases and their use as acellular vaccines is indicated by the increased number of publications in the past decade. One of the main characteristics of gram-negative and positive bacteria is outer membrane vesicle production, which are constantly discharged from the bacterial surface in vitro and in vivo (Jun et al., 2013). Mass spectrometry-based proteomic analyses of OMV have revealed the presence of hundreds of proteins including toxins and other virulence factors. Such studies provide clues which are revealing the pathophysiological function and biogenesis of OMV (Lee et al., 2016). During OMV production by known pathogens various pathogenesis-associated molecules are incorporated into the vesicles including LPS, adhesins and porins that mediate bacterial adhesion and induce inflammatory and other pathogenic responses by target host cells (Olofsson et al., 2010, Ellis et al., 2010). Additionally, OMVs are vectors for other virulence factors such as proteases (Xie, 2015), and toxins (Dutta et al., 2004). These proteins are involved in modulating host immune responses and are associated with the pathogenesis of many diseases. In this regard, this study aimed to identify and characterise the proteomic composition of *F. nucleatum* OMV to better understand how these structures could modulate host cellular response in the context of CRC.

3.2 Hypothesis

In this work, it has been hypothesised that *F. nucleatum* produce OMV in vitro and those OMV modulate colonic epithelial cell responses in a manner that may promote carcinogenesis.

Aims of this study:

- To purify *F. nucleatum* OMV and determine their proteomic composition by mass spectrometry.
- To evaluate the ability of the OMV to degrade host proteins and modulate barrier function in vitro.

3.3 Results

3.3.1 Purification and mass spectrometry analysis of outer membrane vesicles recovered from *F. nucleatum.*

The OMV from *F. nucleatum* (ATCC 25586) were purified as described in Methods and visualised under transmission electron microscopy (TEM) (**Figure 3.1, A**). The OMV ranged in size from approximately 20 nm-250 nm in diameter. The purified OMVs, free from cellular debris as determined by TEM, were subjected to gradient (4-20 %) SDS-PAGE (**Figure 3.1, B**) and the fractionated proteins were excised from the gel in a total of 10 pieces, as indicated in the figure. Each gel piece was processed for analysis by mass spectrometry by Dr. Catherine Botting at the University of St. Andrews.

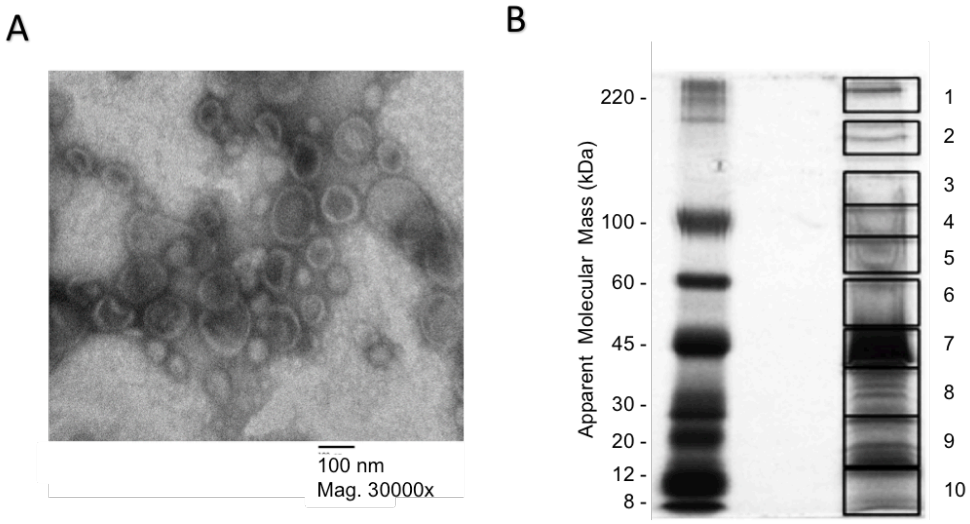

Figure 3.1: Transmission electron micrograph and electrophoretic analyses of OMV from *F. nucleatum*. Panel A: TEM image of purified OMV from *F. nucleatum* (magnification 30,000x; scale bar: 100 nm). **Panel B:** Gradient (4-20 %) SDS-PAGE analysis of *F. nucleatum* OMV. The gel lane was cut into ten separate pieces, as indicated by the boxed areas. The protein constituent in each gel piece was determined by MS analysis as described in Methods. The molecular mass markers are shown on the left-hand side.

A summary of the results of the MS-analysis is shown in **Figure 3.2, A**. The putative subcellular location of the identified proteins was predicted using pSORTb (v3) software tool for the predicted identification of the subcellular location of the OMV constituent proteins (Nancy et al., 2010). Interestingly, the majority of the OMV-associated proteins

are represented by cytoplasmic proteins (52 %), and the remainder was composed of outer membrane proteins (11 %), periplasmic proteins (4 %), cytoplasmic membrane proteins (9 %) with the balance (24 %) represented by proteins of unknown subcellular distribution. The high percentage of cytoplasmic constituents associated with *F. nucleatum* OMV is likely due to the presence of so-called 'inner-outer membrane vesicles' (I-OMVs). These are characterised by the presence of both the inner cytoplasmic membrane and the outer membrane of the parental bacterium. Both I-OMV and single membrane (outer membrane) vesicles were recovered from *F. nucleatum* (**Figure 3.2, B**).

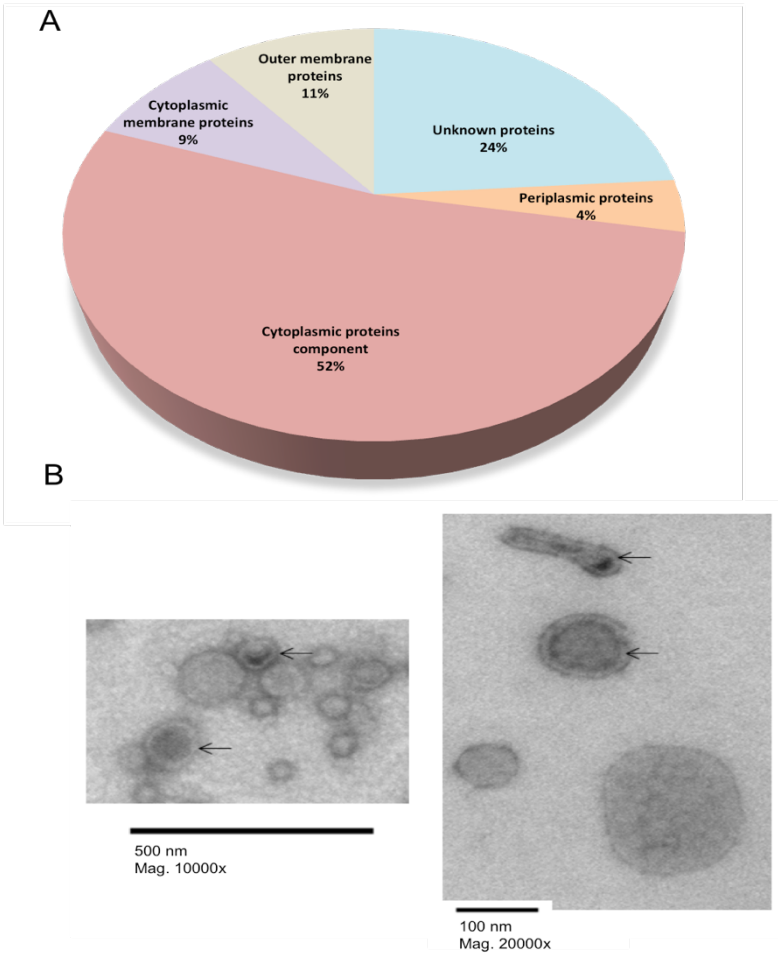

Figure 3.2: Mass spectrometric analysis of *F. nucleatum* OMV proteome. Panel A: Pie-chart summarising the OMV proteome. **Panel B:** TEM images of the OMV preparation indicating the presence of bi-layered I-OMVs (arrows). Left image magnification 10,000x; scale bar: 500 nm and right image magnification 20,000x; scale bar: 100 nm.

The presence of I-OMV provides an explanation for the presence of cytoplasmic proteins in the proteome as these vesicles represent structures that have encapsulated cytoplasmic

constituents in addition to periplasmic proteins. Further, identification of inner membrane proteins in the proteome supports the presence of I-OMV.

A detailed listing of the proteins identified in the MS analysis of the OMV from *F. nucleatum* is shown in **Table 3.1-3.5**. In total, 366 proteins were identified, comparable to the number of proteins identified in the MS analysis of OMV from other species of bacteria. **Table 3.1** lists the cytoplasmic proteins (193), **Table 3.2** lists the cytoplasmic membrane proteins (32), **Table 3.3** lists the periplasmic proteins (15), **Table 3.4** lists outer membrane proteins (37), and **Table 3.5** lists proteins of unknown subcellular location (89).

Table 3.1: Cytoplasmic proteins (193, 52.32 %) identified in *F. nucleatum* OMV.

Accession number (a)	Protein score (b)	% Coverage (c)	Number of peptide matches (d)	Description
gi|19704946	205	42.8	16	50S ribosomal protein L15P
gi|19705258	112	19.4	3	Hypothetical Protein FN1956
gi|19703929	131	43.3	18	(3R)-hydroxymyristoyl-ACP dehydratase
gi|19705272	130	33.5	11	4-amino-4-deoxychorismate lyase
gi|19705330	481	54.5	46	50S ribosomal protein L1
gi|19705329	153	47.1	12	50S ribosomal protein L10P
gi|19704452	139	42.3	15	50S ribosomal protein L21P
gi|19704636	102	25	23	ABC transporter ATP-binding protein
gi|19703509	946	69.4	45	Anhydro-N-acetylmuramyl-tripeptide amidase
gi|19703677	207	25.5	14	Aspartate/aromatic aminotransferase
gi|19703545	329	64.9	18	Biotin carboxyl carrier protein of glutaconyl-COA decarboxylase
gi|19704783	233	33.3	17	Cell division protein FtsZ
gi|19705415	124	16.6	15	DNA gyrase subunit A
gi|19703626	79	4.2	5	DNA polymerase III subunit alpha
gi|19704618	105	29.8	17	DNA-directed RNA polymerase subunit alpha
gi|19704887	3191	84.5	157	Elongation factor Tu
gi|19703700	156	31.8	13	F0F1 ATP synthase subunit beta
gi|19705372	175	14.7	9	Formate tetrahydrofolate ligase
gi|19703370	446	52.6	22	Hypothetical protein FN0018
gi|19704123	100	58.3	13	Hypothetical protein FN0788
gi|19704424	138	31	10	Hypothetical protein FN1089
gi|19704860	249	61	23	Hypothetical protein FN1528
gi|19704939	384	53.1	27	Hypothetical protein FN1618

gi|19705040	1407	76.2	99	Hypothetical protein FN1719
gi|19705282	131	26.2	12	Hypothetical protein FN1986
gi|19705408	234	73.1	29	Hypothetical protein FN2118
gi|19703633	76	34.9	8	Hypoxanthine-guanine phosphoribosyltransferase
gi|19704566	141	37	19	Inosine-5'-monophosphate dehydrogenase
gi|19705114	92	17.7	6	Iron/zinc/copper-binding protein
gi|19704871	155	19.1	5	Iron-sulfur cluster-binding protein
gi|19703419	84	10.3	10	Isoleucyl-tRNA synthetase
gi|19704504	94	18.6	7	L-lactate dehydrogenase
gi|19705000	121	31.4	11	LPS biosynthesis protein WbpG
gi|19704441	96	12.7	4	L-serine dehydratase
gi|19703801	106	22.5	15	lysyl-tRNA synthetase
gi|19703494	571	43.8	30	Malonyl-coa-[acyl-carrier-protein] transacylase
gi|19704917	189	30.8	16	Nitrogen fixation iron-sulphur protein RNFC
gi|19703769	85	13.7	4	Orotate phosphoribosyltransferase
gi|19704711	132	29.2	16	Oxaloacetate decarboxylase
gi|19705105	215	33.7	30	Peptidyl-prolyl cis-trans isomerase
gi|19705412	607	44	63	Phenylalanyl-tRNA synthetase beta chain
gi|19704051	94	15	7	Phophatidylinositol-4-phosphate 5-kinase
gi|19704507	1046	52.8	75	Phosphate acetyltransferase
gi|19703989	245	54	26	Phosphoglycerate kinase
gi|19704064	156	44.3	15	Phosphoglycerate mutase
gi|19704323	90	14.8	6	Phosphoribosylaminoimidazole-succinocarboxamide synthase
gi|492611374	355	48.5	30	Phosphotransacetylase
gi|19705045	87	17	4	Potassium uptake protein KtrA
gi|19704305	107	32	8	Precorrin-8X methylmutase
gi|19705039	271	41.2	72	Preprotein translocase subunit SecA
gi|19704888	487	38.5	51	Elongation factor G
gi|19704622	102	22	2	Protein translation initiation factor 1
gi|492614315	117	33.5	8	PTS glucose transporter subunit IIA
gi|19704250	254	57.3	32	PTS system, N-acetylglucosamine-specific IIA component
gi|19704795	98	36.4	9	Pyridoxal biosynthesis lyase PdxS
gi|19704505	689	27.9	59	Pyruvate-flavodoxin oxidoreductase
gi|19705288	86	16.8	7	Ribose-phosphate pyrophosphokinase
gi|19704944	125	12.6	4	Ribosome recycling factor (RRF)
gi|19704912	168	25.2	8	RNFB-related protein
gi|19704093	124	39.9	22	Rod shape-determining protein MreB

gi|19704013	320	40	18	Ser/Thr protein kinase
gi|19713538	150	32.3	14	Seryl-tRNA synthetase
gi|19703829	189	35	16	Short chain dehydrogenase
gi|19703796	257	60.2	23	Sigma (54) modulation protein
gi|19705007	434	40.2	34	Spore coat polysaccharide biosynthesis protein spsF
gi|19704339	81	22.3	6	TetR family transcriptional regulator
gi|19705322	243	30.3	14	Thiamine biosynthesis lipoprotein apbE
gi|19703445	230	69.9	12	Thioredoxin FN0093
gi|19704743	179	21.5	5	Threonine dehydratase
gi|19703946	98	39.6	8	Threonyl-tRNA synthetase
gi|19703640	240	40.8	11	Transketolase
gi|19705316	372	29.7	27	Translation initiation factor IF-2
gi|19703670	77	29.8	10	Translation initiation factor IF-3
gi|19705269	164	21.9	7	Translation initiation inhibitor
gi|19704701	118	47	13	Triosephosphate isomerase
gi|19705044	143	25	18	tRNA uridine 5-carboxymethylaminomethyl modification enzyme GidA
gi|19705248	94	17.2	9	Tryptophanase
gi|19705010	123	45.2	26	UDP-4-dehydro-6-deoxy-2-acetamido-D-glucose 4-reductase
gi|19703818	198	42.1	19	Uracil phosphoribosyltransferase
gi|19704943	78	31.8	12	Uridylate kinase
gi|19704127	120	18.4	13	Urocanate hydratase
gi|19703623	94	17	5	Xaa-His dipeptidase
gi|19704614	102	16.6	7	Zinc metallohydrolase
gi|19705172	98	28.7	7	Zn-dependent alcohol dehydrogenase and related dehydrogenase
gi|19703553	826	52.9	39	(R)-2-hydroxyglutaryl-CoA dehydratase beta-subunit
gi|19704868	163	24.2	12	(S)-2-hydroxy-acid oxidase chain D
gi|19704796	156	15.3	9	1-deoxy-D-xylulose-5-phosphate synthase
gi|19704772	100	21.3	7	1-phosphofructokinase
gi|19704851	173	28.6	8	23S rRNA methyltransferase
gi|19704840	120	43.6	26	3,4-dihydroxy-2-butanone-4-phosphate synthase
gi|19704967	322	62.1	25	30S ribosomal protein S10P
gi|19704724	276	59.8	16	30S ribosomal protein S16P
gi|19704941	265	47.4	33	30S ribosomal protein S2
gi|19704960	234	52.1	22	30S ribosomal protein S3P
gi|19704951	592	53.8	39	30S ribosomal protein S8P
gi|19705086	291	24.2	38	4-hydroxy-3-methylbut-2-enyl diphosphate reductase/

gi|19704420	89	30.8	7	4-methyl-5 (B-hydroxyethyl)-thiazole monophosphate biosynthesis enzyme
gi|19704956	506	50.8	24	50S ribosomal protein L14P
gi|19703772	470	61.2	46	50S ribosomal protein L19
gi|19703668	323	40.5	21	50S ribosomal protein L20
gi|19704950	284	58.8	33	50S ribosomal protein L6
gi|19705133	151	37.6	13	50S ribosomal protein L9P
gi|19703830	403	49.5	25	acetyl-CoA acetyltransferase
gi|19705279	146	27.7	8	Alkyl hydroperoxide reductase C22 protein
gi|19703392	148	27.5	13	Asparaginyl-tRNA synthetase
gi|19705161	812	65.4	46	Butyrate-acetoacetate CoA-transferase subunit B
gi|19703464	316	31.1	22	Chaperone protein DnaK
gi|19704151	163	66.4	17	Dehydrogenase
gi|19705185	86	41.1	12	Dihydropteridine reductase
gi|19703822	224	41.4	20	D-lactate dehydrogenase
gi|19705416	131	18.9	16	DNA gyrase subunit B
gi|19705326	109	6.8	9	DNA-directed RNA polymerase subunit beta'
gi|19704865	386	45.5	28	Electron transfer flavoprotein subunit alpha
gi|19705083	370	45.6	25	Enolase
gi|19703607	1516	52.2	153	Formate acetyltransferase
gi|19703667	999	74.9	68	Fructose-1,6-bisphosphate aldolase
gi|19705008	151	40.4	16	Gluconate 5-dehydrogenase
gi|19703547	130	31.2	15	Glutaconate coa-transferase subunit A
gi|19704729	90	25	3	Glutaminase
gi|19704192	308	29.3	30	Glycogen phosphorylase
gi|19703422	125	37.3	57	Glycyl-trna synthetase beta chain
gi|19703383	102	21	5	Hypothetical Protein FN0031
gi|19703454	243	55.7	12	Hypothetical Protein FN0106
gi|19703674	124	16.7	6	Hypothetical Protein FN0331
gi|19703891	165	39.3	7	Hypothetical Protein FN0556
gi|19704023	246	41.8	9	Hypothetical Protein FN0688
gi|19704155	223	35.9	23	Mercuric Reductase
gi|19704603	110	10.8	6	Methionyl-trna synthetase
gi|19704036	80	12.2	7	Methyltransferase
gi|496072988	191	27.6	21	Molecular chaperone GroEL
gi|19704011	189	64.4	7	Molecular chaperone GroES
gi|19704060	196	45.7	13	molybdopterin biosynthesis MoeB protein
gi|19705005	208	34.2	12	N-acetylneuraminate synthase
gi|19704414	96	27.1	12	Neutrophil-activating protein A
gi|19704889	236	78.8	25	30S ribosomal protein S7

gi|19704355	149	50	19	3-hydroxybutyryl-coa dehydratase
gi|19705331	661	63.1	38	50S ribosomal protein L11P
gi|19703672	436	69.4	28	50S ribosomal protein L13
gi|19704617	100	45.7	28	50S ribosomal protein L17P
gi|19704961	155	39.6	11	50S ribosomal protein L22P
gi|19704966	572	44.5	30	50S ribosomal protein L3
gi|19704506	254	35.7	21	Acetate kinase
gi|19704638	337	44.3	17	Adenosylcobalamin-dependent diol dehydratase gamma subunit
gi|19704784	126	23	11	Cell division protein ftsa
gi|19705246	126	15.5	17	ClpB protein
gi|19704932	86	13.8	2	Competence protein
gi|19703410	243	45.1	33	Cysteine desulfhydrase
gi|19704555	486	40.8	36	Cysteine synthase
gi|19705146	78	36.1	12	Dihydroxyacetone kinase
gi|19703888	127	24.9	22	D-serine dehydratase
gi|161485655	576	60.3	40	Elongation factor Ts
gi|19703548	214	49.1	19	Glutaconate coa-transferase subunit B
gi|19703987	178	31.9	27	Glyceraldehyde 3-phosphate dehydrogenase
gi|19703666	102	21.3	12	Heat shock protein 90
gi|19704397	90	23.5	8	Hydrolase
gi|19703661	380	70.9	32	Hypothetical protein FN0316
gi|19703794	160	23.8	6	Hypothetical protein FN0459
gi|19704181	470	56.2	40	Hypothetical protein FN0846
gi|19704259	95	12.1	3	Hypothetical protein FN0924
gi|19704948	197	51.8	22	30S ribosomal protein S5P
gi|19704965	368	38.3	31	50S ribosomal protein L4
gi|19705162	864	66.8	60	Acetoacetate:butyrate/acetate coenzyme A transferase
gi|19704633	134	64	15	Adenylate kinase
gi|19703644	121	22	11	Aspartyl-trna synthetase
gi|19704715	99	12.6	6	Citrate lyase beta chain
gi|19703592	684	68.1	45	Cytoplasmic protein
gi|19703911	108	17.1	5	D-amino acid dehydrogenase large subunit
gi|19704284	127	8.4	11	DNA helicase
gi|19704055	106	23.5	7	Elongation factor P
gi|19703519	95	26.1	7	Enoyl-[acyl-carrier-protein] reductase
gi|19704074	99	32.1	8	Formiminotetrahydrofolate cyclodeaminase
gi|492612068	696	56.7	54	Glutamate dehydrogenase
gi|19704076	120	33.3	12	Glutamate formiminotransferase
gi|19705283	99	19.4	7	GntR family transcriptional regulator
gi|19704158	137	21.5	14	GTP-binding protein hflX

gi|19704816	168	26.5	7	Hypothetical protein FN1484
gi|19703947	106	36.7	7	Hypothetical protein FN0612
gi|19704311	114	23.6	8	Hypothetical protein FN0976
gi|19704619	285	62.1	34	30S ribosomal protein S4
gi|19704953	392	60.1	37	50S ribosomal protein L5
gi|19704656	594	36.7	41	Acetoacetate metabolism regulatory protein atoC
gi|19704118	303	38.3	30	Acyl-coa dehydrogenase
gi|19705363	173	33.3	11	Adenine phosphoribosyltransferase
gi|19705019	253	30.9	17	dTDP-4-dehydrorhamnose reductase
gi|19704119	806	56.5	49	Electron transfer flavoprotein subunit beta
gi|19703787	271	29.2	24	Glucosamine--fructose-6-phosphate aminotransferase [isomerizing]
gi|19704881	191	47.6	20	Hypothetical protein FN1549
gi|19704963	281	43.5	19	50S ribosomal protein L2
gi|19703702	132	22.8	14	F0F1 ATP synthase subunit alpha
gi|19703594	127	42	8	Hypothetical protein FN0249
gi|19705178	190	54.5	9	Bis(5'-nucleosyl)-tetraphosphatase
gi|19703549	585	35.6	50	Glutaconyl-coa decarboxylase A subunit
gi|19703914	2000	46.9	143	Cytoplasmic protein

(a) Accession number: a unique identifier assigned to the protein by FASTA database[2]; **(b) Protein score:** the protein score is the sum of the highest ions score for each distinct sequence[3]; **(c) % Coverage:** The percentage of all the amino acids in the protein sequence that were covered by identified peptides detected in the sample, It is calculated from the length and the set of peptides assigned to the protein[4]; **(d) Number of peptide matches**: The number of distinct peptide sequences in the protein group.

Table 3.2: Cytoplasmic membrane proteins (32, 8.72 %) identified in the proteome of *F. nucleatum* OMV.

Accession number	Protein score	% Coverage	Number of peptide matches	Description
gi|19704586	84	5.8	2	High-affinity iron permease
gi|19704003	149	39.1	16	High-affinity zinc uptake system protein znua precursor
gi|19703356	158	20.5	11	Inner membrane protein
gi|19703718	151	17.3	8	Iron ABC transporter ATP-binding protein sfuC

[2] http://www.matrixscience.com/training/2.5/K._Sequence_Database_Administration.pdf

[3] http://www.matrixscience.com/help/interpretation_help.html

[4] http://www.matrixscience.com/help/export_help.html

gi|19704697	111	14.6	3	Peptide ABC transporter ATP-binding protein
gi|19704540	404	46.8	36	Protease FN1205
gi|19704606	121	13.3	14	Protease IV FN1271
gi|19703684	130	7.7	3	Transport protein FN0341
gi|19704462	230	20.5	15	Hypothetical protein FN1127
gi|19705281	164	19.4	10	Hypothetical protein FN1985
gi|19704202	78	20.2	21	Long-chain-fatty-acid-Coa ligase
gi|19705321	255	15.9	14	Membrane-bound proton-translocating pyrophosphatase
gi|19704863	116	17.6	6	Negative regulator of murein hydrolase
gi|19704034	78	14.1	5	Protein translocase subunit SecD
gi|19704670	138	40.4	9	Protein translocase subunit YajC
gi|492609716	119	12.4	9	PTS fructose transporter subunit IIC
gi|19704773	295	19.7	20	PTS system, fructose-specific IIABC component
gi|19705163	167	5.2	6	Short-chain fatty acids transporter
gi|19703712	82	39.5	15	Signal peptidase I
gi|19705203	119	20.3	12	Sugar transport ATP-binding protein
gi|19703496	293	35.8	17	3-oxoacyl-[acyl-carrier-protein] synthase
gi|19704546	126	15.5	10	Cell division protein Fts I
gi|19703805	113	4.3	3	Efflux pump component MtrF
gi|19704501	151	21.6	14	Galactose/methyl galaxtoside transporter ATP-binding protein
gi|19703566	134	11	9	Carbon starvation protein A
gi|19704710	85	12.3	4	Citrate-sodium symport
gi|496295757	152	37	18	Gtpase Der
gi|19704112	280	26.7	26	GTP-binding protein lepA
gi|19704050	184	25.5	12	Hypothetical protein FN0715
gi|19703521	666	76.9	52	Cell division inhibitor MinD
gi|19703515	86	20.5	13	GTP-binding protein EngA
gi|19703726	86	10.1	5	Hypothetical protein FN0384

Table 3.3: Periplasmic proteins (15, 4.09 %) identified in the proteome of *F. nucleatum* OMV.

Accession number	Protein score	% Coverage	Number of peptide matches	Description
gi|19704500	1261	68.9	73	D-galactose-binding protein
gi|19704333	527	52	40	Dipeptide-binding protein FN0998
gi|19703738	3874	67.9	252	Dipeptide-binding protein FN0396

gi|19704855	380	37.5	35	Dipeptide-binding protein FN1523
gi|19704446	92	14.7	8	Dipeptide-binding protein FN1111
gi|19703717	1145	57.7	66	Iron(III)-binding protein
gi|19704730	92	8.1	4	Amino acid carrier protein AlsT
gi|19704522	91	16.3	4	Amino acid-binding protein
gi|19705117	76	12.6	3	Manganese-binding protein
gi|19704804	112	20.8	7	N-acetylneuraminate-binding protein
gi|19704836	528	37	34	Nickel-binding protein
gi|19704648	95	10.3	5	Oligopeptide-binding protein oppa FN1313
gi|19704973	176	17.8	10	Oligopeptide-binding protein oppa FN1652
gi|19704470	91	37.9	20	Phosphonates-binding protein
gi|19703953	225	47.4	22	Spermidine/putrescine-binding protein

Table 3.4: Outer membrane proteins (37, 10.62 %) identified in the proteome of *F. nucleatum* OMV.

Accession number	Protein score	% Coverage	Number of peptide matches	Description
gi|19705267	628	42	39	Hemin receptor
gi|19704535	581	78	44	Hypothetical protein FN1200
gi|19704858	8168	64.1	509	Hypothetical protein FN1526 (RadD)
gi|19704886	1739	35.4	119	Hypothetical protein FN1554
gi|19705337	3966	56.2	177	Hypothetical protein FN2047
gi|19703624	130	22.7	6	Lipoprotein 1
gi|492606366	2510	59.2	138	Membrane protein
gi|495968818	1032	30.4	115	Membrane protein
gi|492611696	6201	52.3	327	Membrane protein
gi|492656580	678	26	48	Outer membrane autotransporter barrel domain-containing protein
gi|19703678	920	76.2	50	Outer membrane porin F FN0335
gi|19703598	241	48.4	18	Outer membrane protein FN0253
gi|19704600	1264	36.1	40	Outer membrane protein FN1265
gi|19703736	495	53.3	51	Outer membrane protein FN0394
gi|19705216	2099	58	234	Outer membrane protein FN1911
gi|19704338	2548	76.2	117	Outer membrane protein P1 precursor FN1003
gi|19704608	1054	67.1	62	Outer membrane protein TolC
gi|496078626	3270	24.4	276	Outer membrane protein, partial
gi|492609940	157	23.4	8	Cell wall endopeptidase M23
gi|530296	21135	78.3	1456	Porin (FomA) FN1859
gi|19705025	114	17.9	13	Serine protease FN1074
gi|19705252	521	30.9	41	Serine protease FN1950
gi|19704758	5430	53.2	262	Serine protease FN1426
gi|492596693	841	12.3	73	Serine protease

gi|19703893	776	45.4	38	TraT complement resistance protein precursor
gi|492611516	2806	45.1	174	*Fusobacterium* outer membrane protein, partial
gi|19703727	127	28.1	11	Hypothetical protein FN0385
gi|19704200	639	71	54	Hypothetical protein FN0865
gi|492609727	205		22	*Fusobacterium* outer membrane protein
gi|496079010	1215	21	77	*Fusobacterium* outer membrane protein family
gi|19704402	366	60.8	24	Hypothetical protein FN1067
gi|19704070	2032	35.3	71	Cell surface protein FN0735
gi|492611840	1249	23.3	87	*Fusobacterium* outer membrane protein, partial
gi|19705141	4724	73.3	294	Hypothetical protein FN1836
gi|19703800	1207	68.5	92	Hypothetical protein FN0465
gi|19703945	937	49.8	121	Hypothetical protein FN0610
gi|19703599	2814	45.6	173	Hypothetical protein FN0254

Table 3.5: Proteins of unknown subcellular location (89, 24.25 %) identified in the proteome of *F. nucleatum* OMV.

Accession number	Protein score	% Coverage	Number of peptide matches	Description
gi|19704160	173	45.1	25	Cytoplasmic protein
gi|19703402	968	63.6	71	Fumarate reductase flavoprotein subunit
gi|492614404	4560	51.1	199	*Fusobacterium* outer membrane protein, partial
gi|19704587	270	49.6	18	34 kDa membrane antigen precursor
gi|19703581	184	40.7	14	ABC transporter substrate-binding protein
gi|66268805	87	35.1	5	Apoptosis inducing membrane protein
gi|19704593	453	46.2	33	C4-dicarboxylate-binding protein
gi|19703520	122	18.1	6	Cell division inhibitor minC
gi|19703522	112	47.5	11	Cell division inhibitor minE
gi|19704831	3558	50.9	141	Cell surface protein FN1499
gi|19704473	226	39.9	11	Cytoplasmic protein FN1138
gi|19703554	235	36	17	Cytoplasmic protein
gi|19704109	396	66.5	27	Cytoplasmic protein
gi|19705232	299	32.7	45	DEGV protein
gi|19703807	205	19.8	19	Flavodoxin flda
gi|496296672	1342	21.4	92	*Fusobacterium* outer membrane protein family, partial
gi|492611534	2863	35.7	157	*Fusobacterium* outer membrane protein, partial

gi|19705213	587	41.6	33	Glycerophosphodiester phosphodiesterase
gi|19703378	117	32.9	5	Hypothetical protein FN0026
gi|19703401	434	43.2	20	Hypothetical protein FN0049
gi|19703593	1034	68.8	56	Hypothetical protein FN0248
gi|19703609	1101	62	53	Hypothetical protein FN0264 (Fad A)
gi|19703625	268	27.2	15	Hypothetical protein FN0280
gi|19703694	388	70.8	20	Hypothetical protein FN0351
gi|19703713	206	23.9	12	Hypothetical protein FN0371
gi|19703732	103	52.6	10	Hypothetical protein FN0390
gi|19703749	917	69.5	54	Hypothetical protein FN0407
gi|19703892	130	26.2	12	Hypothetical protein FN0557
gi|19703936	106	50.4	15	Hypothetical protein FN0601
gi|19703972	96	45.3	13	Hypothetical protein FN0637
gi|19703990	141	50	12	Hypothetical protein FN0655
gi|19704024	459	62.1	29	Hypothetical protein FN0689
gi|19704053	170	14.7	5	Hypothetical protein FN0718
gi|19704066	243	63.8	30	Hypothetical protein FN0731
gi|19704156	693	45.1	71	Hypothetical protein FN0821
gi|19704167	282	74	11	Hypothetical protein FN0832
gi|19704240	254	65.3	24	Hypothetical protein FN0905
gi|19704251	1971	62.4	111	Hypothetical protein FN0916
gi|19704282	180	38.5	12	Hypothetical protein FN0947
gi|19704329	101	33.6	9	Hypothetical protein FN0994
gi|19704340	252	45.9	20	Hypothetical protein FN1005
gi|19704352	240	61.4	34	Hypothetical protein FN1017
gi|19704408	177	28	5	Hypothetical protein FN1073
gi|19704413	189	45.3	13	Hypothetical protein FN1078
gi|19704479	1240	67.1	100	Hypothetical protein FN1144
gi|19704488	183	32.1	5	Hypothetical protein FN1153
gi|19704548	253	33.3	16	Hypothetical protein FN1213
gi|19704588	1803	75.9	64	Hypothetical protein FN1253
gi|19704668	106	31.9	5	Hypothetical protein FN1333
gi|19704859	711	51.9	33	Hypothetical protein FN1527 (Fad I)
gi|19704861	81	45.5	2	Hypothetical protein FN1529
gi|19704892	129	25.1	5	Hypothetical protein FN1560
gi|19704968	2984	38.8	106	Hypothetical protein FN1647
gi|19705089	201	41.7	11	Hypothetical protein FN1784
gi|19705090	146	24.8	5	Hypothetical protein FN1785
gi|19705097	2107	73.6	74	Hypothetical protein FN1792
gi|19705112	639	62.1	31	Hypothetical protein FN1807
gi|19705130	313	51.7	21	Hypothetical protein FN1825
gi|19705140	84	23.1	3	Hypothetical protein FN1835
gi|19705157	172	40.5	11	Hypothetical protein FN1852
gi|19705198	4248	51.1	207	Hypothetical protein FN1893
gi|19705215	358	61.1	36	Hypothetical protein FN1910

gi|19705244	92	24.8	9	Hypothetical protein FN1939
gi|19705348	2281	37.2	119	Hypothetical protein FN2058
gi|19705411	541	51.7	40	Hypothetical protein FN2121
gi|523655036	1502	21	90	Hypothetical protein, partial
gi|19704460	170	51.9	14	LemA protein
gi|19705204	467	35.8	25	Lipoprotein 2
gi|492614725	554	50.1	32	Membrane protein
gi|496075624	1770	22.8	114	Membrane protein
gi|492610276	937	20.4	69	Membrane protein
gi|496075749	4243	39.5	238	Membrane protein
gi|496078982	4413	38.1	248	Membrane protein
gi|496070514	1312	18.7	84	Membrane protein
gi|492586863	1129	12.1	71	Membrane protein
gi|492647631	287	4.2	23	Membrane protein
gi|492564676	2158	15	187	Membrane protein
gi|492614450	8468	41.6	522	Membrane protein
gi|495977401	2768	19.3	257	Membrane protein
gi|19704915	100	24.9	5	Nitrogen fixation protein RNFG
gi|19703860	756	39.7	66	Penicillin-binding protein
gi|19705393	2071	84.1	106	RecA protein
gi|19704409	120	26.7	8	Signal recognition particle receptor ftsy
gi|492611783	556	46.5	64	Stage II sporulation protein spoiid
gi|19704458	113	36.9	6	Thioredoxin-like protein FN1123
gi|492607145	206	27.2	15	Von Willebrand factor A
gi|19704354	314	24	11	3-hydroxybutyryl-coa dehydrogenase
gi|492620353	801		48	Galactoside ABC superfamily ATP binding cassette transporter, binding protein
gi|19704016	92	32.4	14	MarR family transcriptional regulator

As expected, the protein profile of the OMV and whole bacteria were dissimilar, as judged by SDS-PAGE (**Figure 3.3, A**), reflecting the differential protein composition. There appears to be enrichment of high molecular mass species in OMV in comparison to the whole cell whereas the distribution of low molecular mass species appears to be similar in both, but are not necessarily the same proteins. Analysis of the OMV proteins also revealed the presence of several classes of proteases, and these are summarized in **Table 3.6**. As indicated, these incorporated proteases and peptidases whose theoretical subcellular distribution was predicted to include both cytosolic and outer membrane locations. The presence of five proteases/peptidases in the outer membrane was of particular interest given the potential for these to interact with host cells due to their predicted location on

the surface of both the OMV and the intact bacteria. In order to determine whether or not these proteases were biologically active in the OMV, a preparation of OMV was subjected to analysis by zymography using gelatin as the substrate. It is clear from **Figure 3.3, B** that there are multiple biologically active proteases associated with the OMV. Of interest was the observation that only weak proteolytic activity was found associated with the whole bacteria, at least under the conditions tested, suggesting that proteases may be differentially sorted and concentrated into OMV.

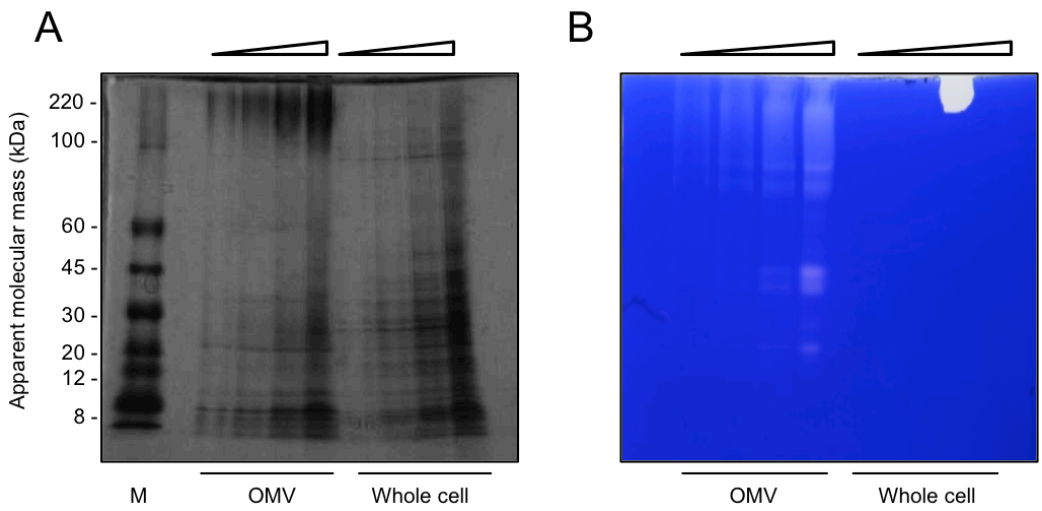

Figure 3.3: SDS-PAGE and zymographic analyses of OMV from *F. nucleatum*. Panel A: Gradient SDS-PAGE (4-20 %) gel loaded with increasing amounts (indicated by the gradient above the figure) of OMV and whole *F. nucleatum*. **Panel B:** Zymographic analysis of *F. nucleatum* whole cells and *F. nucleatum* OMV. A gradient acrylamide gel (4-20 %) containing 0.1 % gelatin was used. Zones of clearance in the zymogram indicate areas of proteolytic activity. Molecular mass markers are shown on the left-hand side.

Table 3.6: Proteases identified in the *F. nucleatum* OMV proteome.

Accession number	Protein score	% Coverage	Number of Peptide matches	Description	Subcellular location
gi|19704540	404	46.8	36	Protease FN1205	Cytosol
gi|19704606	121	13.3	14	Protease IV FN1271	Cytosol
gi|19705025	114	17.9	13	Serine protease FN1074	Outer Membrane
gi|19705252	521	30.9	41	Serine protease FN1950	Outer Membrane
gi|19704758	5430	53.2	262	Serine protease FN1426	Outer Membrane
gi|492596693	841	12.3	73	Serine protease	Outer Membrane
gi|19703623	94	17	5	Xaa-His dipeptidase	Cytosol
gi|49609940	157	23.4	8	Cell wall endopeptidase M23	Outer Membrane
gi|19703712	82	39.5	15	Signal peptidase	Cytosol

In addition to the proteases identified, there are many proteins in *F. nucleatum* OMV with potential virulence functions including NapA, FomA, FadA, FadD, Fad-I, ClpB, GroEL, and TraT that will be considered in the discussion section.

Given the apparent abundance of proteolytic activity associated with the OMV compared to the whole cells it was of interest to evaluate this observation further to determine if these activities could have an impact on host cells. In the first instance, attempts were made to determine and verify the subcellular distribution of the various protease activities by fractionating whole bacteria rather than OMV given the small amount of OMV available. In particular, it was of interest to identify *F. nucleatum* outer membrane-associated proteases, as these would likely interact with host cell proteins.

Thus, proteins loosely associated with the bacterial surface were recovered from intact *F. nucleatum* by gently vortexing the bacteria in PBS at room temperature (fraction S1). Following centrifugation, the pellet of cells was suspended in Tris-buffer (25 mM, pH 7.4) supplemented with KCL (0.5 M) and treated similarly to extract more strongly associated cell surface proteins (fraction S2). The residual pellet was subjected to sonication followed by ultracentrifugation to recover the largely cytoplasmic proteins (fraction S3) and the membrane bound proteins were finally recovered by solubilisation of the pellet resulting

from the ultracentrifugation step using 25 mM Tris containing 0.2 % v/v Triton X-100 (fraction S4) as described in the Methods section. Each fraction was subjected to SDS-PAGE (**Figure 3.4, A**) and zymography (**Figure 3.4, B**) to evaluate the complexity of proteins in each fraction and the subcellular distribution of proteolytic activities, respectively. Microscopic analysis of the bacteria after the PBS and KCL washes indicated that the bacteria remained intact (**Figure 2.1** in the Methods section).

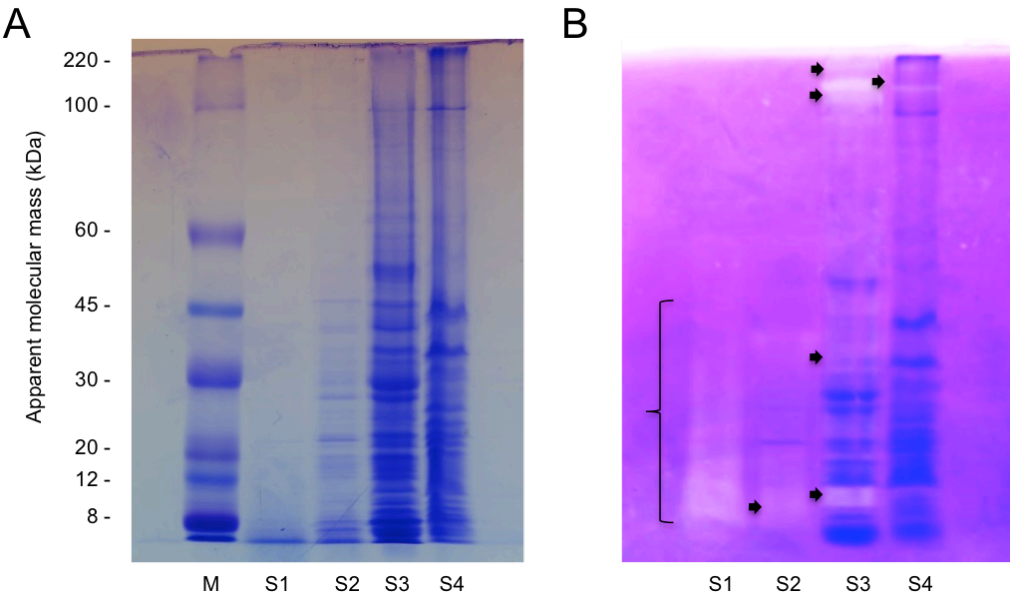

Figure 3.4: Subcellular fractionation of *F. nucleatum* protease activities. Fraction S1: PBS extract of whole cells. Fraction S2: 0.5 M KCL wash of PBS-treated cells. Fraction S3: cytoplasmic extract. Fraction S4 solubilized membrane extract. **Panel A:** Gradient SDS-PAGE (4-20 %) gel of all extracts. **Panel B:** Zymogram showing the protease activity present in each fraction (as indicated by arrows) using 4-20 % gradient SDS-PAGE containing 0.05 % gelatin. M= molecular size markers.

As the PBS extracted material appeared to contain a large proportion of the protease activity associated with whole cells (**Figure 3.4, B, lane S1**) this fraction was scaled up and subjected to ion-exchange chromatography (**Figure 3.5**) in an attempt to further separate the activities therein as there appeared to be multiple species present as shown by the bracket in **Figure 3.4, B (lane S1)**. This indicates that the PBS wash extracted most of the proteases found associated with the *F. nucleatum* outer membrane (loosely attached) whereas the KCL wash recovered fewer protease activities (strongly attached) (**Figure 3.4, B, lane S2**).

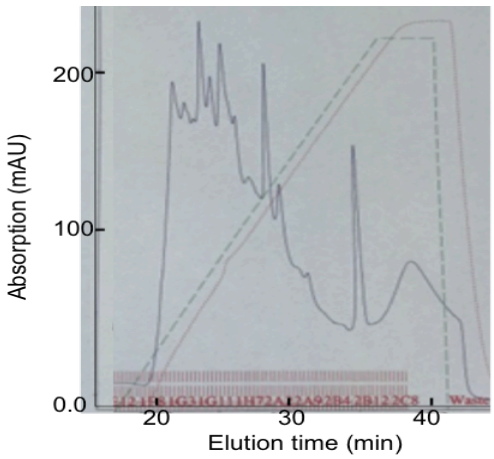

Figure 3.5: Ion-exchange chromatography (IEC) of PBS extract. The *F. nucleatum* PBS wash was recovered at room temperature. This was followed by dialysis against Tris (25 mM, pH 7.4). The resulting solution was subjected to ion-exchange chromatography using mono Q, HR 5/5 column and the bound proteins were eluted with a liner salt gradient (0.5 M KCL). The resulting protein fractions were collected in 96-well plates.

This fractionation was only attempted with whole bacteria, as there was insufficient biomass with a typical OMV preparation (approx. 3 mg). In an attempt to identify active fractionated protease activity, two different chromogenic protease substrates were used, azocasein and azoalbumin (**Figure 3.6**). This study revealed that the surface attached proteases exhibited differential activity towards the two substrates.

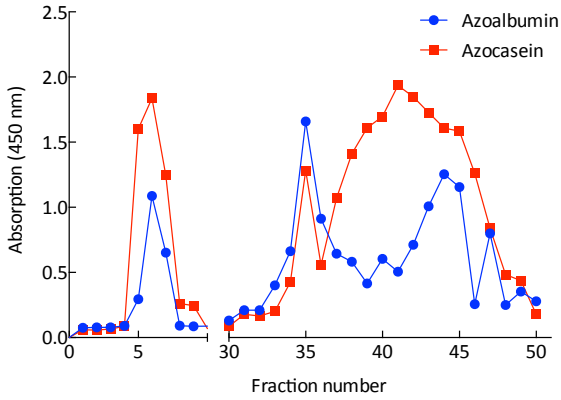

Figure 3.6: Analysis of the proteolytic activity obtained from *F. nucleatum* PBS wash toward azoalbumin and azocasein. The *F. nucleatum* PBS wash was separated by IEC before the proteolytic activity of various fractions was tested using azoalbumin and azocasein as substrates.

The PBS-extracted proteins (**Figure 3.7, A**) were analysed by SDS-PAGE (**Figure 3.7, B**) which revealed the presence of multiple protein species, many of which were proteolytically

active towards the gelatin (**Figure 3.7, C**). This suggests that proteases that were active against azoalbumin would not necessarily be active against gelatin as demonstrated by the lack of gelatinase activity in fraction 3 and 4.

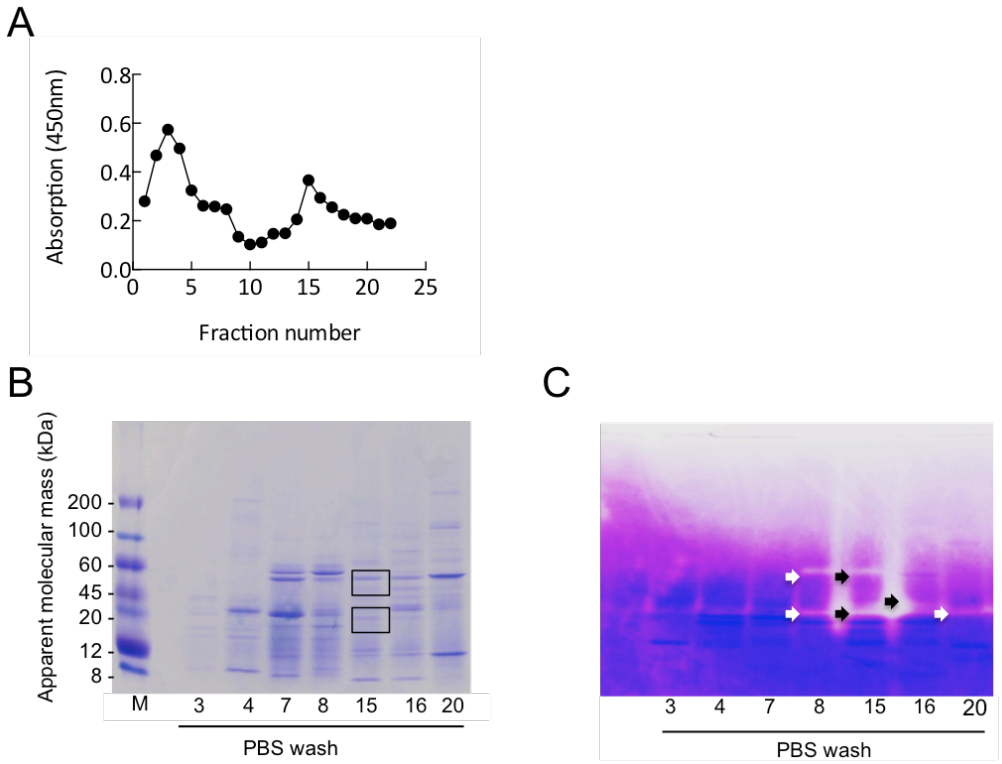

Figure 3.7: Analysis of the proteolytic activities obtained from *F. nucleatum* PBS wash. Panel A: Shows the proteolytic activity of the various fractions separated by IEC towards azoalbumin. **Panel B:** Coomassie blue stained gradient (4-20 %) acrylamide gel of the fractions indicated beneath the gel. **Panel C:** Zymography of the fractions shown in panel B using 4-20 % gradient SDS-PAGE containing 0.05 % gelatin. The proteolytic activity indicated by the arrows.

The protein species exhibiting protease activity in fraction 15 (**Figure 3.7, C**) were excised for MS-analysis from the corresponding Coomassie-stained gel as indicated by small boxes (**Figure 3.7, B**). The LC-MS/MS analysis revealed the presence of three proteolytic enzymes (**Table 3.7**). Of these three proteases, two (FN1205, and FN0278) were detected previously in the MS analysis of the OMV proteome. Interestingly, pSORTb analysis of these proteins showed that they are likely to be cytosolic proteins, thus suggesting that, if truly cytosolic, they can adhere to the surface of the bacterium when present extracellularly as they were recovered from a PBS wash of intact bacteria. Phase contrast microscopy indicated that the bacteria remained intact after both the PBS and subsequent KCl washes indicating that no lysis occurred (**Figure 2.1**, in the Methods section). Comparing the proteolytic activities

recovered from *F. nucleatum* and OMV the majority of the surface associated proteinases recovered by the PBS wash were of low molecular mass (≤ 40 kDa) whereas the proteolytic activity recovered from OMV was represented mostly by high molecular mass species (> 40 kDa).

Table 3.7: Mass spectrometry identification of proteases identified by zymography In Figure 3.7, B.

Protease identified	Accession number	% Coverage	Mass	Subcellular location	Identified in OMV
Peptidase T (FN0733)	gi|19704068	18	46356	Cytoplasm	Not detected
Protease (FN1205)	gi|19704540	12	47474	Cytoplasm	Yes
Xaa-His dipeptidase (FN0278)	gi|19703623	59	50397	Cytoplasm	Yes

3.3.2 *F. nucleatum* proteases degrade host cell proteins

One study has shown that a serine protease activity recovered from *F. nucleatum* OMV was apparently able to degrade fibronectin, fibrinogen, and IgA (Bachrach et al., 2004). Thus, it was of interest to extend these studies and evaluate the ability of proteases associated with the cell surface and OMV to degrade other host proteins. In this regard, the effect of *F. nucleatum* proteolytic activity on E-cadherin, a protein involved in maintenance of the epithelial cell barrier function, was examined (**Figure 3.8**). Recombinant human E-cadherin was incubated with *F. nucleatum* and OMV and degradation was monitored by Western blotting. Evidence of degradation was indicated by the appearance of low molecular mass peptides (56, 50.8, 42.2, 23, 19.8, and 16.4 kDa). Immunofluorescence analysis also showed a decrease in E-cadherin expression following treatment of the colonic cell line SW-480 with *F. nucleatum* and OMV (**Figure 3.9**).

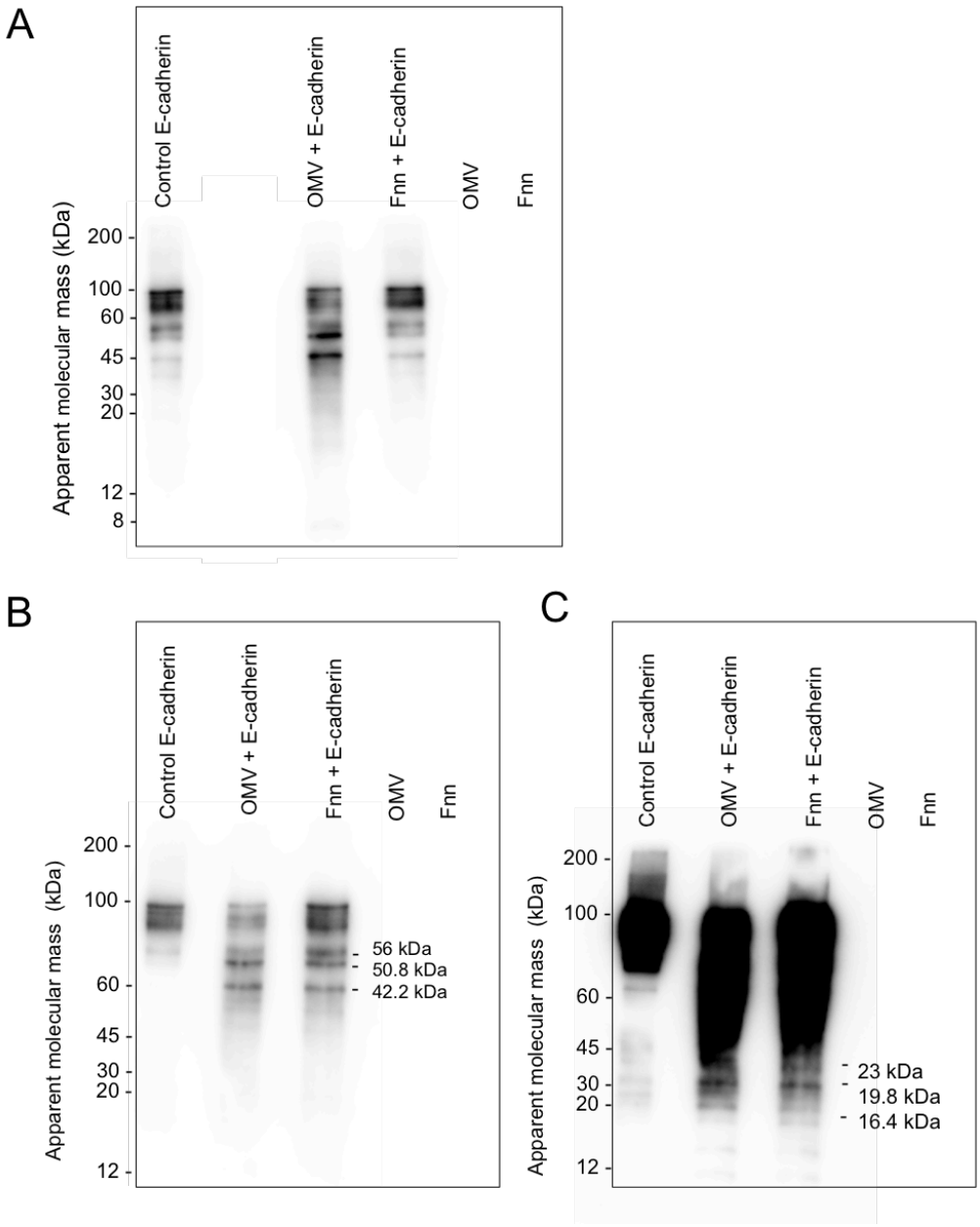

Figure 3.8: Effect of *F. nucleatum* and *F. nucleatum* OMVs on E-cadherin. Panel A: Purified E-cadherin was treated for 24 h with whole *F. nucleatum* (10^6 bacteria/ml) and *F. nucleatum* OMV (1.8 µg/ml). Samples were separated on 12.5 % SDS-PAGE gel, and corresponding Western Blots were probed with anti-E-cadherin. **Panel B and C:** Purified E-cadherin was treated for 48 h with whole *F. nucleatum* ($4.3X10^6$ bacteria/ml) and *F. nucleatum* OMV (0.59 µg/ml). Panel C shows an overexposed image of the blot shown in panel B to visualise the very low molecular mass peptides generated. Molecular mass marker are shown on the left-hand side of each panel.

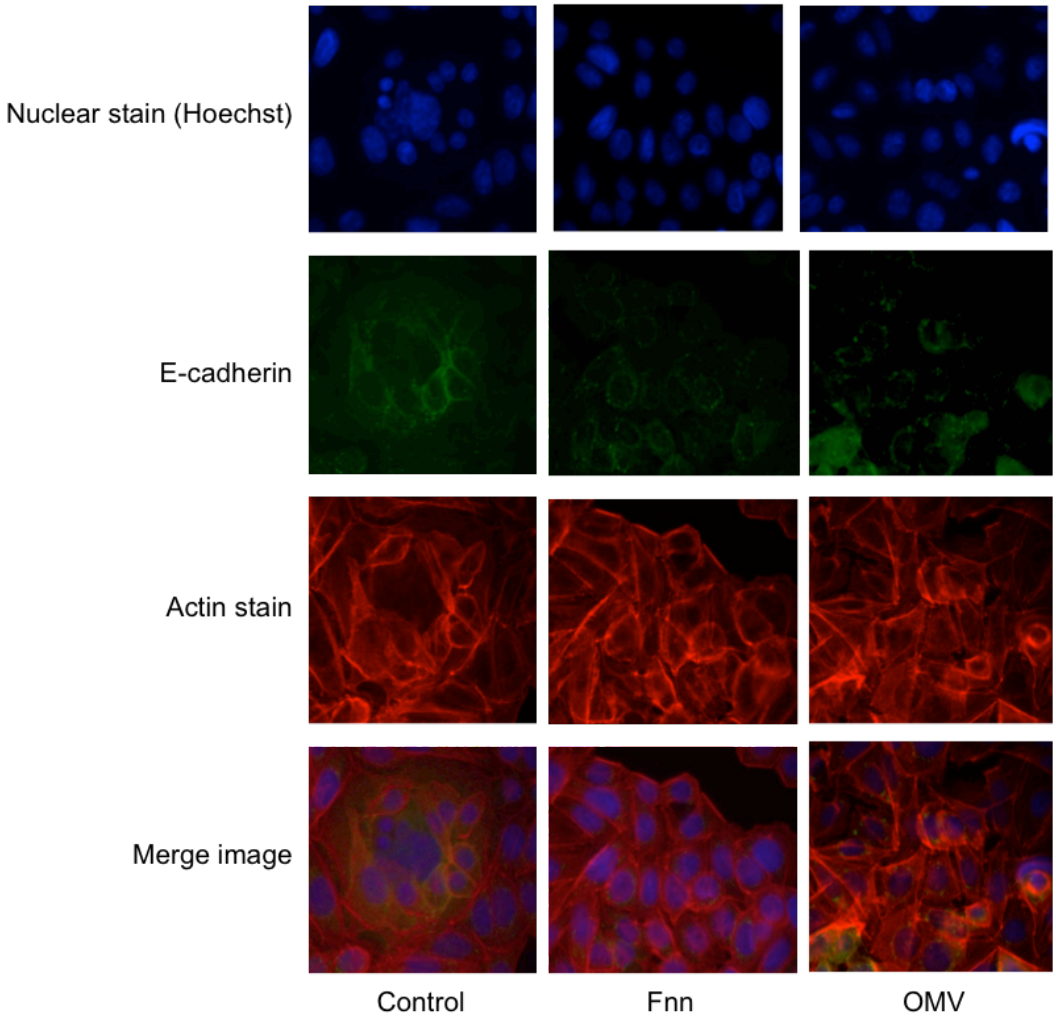

Figure 3.9: E-cadherin expression on SW-480 cells. SW-480 cells were treated with *F. nucleatum* (MOI 100:1) and OMV (50 μg/ml) for 24 h. SW-480 cells were washed with PBS prior to fixation and detection of E-cadherin expression by immunofluorescence (magnification 40 X).

Attempts were made to evaluate the ability of OMV and partially purified protease activities to degrade other host proteins including mucin, immunoglobulin (IgA), and the ECM component laminin. Under the conditions tested, degradation of mucin or IgA was not apparent (data not shown). However, evidence of degradation of laminin due to incubation with partially purified proteases from *F. nucleatum* (F5, obtained from fractionation of PBS wash by IEC) is evident due to the appearance of low molecular mass peptides when compared with the untreated protein control (**Figure 3.10**). OMV proteases also degrade laminin as indicated by the accumulation of 60 kDa material, which appears to be more intense than the corresponding band in the control sample. However, the pattern of laminin degradation by *F. nucleatum*-associated proteases activity and the OMV-associated

proteases was different suggesting that the OMV harbour qualitatively different proteases activities.

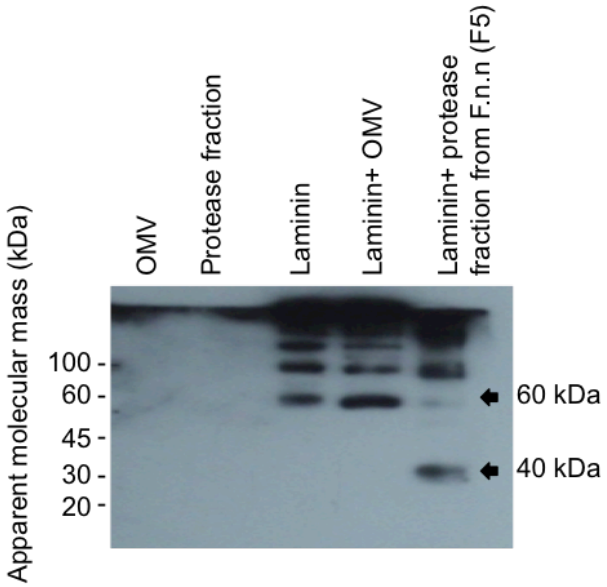

Figure 3.10: Effect of *F. nucleatum* OMV and partially purified proteases on laminin. Purified laminin was treated for 36 h with OMV (2 μg /ml) and F5 (a partially purified protease fraction (5 μg/ml)) obtained from a PBS wash of whole *F. nucleatum*. Samples were separated on a 12.5 % SDS-PAGE gel and laminin degradation was evaluated by Western blotting. Molecular mass markers are shown on the left-hand side.

3.3.3 *F. nucleatum* and OMV modulate epithelial barrier function of colonic epithelial cells

Given the amount of proteolytic activity associated with the OMV and their ability to degrade E-cadherin it was of interest to determine whether or not they could modulate colonic epithelial barrier function. To address this question, an in vitro model of the colonic epithelial barrier was used employing the colonic epithelial cell lines Caco-2 and T-84. Once a functional monolayer was formed in vitro, as evidenced by stable high trans-epithelial electrical resistance, the monolayer was exposed to both OMV and the intact bacterium. The data demonstrate that OMV could modulate the TER of Caco-2 monolayers in a dose-dependent manner (**Figure 3.11, A**) and were more efficient than the intact bacteria in doing so. **Figure 3.11, B** shows the percentage change in the TER value with time.

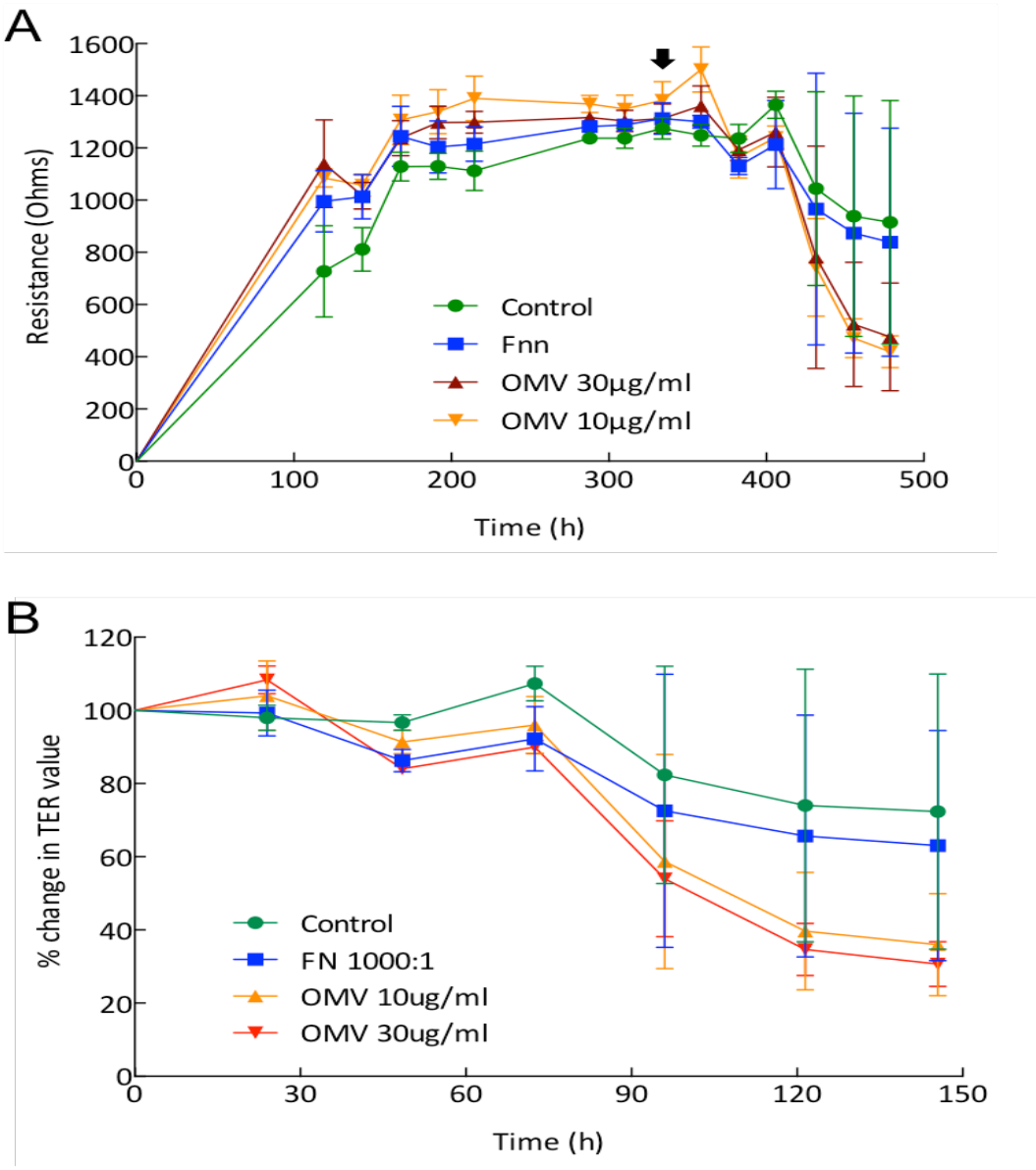

Figure 3.11: Effect of *F. nucleatum* and OMV on barrier integrity. Caco-2 cells were grown until a stable TER was achieved (approx. 13 days) and then subjected to serum deprivation for 24 h prior to sample addition. Cells were incubated with *F. nucleatum* (MOI 1000:1), and OMV (10 and 30 µg/ml). The results show the mean ± SEM (n=3). OMV (10 and 30 µg/ml) significantly decrease the TER ($p < 0.03$ and $p < 0.02$, respectively). **Panel A:** resistance value (Ohms). **Panel B:** the percent change in TER value after sample addition. Arrow indicates sample addition (bacterial cells and OMV).

Similarly, there was an OMV-dependent reduction in the TER across T-84 monolayers (**Figure 3.12**). Interestingly, a more rapid reduction in the TER was observed in the case of the T-84 cell model compared with the Caco-2 cell monolayer. Also in both cases *F. nucleatum* whole cells were unable to induce a significant reduction in the TER, unlike the OMV.

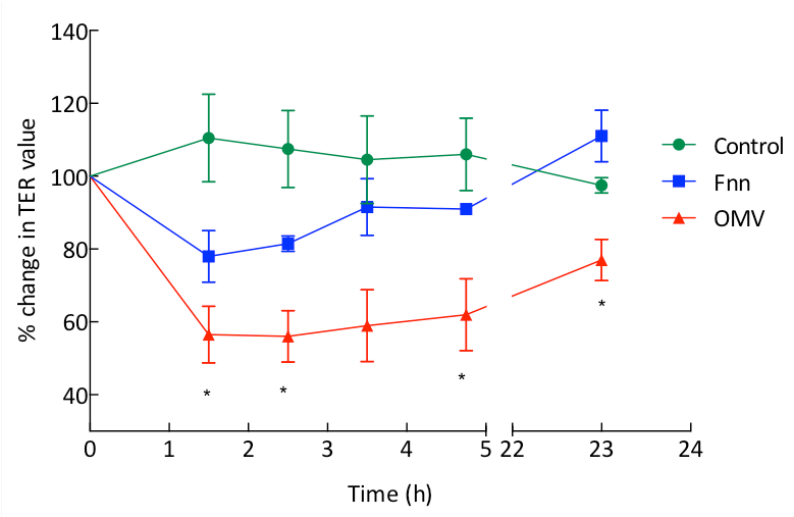

Figure 3.12: Effect of *F. nucleatum* and OMV on T-84 cells. T-84 cells were incubated with *F. nucleatum* (MOI 200:1), OMV (10 µg/ml). OMV significantly reduce the TER (* = P < 0.05 when compared with the same time point of the control) n=3.

Using the Caco-2 monolayer model the effect of incubating with different subspecies of *Fusobacterium* (*nucleatum*, *polymorphum*, and *vincentii*) on E-cadherin expression was evaluated. The data demonstrate that all subspecies reduced E-cadherin expression following a prolonged co-incubation (9 days) (**Figure 3.13**). Taken together, these data suggest that *F. nucleatum* OMV were able to modulate the TER, likely due to a reduction in the tight junctional protein E-cadherin.

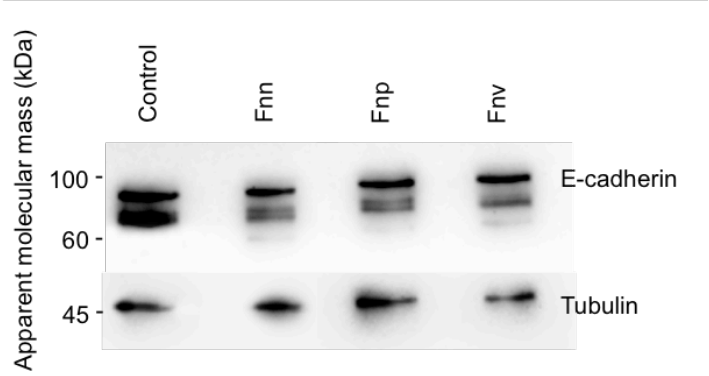

Figure 3.13: Effect of different *Fusobacterium* species on Caco-2 E-cadherin expression. Western blot of the lysed Caco-2 cells were probed using anti-E-cadherin after prolong exposure to Fusobacterial spices (MOI 2000:1).

Visualisation of the interaction of the OMV with colonic cell lines (T-84 and SW-620) by confocal microscopy indicated that the vesicles appear to be possibly internalized by both cell lines (**Figure 3.14**). Also, the OMV appear to coalesce at the cell-cell boundaries as these become clearly visible when co-incubated with fluorescently labelled OMV (**Figure 3.14, C**).

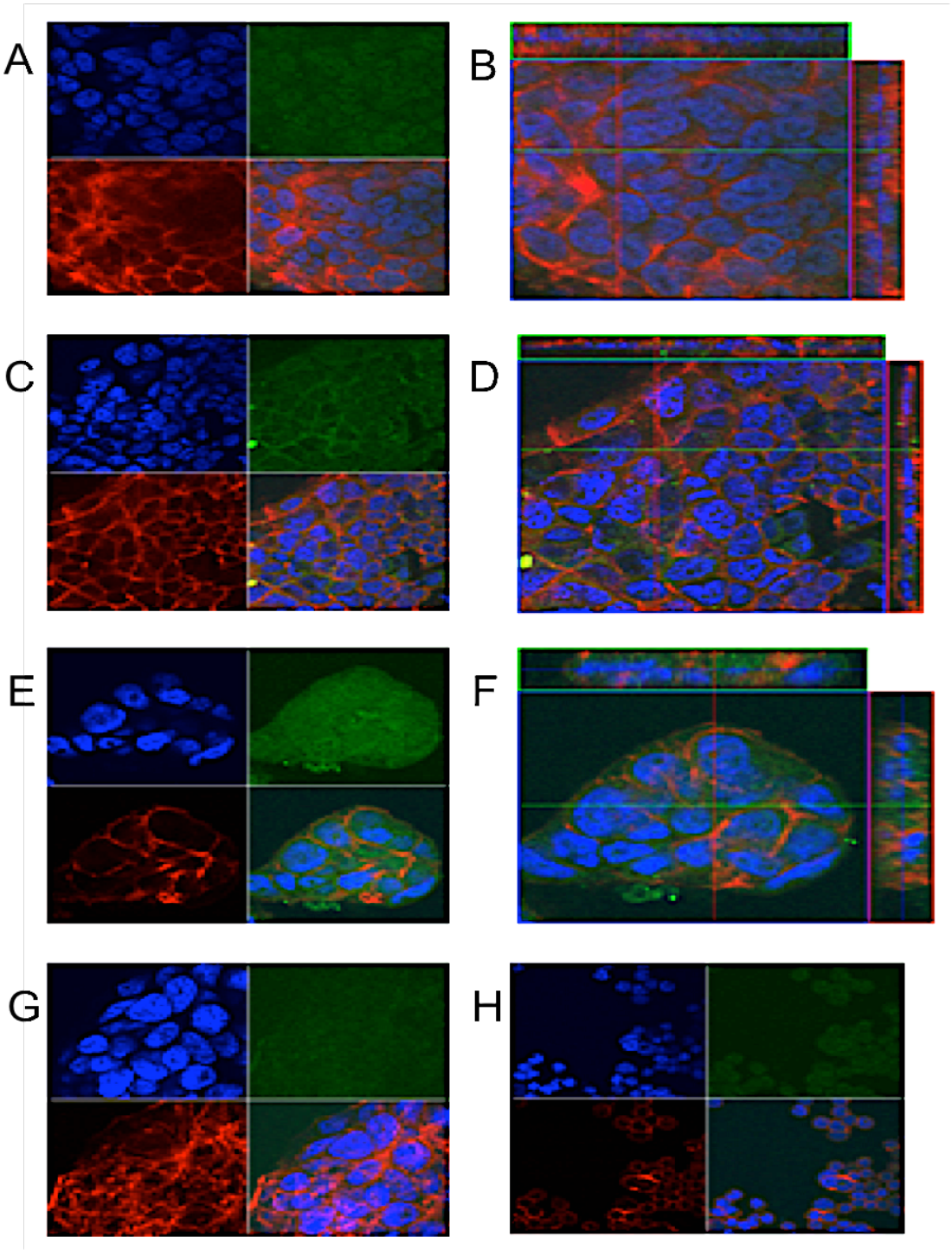

Figure 3.14: *F. nucleatum* OMV appear to bind to and be internalised by colonic epithelial cells. Panel A: T-84 cells incubated with Alexa488-OMV for 1 h; **Panel B:** z-stack of A; **Panel C:** T-84 cells incubated with Alexa488-OMV for 4 h; **Panel D:** z-stack of C; **Panel E:** T-84 cells incubated with Alexa488-OMV also for 4 h; **Panel F:** z-stack of E; **Panel G:** Control T-84 stained with phalloidin and hoechst and images captured in each channel; **Panel H:** SW-620 cells incubated with Alexa488-OMV for 4 h. Panels A, C, E, G and H show the individual fluorescence channels for the nuclear stain (top left quadrant), the green channel (top right quadrant), the actin stain (bottom left quadrant) and the merged image (bottom right quadrant).

Based on these observations it was hypothesised that the OMV-associated protease activity may be responsible in whole or in part for the observed effect on the TER, particularly if they could access the intracellular junctional space. To test this possibility attempts were made to inhibit protease activity with a pan-protease inhibitor cocktail. However, co-incubation of the cells with inhibitors alone resulted in a decrease in the TER (data not shown). The alternative strategy of pre-incubating the OMV with the inhibitors followed by removal of the inhibitors by ultra-centrifugal washing failed to yield useful data due in part to the fact that much of the OMV material was lost as a consequence this treatment as confirmed by SDS-PAGE (data not shown).

3.4 Discussion

The work described in this chapter aimed to isolate *F. nucleatum* OMV, determine the protein composition of these OMV, and undertake studies to identify potential properties of the OMV that may impact on host colonic cells. Transmission electron microscopy of the OMVs revealed the presence of two types of OMV, those with a bilayer structure and vesicles with a single membrane. Together, the MS analysis of the OMV identified 366 proteins. Interestingly, OMVs were found to be enriched in protease activity compared with the parental bacterium and the OMV-associated proteolytic activity was able to degrade the important host components E-cadherin and laminin. In addition, subcellular fractionation/purification of the loosely attached proteins indicated several proteolytic activities are associated with the surface of the bacterium. However, in this study, the protocol used to prepare the OMV involved a PBS wash prior to storing the OMV at -80^{o}C. Thus, it is most likely that the whole cell-associated proteases are washed off the surface of the OMV. This likely explains why difference are seen in the degradation pattern of laminin, for example, when treated with OMV and PBS-extracted proteases from whole *F. nucleatum*.

Many gram-negative pathogens utilise OMV as an important secretion mechanism, which enables them to deliver virulence factors into host cells at distal and local sites (Ellis and Kuehn, 2010). The vesicle structure protects multiple factors from digestion by host proteases (Kuehn and Kesty, 2005) such as protein adhesins that could facilitate invasion and transformation of the host (Renelli et al., 2004). OMVs are important virulence factors that contribute to the pathogenicity of bacteria. For example, it has been shown that *H. pylori* OMV simulate gastric epithelial cells to produce IL-8 (Ismail et al., 2003). *E. coli* OMV induce a lethal sepsis-like syndrome in mice that is linked to systemic induction of IL-6 and TNF-α (Park et al., 2010b). Similarly, *C. jejuni* is associated with gastroenteritis and produces cytotoxic OMV capable of inducing IL-8 by intestinal epithelial cells (Elluri et al., 2014). Generally, studies such as those demonstrate that pathogenic gram-negative bacteria commonly use OMV to enhance the pathology associated with the infection.

The proteomic composition of *F. nucleatum* OMV demonstrates that they are composed of outer and inner membrane proteins, in addition to periplasmic and cytoplasmic constituents. Further, the recovery of both bilayer OMV and monolayer OMV from *F. nucleatum* raises the issue of differentiating between the functions of these vesicle types and whether they differentially interact with host cells. Curiously, some publications do not identify cytoplasmic proteins associated with OMV preparations (e.g. Sharpe et al. (2011)). A recent careful electron microscopy study concluded that the presence of a bilayer structure indicates the presence of both outer and inner membranes in populations of OMV isolated from different species of bacteria thus accounting for the presence of cytoplasmic proteins therein (Pérez-Cruz et al., 2015). Prior to this, there was generally the mistaken assumption that OMV arose as a consequence of budding of the outer membrane alone resulting in the encapsulation of periplasmic proteins only. OMV are thus composed of outer membrane proteins, LPS, phospholipids, periplasmic proteins, plasma membrane components, and cytoplasmic constituents (Beveridge, 1999, Pérez-Cruz et al., 2015). Cytoplasmic proteins are found in OMV from many different species including *N. meningitidis* (Lappann et al., 2013), *Francisella novicida* (Pierson et al., 2011), *H. pylori* (Mullaney et al., 2009), *A. baumannii* (Kwon et al., 2009), *S. flexneri* (Chen et al., 2014), *K. pneumoniae* (Cahill et al., 2015) and *P. aeruginosa* (Choi et al., 2011).

Recently, MS-based proteomic analysis of gram-negative OMV improved knowledge about their pathophysiological functions (Lee et al., 2016). An interesting observation arising from the MS analysis of the *F. nucleatum* OMV was the present of multiple active proteases. Microorganisms generally utilize proteases for degrading host tissues and for acquiring nutrients and have been shown to be important for survival in the host (Bamford et al., 2007). It has been shown that *F. nucleatum* may contain more than one peptidase (Rogers et al., 1998). The result presented in the current study, provide evidence of multiple proteolytic activities capable of using azocasein and azoalbumin as substrates. Two studies have identified a Fusobacteria serine protease (65 kDa) (Doron et al., 2014, Bachrach et al., 2004). This serine protease has been shown to be capable of inactivating host defense effectors and degrading components of periodontal tissue (Bachrach et al., 2004). OMV from different species of bacteria have been documented to contain active proteases which can be delivered to host cells tissue (Zhong, 2011). For example, proteases are

preferentially and selectively packed into OMV as in case of *B. fragilis* and *B. thetaiotaomicron* (Elhenawy et al., 2014). Proteases interact with host cells and modulate host immune system (Rompikuntal et al., 2015), in addition to being cytotoxic, and inducing inflammatory responses (Mondal et al., 2016). Thus, proteases are considered to be important virulence factors of gram-negative bacteria in which they play a role in cytotoxicity, induction of inflammatory responses and facilitate bacterial invasion (Mondal et al., 2016).

Serine proteases are considered to be the most functionally diverse and abundant group of proteolytic enzymes in prokaryotic and eukaryotic organisms (Page and Di Cera, 2008). Extracellular secretion of serine proteases has been identified in many pathogenic gram-negative bacteria species including *Shigella*, *Neisseria*, *E. coli*, *Salmonella*, *Citrobacter rodentium*, *Edwardsiella* species (Ruiz-Perez and Nataro, 2014) and *V. cholerae* (Mondal et al., 2016). Possessing the ability to secrete serine proteases enables microorganisms to hydrolyse host extracellular and intracellular protein substrates, resulting in cytoskeleton destruction (Canizalez-Roman and Navarro-García, 2003), degradation of E-cadherin (Schmidt et al., 2016), impaired immunity (Orth et al., 2010) or induction of autophagy (Moal et al., 2011) and thus clearly have a major role in infection induced pathology.

A Xaa-His dipeptidase (**Table 3.1**) was identified in the *F. nucleatum* OMV. Xaa-His dipeptidase is a broad activity dipeptidase belonging to metallopeptidase M20 family (Chang et al., 2010). This dipeptidase is utilised by *Streptococcus equi* species and is 34 % identical to *F. nucleatum* enzyme. Generally, such dipeptidase have broad substrate specificity and in the case of *P. gingivalis*, contribute to periodontal disease (Aoki et al., 2012).

Genome sequencing of *F. nucleatum* performed by the J. Craig Venter Institute identified 2068 genes including 21 proteases by mass spectrometry analysis (**Table 3.8**). The mass spectrometry analysis in this study of *F. nucleatum* OMV identified 366 proteins including 9 proteases (**Table 3.6**). *F. nucleatum* OMVs appear to be enriched in protease activity compared with the parental bacterium, as judged by zymography, suggesting that these proteins are differentially sorted into the vesicles. Other studies also indicate that proteins can be selectively loaded into OMV including proteases, in *P. gingivalis* (Haurat et al., 2011),

V. cholerae (Mondal et al., 2016), *H. pylori* (Olofsson et al., 2010), and *K. pneumoniae* (Cahill et al., 2015).

Table 3.8: Proteases identified in *F. nucleatum* genome.

Gene symbol	Description	Found in OMV (in this study)
FN1322	Membrane metalloproteases	Not detected
FN1426	Serine protease	Detected
FN1704	Serine protease	Detected
FN1826	Protease	Not detected
FN1931	Protease	Not detected
FN1950	Serine protease	Detected
FN2014	ATP-dependent protease La	Not detected
FN2015	ATP-dependent clp protease ATP-binding subunit clpX	Not detected
FN2016	ATP-dependent Clp protease proteolytic subunit	Not detected
FN0508	Serine protease	Not detected
FN0873	Protease IV	Not detected
FN0920	Protease htpX	Not detected
FN0946	Zinc metalloprotease	Not detected
FN1029	Zinc protease	Not detected
FN1121	ATP-dependent Zn proteases	Not detected
FN1205	Protease	Detected
FN1271	Protease IV	Detected
FN1280	Serine protease, V8 family	Not detected
FN1281	Cysteine protease	Not detected
FN0733	Peptidase T	Not detected

F. nucleatum FadA (FN0264), which was identified in the OMV in the current study (**Table 3.5**) is required by *F. nucleatum* for attachment and cell invasion (Fardini et al., 2011). FadA binds to E-cadherin on CRC and non-CRC cells and mediates the attachment and invasion of bacteria into the epithelial cells (Rubinstein et al., 2013). Another possibility that enables microorganisms to invade and modulate epithelial barrier function is by degrading host proteins including E-cadherin. In this study, it has been found that *F. nucleatum* and *F. nucleatum* OMV proteases are capable of degrading human E-cadherin. It has been reported that *B. fragilis* secretes proteases, which can cleave E-cadherin, and it also secretes metalloproteinase that facilitates cell binding to E-cadherin (Remacle et al., 2014). It has been demonstrated that a serine protease secreted by *H. pylori* is important in the cleaving of E-cadherin, resulting in disruption of the epithelial barrier (Hoy et al., 2010) and facilitates invasion through the polarized epithelium (Schmidt et al., 2016). Similarly, other pathogens proteinases cleave E-cadherin such as *E. coli*, *S. flexneri, N.*

gonorrhoeae, C. jejuni (Hoy et al., 2012), *P. gingivalis* (Katz et al., 2000), and *S. aureus* (Inoshima et al., 2011). Furthermore, some microorganisms adhere to E-cadherin, which facilitates colonization of the host, including *Streptococcus pneumoniae* (Anderton et al., 2007), and *C. botulinum* (Sugawara et al., 2010).

In addition, in this study immunofluorescence analysis showed that *F. nucleatum* and OMV treatment resulted in a reduction in E-cadherin expression in colorectal epithelial cells, which would be associated with weakening of the protective epithelial barrier function. Attempts to inhibit the *F. nucleatum* and *F. nucleatum* OMV protease to confirm their role in this process using broad-spectrum protease inhibitors designed to inhibit serine, cysteine, and metalloproteases was not successful (data not shown).

In the case of laminin, protease activity from *F. nucleatum* and OMV were able to partially degrade laminin. Laminin is a large molecular weight glycoprotein that forms the basement membrane of cells (Aumailley, 2013). Other pathogens including *H. pylori* upregulate the expression of MMPs, which are involved in the degradation of ECM proteins including laminin (Sougleri et al., 2016). Also, *Haemophilus influenzae* (Fink et al., 2002) and *Bacillus anthracis* (Wang et al., 2016c) interact with laminin, which facilitates bacterial invasion and colonisation. Taken together, it is likely that the ability of *F. nucleatum* and its OMV to degrade E-cadherin contribute to the loss of barrier function, as judged by the decrease in TER. Further it is also likely that the small size of the OMV enable them to more easily access the adherens functional proteins to initiate their proteolysis.

In addition to the protease activities, several other proteins with potential virulence attributes were identified in the OMV proteome. For example, a Neutrophil-activating protein A (NapA) (**Table 3.1**) was identified in the *F. nucleatum* OMV, which is 30 % identical to *H. pylori* NapA. It is a conservative protein in *F. nucleatum* and in *H. pylori* it is considered a major virulence factor that activates NADPH oxidase of human leukocytes inducing reactive oxygen production (Satin et al., 2000). In addition, NapA have immunomodulatory properties, may function as adhesion protein, and able to protect *H. pylori* from oxidative stress (protects DNA from damage). Although little is known about its role in *F. nucleatum* it would be interesting to investigate potential similarities.

RadD (**Table 3.4**) is classified as type Va autotransporter that induces cell death by a contact-base process, but the exact mechanism is not understood (Kaplan et al., 2010). RadD is a large Fusobacterial OMP, which was identified as an arginine inhibitable adhesin that mediates adhesion of *F. nucleatum* to *S. sanguinis* (Kaplan et al., 2009). Also, RadD is associated with biofilm formation and induces apoptosis in immune cells (Kaplan et al., 2010), so it may possibly serve a similar function in *F. nucleatum* OMV. RadD is necessary for interspecies adherence and multiple species biofilm formation (Kaplan et al., 2009). Rad D is responsible for coaggregation of *F. nucleatum* with *S. sanguinis,* which influences gene regulation in *F. nucleatum* (Simanian, 2013).

Various studies demonstrate that OMV play a major role in biofilm formation and also participate in forming part of the biofilm matrix in some bacteria, including *H. pylori* (Yonezawa et al., 2011), *Francisella* (van Hoek, 2013), *P. aeruginosa* (Murphy et al., 2014), *V. cholerae* (Altindis et al., 2014), and *P. putida* (Baumgarten et al., 2012). It is possible that OMV contribute to biofilm formation according to their content, which differ according to bacterial species. *P. aeruginosa* OMV, for example, contain polysaccharide on the vesicles surface, which provides a structural support for biofilms and confers immune protection and bacterial persistence (Murphy et al., 2014).

FomA (FN1859, **Table 3.4**), a major outer membrane protein of *F. nucleatum* that functions as non-specific porin (Kleivdal et al., 1995) is also present in OMV. FomA is involved in the binding between *Fusobacterium* and other species including *S. sanguis* and *P. gingivalis* in oral infections (Kinder and Holt, 1993, Liu et al., 2010b). The transmembrane domain of FomA is inserted and folded into the phospholipid bilayers similar to *E. coli* OmpA (Pocanschi et al., 2006). *F. nucleatum* FomA is 32 % identical to *E. coli* OmpA and 60 % identical to *H. pylori* OmpA. *F. nucleatum* FomA is also as a potent immunogenic protein (Bakken et al., 1989, Toussi et al., 2012a), which facilitates bacterial evasion of the host immune system (Kleivdal et al., 1999). FomA could thus serve as a receptor involved in coaggregation with various oral pathogens that make it a potential vaccination target to prevent bacterial coaggregation (Liu et al., 2010b), similar to targeting RadD. Indeed, using both RadD and FomA as vaccine candidates could have a synergistic effect.

Although, it is not known what the function of *Fusobacterium*-associated defensin inducer (Fad-I) (FN1527, **Table 3.5**) is in *F. nucleatum* or its OMV it is likely that *F. nucleatum* gain a selective advantage in its niche by stimulatory production of an antibacterial peptide. It has been shown that *F. nucleatum* OMVs are potent stimulators of hBD_2 (DEFB4A) and hBD_3 (DEFB103A) in colonic epithelial cells (McGrory et al, unpublished).

The ATP-dependent chaperone ClpB (**Table 3.1**), also present in OMV, is involved in the virulence of many pathogens and it is part of the oxidative stress associated chaperone system that includes DnaK that elevated in *F. nucleatum* (Doyle and Wickner, 2009, Steeves et al., 2011). Also, ClpP has been reported to be involved in bacterial attachment in *Y. enterocolitica* and involved in preventing growth and survival of macrophages by *S. enterica* (Hensel et al., 1995, Yamamoto et al., 2001). *F. nucleatum* ClpB is highly conserved with 97 % identical to *F. vincentii*, but only 24 % identity to *F. polymorphum* and *F. animalis*. Also, *F. nucleatum* ClpB is 51 % identical to *E. coli*, 47 % identical to *H. pylori*, 51 % identical to *Y. enterocolittica,* 51 % identical to *S. enterica*, 53 % identical to *P. gingivalis,* and 54 % identical to the variant in *L. monocytogenes.* In all cases the chaperone has been shown to be involved in the virulence (e.g. invasion) of these pathogens (Chastanet et al., 2004).

GroEl (**Table 3.1**) is a potent immunogenic heat shock protein involved in T-cell activation (Wick et al., 2001, Lee et al., 2012a) and GroEL serum antibody levels positively correlate with periodontitis (Tabeta et al., 2000). It has been reported that GroEL promoted inflammation by pathogens including *F. nucleatum, A. actinomycetemcomitans, P. intermedia, P. gingivalis,* and *T. forsythia* in atherosclerotic lesions (Ford et al., 2006). *F. nucleatum* GroEL is 99 % identical to *F. polymorphum*, and *F. vincentii* homologues and 98 % to *F. animalis*. GroEL of *F. nucleatum* participates in chronic inflammation in oral infections and is associated with systemic disease such as atherosclerosis. GroEL induces chemokines expression including IL-8, IL-6 and monocyte chemoattractant protein-1 as well as its ability to act as an adhesion molecule (Lee et al., 2012a, Hinode et al., 1998). Also, it has been observed that GroEL interacts with LPS and elicits a synergistic response in macrophages and stimulates IL-8 secretion (Noah et al., 2010).

TraT (FN0558, **Table 3.4**) confers resistance to complement (Sukupolvi and O'Connor, 1990) and thus could enable *F. nucleatum* evade destruction by the immune system. Again, TraT is highly conserved among the *Fusobacterium* species *vincentii* and *polymorphum* at 92 %.

The intestinal epithelial layer is an important barrier between the host and luminal contents thus maintenance of this barrier function is crucial. Alteration in barrier function has been connected to diseases including IBD, Celiac disease, and irritable bowel syndrome (Camilleri et al., 2012). The space between intact epithelial cells is sealed (paracellular space) a function mediated by the apical junctional complex that is composed of adherens junctions and tight junctions (Turner, 2009) and the adherens junctions provide adhesive bonds, which maintain cellular proximity. The tight junctions are composed of transmembrane proteins including claudin, occludin, and zonula occludens (ZO-1, and ZO-2) (Turner, 2009).

A convenient measure of the integrity of model epithelial monolayer is to determine the TER. High TER values reflect the integrity of filter grown epithelial cells and can be used to measure perturbation of the barrier integrity. The results of this study show that only OMVs modulate the TER by decreasing the electrical resistance across polarized epithelial cells monolayers in a time-dependent manner suggesting that this barrier function is compromised. However, only the OMV induced a significant reduction in TER.

Three possibilities may account for the reduction effect on TER. Firstly, proteases associated with the OMV may participate in this process by degrading adherens junction or tight junction proteins. Efforts to inhibit the OMV-associated protease activity by using protease inhibitors were unsuccessful and resulted in de-attachment of the cells from the substratum. Alternative strategies involved pre-incubating the OMV with inhibitor then removing the inhibitors by ultracentrifugation, which was unsuccessful also due to unacceptable loss of OMV.

Secondly, the MS analysis of the OMV demonstrated that FadA is present therefore it is likely that this can bind to E-cadherin on the colonic cells. FadA is a conserved adhesin of Fusobacteria and an important virulence factor known to engage with E-cadherin and induce dissociation of β-catenin in CRC (Han et al., 2005, Rubinstein et al., 2013, Fardini et

al., 2011). It has been cited by Whitmore and Lamont (2014) that gene levels of FadA in CRC is 10 time higher than normal individuals.

In support of this hypothesis, the data presented here demonstrates that OMV possibly bind to colonic cells at the cell periphery, the cellular location of E-cadherin. In addition, a consequence of this co-incubation is a decrease in the protein expression levels of E-cadherin. This latter data suggests that the observed decrease in E-cadherin expression could be responsible, in part, for the decrease seen in the TER. Thirdly, reduced E-cadherin gene expression elicited by co-incubation of colonic cell lines with *F. nucleatum* and OMV would also compromise barrier integrity as E-cadherin is functionally linked to the generation of a polarized epithelial phenotype. Any disruption to this role would likely disrupt barrier function.

The passage between the tight junctions is too narrow for bacteria to cross (Turner, 2009), and the translocation of the bacteria is a sign of impairment of intestinal integrity via tight junction pathway. EPEC decrease TER across polarized monolayers of Caco-2 by disrupting a transcellular pathway instead of disrupting intercellular tight junction (paracellular) (Canil et al., 1993). Also, It is possible that *F. nucleatum* OMVs were able to coalesce at the cell-cell boundaries, the subcellular location of E-cadherin. Several bacteria use different tactics to commandeer junctional structures for their advantage, which enables them to enter host cells. For example, *H. pylori* OMV can enter gastric cells via clathrin-dependent and independent means (Olofsson et al., 2014). However, it appears that *F. nucleatum* enter colonic cells in a clathrin-dependent manner (Rubinstein et al., 2013).

Caco-2 and T-84 cell lines have been used as a model for studying colon cancer (Pageot et al., 2000), and intestinal epithelium development (Bernet-Camard et al., 1996). During active inflammation, such as in IBD, increased permeability of the epithelium can arise as a result of deterioration of the epithelial barrier function (Hollander, 1988). This contributes to pathogenesis of the disease and triggers the inflammation cascade (Sanders, 2005).

In summary, to date, the role of *F. nucleatum* OMV in pathogenesis has not been investigated. The results presented here provide evidence of a functional role for these vesicles in modulating host cell response, but that are also distinct. It is recognized that *F.*

nucleatum cause diseases, but it's emerging that OMV separately cause damage to host tissues (as demonstrated in this work that OMV induce a significant reduction in TER and were efficient in degrading E-cadherin). Thus, OMV are likely important virulence factors that associate with pathogenesis and could produce damage to host cell. The MS analysis confirmed the presence of multiple proteolytic activities associated with OMV and those proteases were proteolytically active and can cause damage to host proteins. It is likely that the other immunogenic proteins identified in the OMV are also likely involved in the provocation of strong inflammatory responses, but further work is required to study these molecules further.

CHAPTER FOUR

The Contribution of *F. nucleatum* and OMV to the Induction of Pro-Inflammatory Cytokine/Chemokine Expression in Colonic Epithelial Cells

4.1 Introduction

OMVs are similar to their parental bacteria and like these they adhere to epithelial cells. For example, *H. pylori* OMV contain many OMPs and toxins that are able to bind and invade gastric epithelial cells (Parker et al., 2010). Similarly, *Legionella pneumophila* OMV bind to lung epithelial cells (Galka et al., 2008) where they can modulate cytokine production. Likewise, *F. nucleatum* has been reported to provoke a potent inflammatory response as evidenced by its ability to promote pro-inflammatory cytokine production by colonic and oral epithelial cells (Kang et al., 2009, Tang et al., 2016). However, little is known about *F. nucleatum* OMV in this regard. MS analysis of *F. nucleatum* OMV identified the present of FadA, many active proteases, and other potential pro-inflammatory molecules. FadA facilitates bacterial internalisation and invasion into host cells. Additionally, MS analysis of OMV revealed the presence of other proteins with potential virulence activities including NapA, FomA, Fad-I, and GroEL, in addition to LPS, which are associated with the initiation of strong immune responses and stimulate the production of pro-inflammatory cytokines. The inflammatory state of the colon greatly influences the development of dysplasia and CRC. In patients with IBD, chronic and severe inflammation of the colon significantly increases the risk of developing CRC (Beaugerie and Itzkowitz, 2015). In this study, the ability of *F. nucleatum* and OMV to interact with colonic epithelial cells in vitro and modulate the expression of pro-inflammatory cytokines were explored.

4.2 Hypothesis

In this work, it has been hypothesised that *F. nucleatum* and OMV are able to modulate pro-inflammatory cytokine expression in colonic epithelial cells.

Aims of the study:

To examine the ability of *F. nucleatum* and OMV to induce pro-inflammatory cytokine expression in colonic epithelial cells in vitro.

4.3 Result

4.3.1 *F. nucleatum* and OMV induce pro-inflammatory cytokine gene expression

NF-κβ is a transcription factor and is involved in the expression of almost 400 different genes and is linked to a variety of human diseases (Serasanambati and Chilakapati, 2016). Its activation is induced by many factors, including inflammation and cytokines and is associated with cytokine/chemokine regulation including IL-1 β, IL-6, IL-8, TNF-α, COX-2, and MMPs (Hayden and Ghosh, 2012). It was thus of interest to examine the effect of *F. nucleatum* and OMV on NK-κβ gene expression in SW-480 cells as no data were available on the effects of *F. nucleatum* OMV in this regard.

F. nucleatum and OMV induce expression of NK-κβ subunit 1 and 2 in SW-480 cells. Over 4 h of incubation, *F. nucleatum* induced approximately a 4-fold increase in NF-κβ subunit 1, which then reduced to 2.5-fold within a 24 h period. Also, OMV induced 8.5-fold increase in NF-κβ subunit 1 gene expression over a 4 h period, which reduced with time also (**Figure 4.1**). *F. nucleatum*, induced a 6-fold increase in NF-κβ subunit 2 gene expression over 4 h which reduced by 2-fold within 24 h, whereas OMV induced a 10-fold increase in NF-κβ subunit 2 gene expression over 4 h that decreased to 1.5-fold after 24 h. OMV induced strong expression of both subunits of NF-κβ that almost reduced to control levels within 24 h, whereas *F. nucleatum* induced a lower increase in both NF-κβ subunits but sustained higher expression levels than OMV at 24 h (**Figure 4.1**).

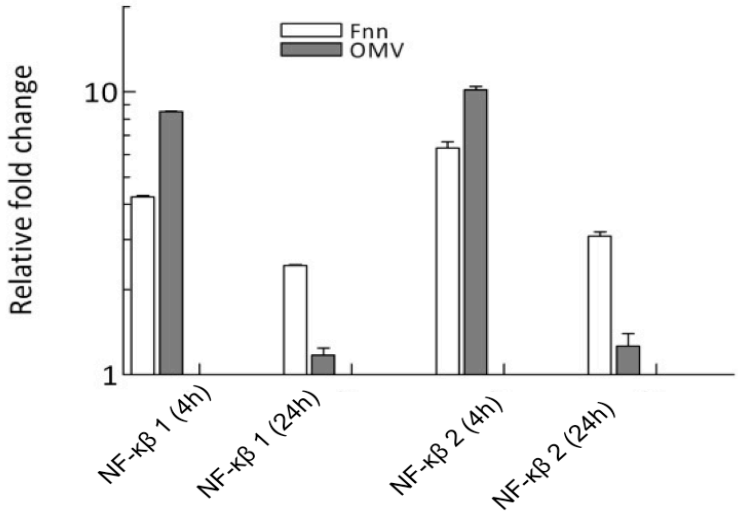

Figure 4.1: Effect of *F. nucleatum* and OMV on the expression of NF-κβ in SW-480 cells. SW-480 cells were treated with *F. nucleatum* (MOI 100:1) and OMV (10 μg/ml) over a 4 and 24 h (n=2).

The IL-6/STAT-3 signalling pathway is involved in the regulation of intestinal cell proliferation and survival, which is associated with IBD and CRC (Waldner et al., 2012). STAT-3 and NF-κβ are important transcription factors that contribute to oncogene activation. STAT-3 plays an essential role in the regulation of immune responses in tumours and has been associated with cancer cell proliferation, survival, and invasion, in addition to its role in tumour inflammation and immunity (Yu et al., 2009a). In order to examine the ability of *F. nucleatum* and OMV to induce STAT-3 phosphorylation, SW-480 cells were treated with *F. nucleatum* and OMV over 10, 30, and 60 min periods. Western blotting was performed on the cell lysates and probed with anti-STAT-3 and anti-phosphorylated STAT-3 (pSTAT-3) and tubulin was used as a loading control (**Figure 4.2, A**). Normalisation of the pSTAT-3 expression levels using total STAT-3 indicated that *F. nucleatum* and OMV-induced STAT-3 phosphorylation by 2-fold and 3-fold, respectively, over 10 min, after which time the pSTAT-3 expression level started to reduce until it reached the control level within 1 h (**Figure 4.2, B**).

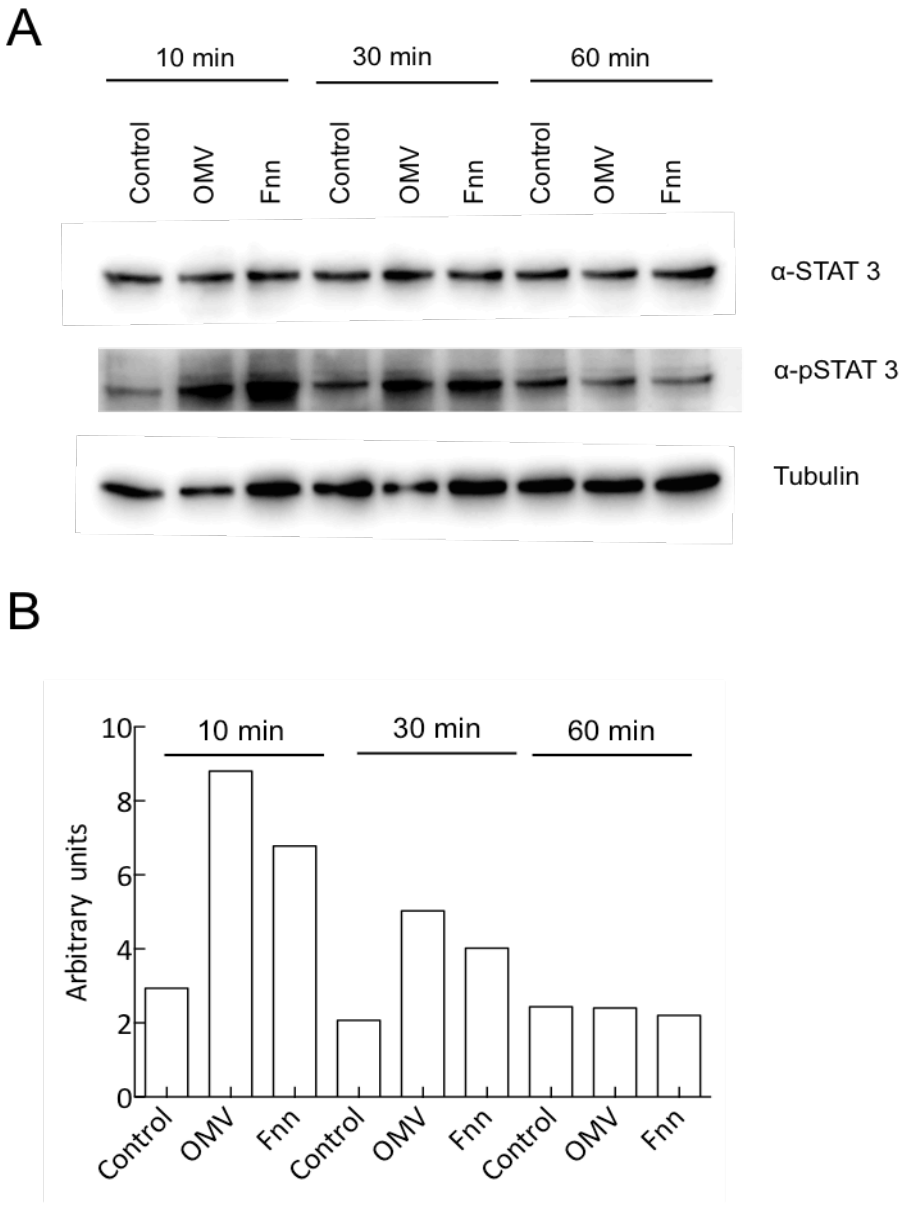

Figure 4.2: Western blot showing the effect of *F. nucleatum* and OMV on STAT-3 phosphorylation in SW-480 cells. SW-480 cells were co-cultured with *F. nucleatum* (MOI 200:1), and *F. nucleatum* OMV (30 μg/ml) for 10, 30, and 60 min. **Panel A:** SW-480 cells were lysed and whole cells extracts were resolved by 12.5 % SDS-PAGE and electrotransferred to PVDF. Total membrane, STAT-3 expression (upper panel), phosphorylated STAT-3 expression (middle panel), and tubulin expression was used as loading control (lower panel). **Panel B:** Normalisation of pSTAT-3 expression in SW-480 using total STAT-3.

NF-κβ regulates the production of pro-inflammatory cytokines and chemokines including IL-8, a common chemokine produced in response to inflammation such as that encountered during bacterial infection. Dose response studies were undertaken for *F. nucleatum* and extended for OMV to determine the minimum amount of each that would

be required to elicit IL-8 gene expression. SW-480 cells were treated with an increasing MOI of *F. nucleatum* : colonic cells (1:1, 10:1, 100:1, and 1000:1) and increasing amounts of OMV (0.01, 0.1, 1, and 10 μg/ml). *F. nucleatum* induced a statistically significant increase in IL-8 gene expression at every MOI tested relative to the control cells (**Figure 4.3, A**) with a small decrease in gene expression observed at an MOI 1000:1. Likewise, significant IL-8 gene expression was apparent in response to increasing amounts of OMV up to 10 μg/ml relative to control cells (**Figure 4.3, B**). With an MOI of 1.0 and a small amount of OMV (0.01 μg/ml), a significant amount of IL-8 was expressed, which makes them potent inducers of *IL-8* expression.

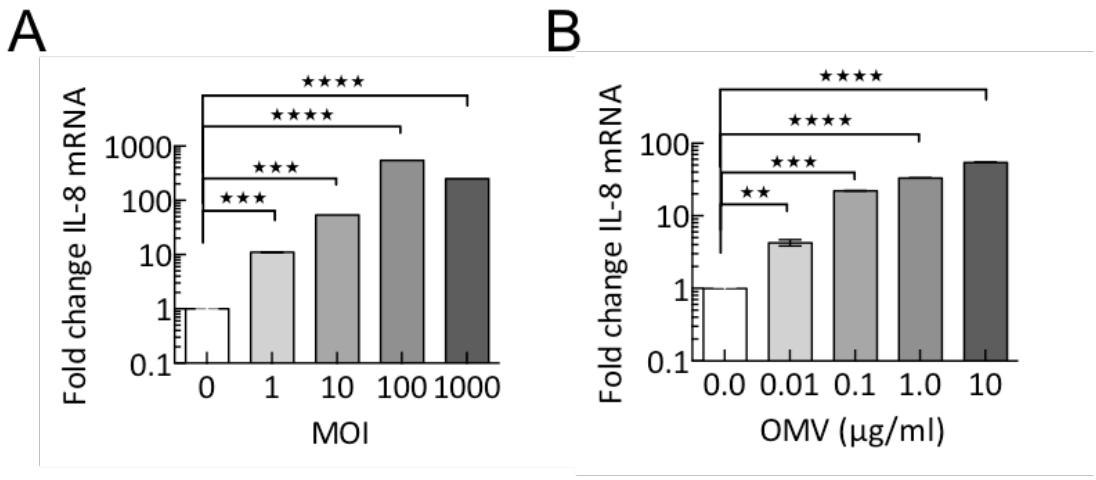

Figure 4.3: *F. nucleatum* and OMV modulate IL-8 gene expression in a dose-dependent manner. SW-480 cells were treated with *F. nucleatum* (MOI 1:1, 10:1, 100:1, and 1000:1) and OMV (0.01, 0.1, 1, and 10 μg/ml). Cells were incubated for 4 h. The results are represented as the mean ± SEM (n=3). **Panel A:** IL-8 mRNA dose response to MOI of *F. nucleatum*. **Panel B:** IL-8 mRNA dose response to OMV. ** = $p < 0.01$, *** = $p < 0.001$, and **** = $p < 0.0001$.

In order to examine the effect of different *Fusobacterium* species (*F. nucleatum*, *F. vincentii*, and *F. polymorphum*) and their OMV on IL-8 production in SW-480 cells, the cells were treated with the different species and an increasing amount of OMV over 24 h (**Figure 4.4**). As expected, all the *Fusobacterium* species and their OMV induced IL-8 secretion by SW-480 cells. *F. vincentii* and *F. polymorphum* induced more IL-8 secretion when compared with *F. nucleatum*. Also, *F. polymorphum* OMV induced the highest amount of IL-8 followed by *F. vincentii* OMV, then *F. nucleatum* OMV in a dose-dependent manner (**Table 4.1**).

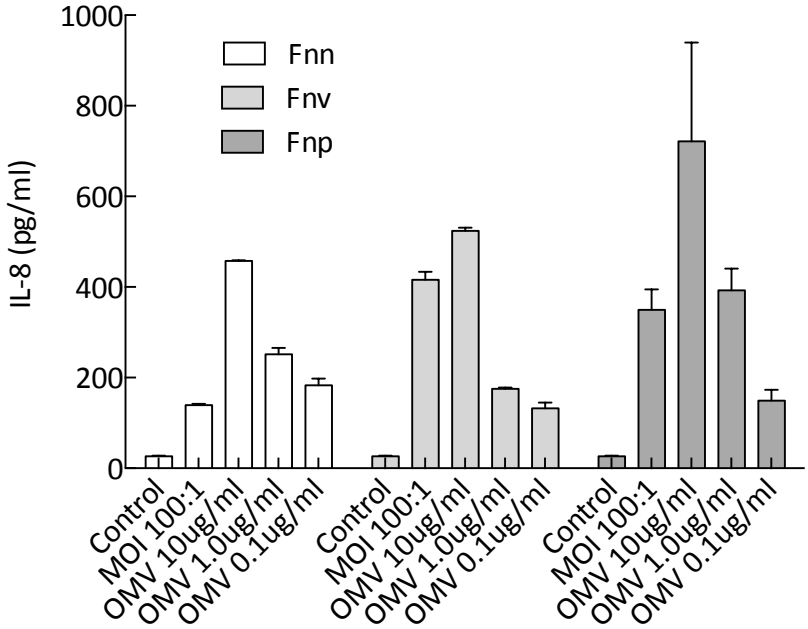

Figure 4.4: The effect of *F. nucleatum*, *F. vincentii*, and *F. polymorphum* and their OMV on IL-8 production in SW-480 cells. SW-480 cells were infected with *F. nucleatum*, *F. vincentii*, and *F. polymorphum* (MOI 100:1) and OMV (0.1, 1.0, and 10 μg/ml) over 24 h. IL-8 production was measured in the supernatant by ELISA (n=2).

Table 4.1: IL-8 production in SW-480 cells (pg/ml).

***F. nucleatum* species**	**Control**	**Bacteria**	**OMV (μg/ml)**		
			0.1	1.0	10
F. nucleatum	26.5 ± 1.2	140 ± 2.5	182.8 ± 15	252 ± 14	457 ± 1.4
F. vincentii	26.5 ± 1.2	415 ± 18	132 ± 13	176 ± 2.6	524 ± 7
F. polymorphum	26.5 ± 1.2	350 ± 44	150 ± 24	393 ± 48	722 ± 217

To examine the temporal effect of *F. nucleatum* and OMV on IL-8 gene expression in colonic epithelial cells a time course study was performed with SW-480 cells. The treatment of SW-480 cells with *F. nucleatum* and OMV resulted in a statistically significant increase in IL-8 gene expression at every time point tested relative to the control cells (**Figure 4.5**) with a 167-fold increase in *IL-8* expression observed after 6 h ($p < 0.001$). Over time, *IL-8* expression decreases gradually but remained significantly higher than that of the control cells at 72 h. *F. nucleatum* induced a 31-fold increase and OMV induced a 13-fold increase ($p < 0.001$ for both).

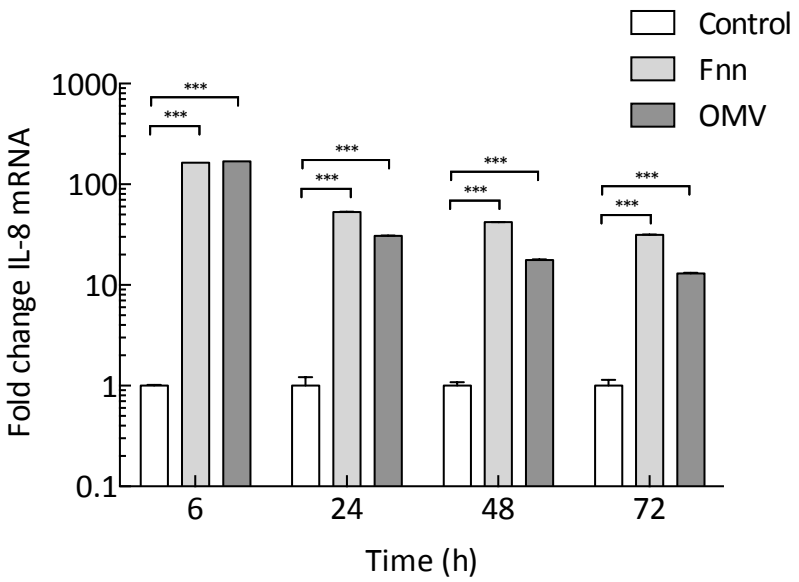

Figure 4.5: Time course of the effect of *F. nucleatum* and OMV in the induction of IL-8 gene expression in SW-480. SW-480 cells were treated with *F. nucleatum* (MOI 500:1) and OMV (30µg/ml) over the times indicated (n=3). *** = $p < 0.001$.

To evaluate the effect of *F. nucleatum* and OMV on colonic epithelial cell IL-8 secretion SW-480 cells were treated with *F. nucleatum* and OMV over two-time points (6 h and 24 h). In the first instance, the amount of IL-8 secretion in response to *F. nucleatum* and OMV was measured by ELISA. Treatment resulted in the induction of IL-8 secretion from SW-480 cells relative to control. The amount of IL-8 secreted reached approximately 800 pg/ml within 6 h and continued to increase to approximately 1600 pg/ml by 24 h (**Figure 4.6**). Thus, both IL-8 protein and gene expression were sustained over time that permitted a large amount of IL-8 to be produced by colonic cells in response to infection by both *F. nucleatum* and OMV.

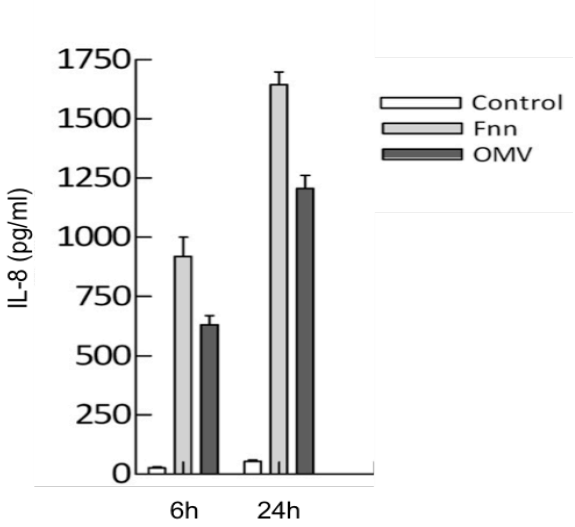

Figure 4.6: Effect of *F. nucleatum* and OMV on IL-8 in SW-480. SW-480 cells were treated with *F. nucleatum* (MOI 200:1) and OMV (5 µg/ml) over a 6 and 24 h period. Secreted IL-8 was detected by ELISA following treatment of cells (n=2).

To examine the effect of *F. nucleatum* and OMV on IL-8 production by other colonic cells (SW-620, Caco-2, and T-84) time course studies were performed. The cells were treated with *F. nucleatum* and OMV. Similar to SW-480 cells *F. nucleatum* and OMV induce an increase in *IL-8* expression in SW-620, Caco-2, and T-84 cells that was approximately 10 times less than that of SW-480 (**Figure 4.7, A, B, and C**). Similarly, *F. nucleatum* and OMV induced IL-8 production in SW-620 and T-84 cell lines. In Figure 4.8 there is an increase amount of IL-8 induce in control which is unusual or indicate a problem with assay. However, SW-620 represent a late stage of metastases and their reaction unpredictable. It is noticeable that SW-480 had the lowest IL-8 background in addition to its ability to induce the highest amount of IL-8 following the treatment.

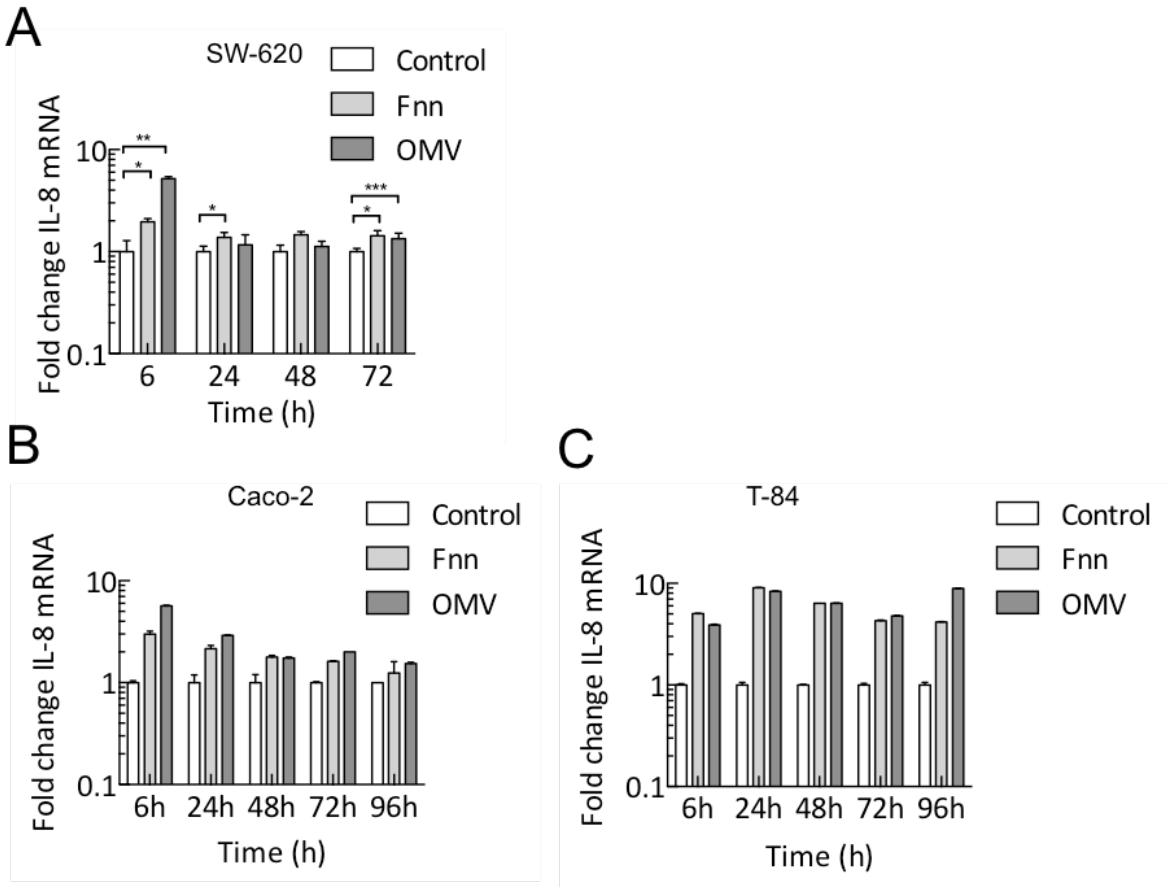

Figure 4.7: Time course of the effect of *F. nucleatum* and OMV on the induction of IL-8 gene expression in SW-620, Caco-2, and T-84 cell lines. Different cell lines were treated with *F. nucleatum* (MOI 500:1) and OMV (30µg/ml) over the times indicated. **Panel A:** IL-8 gene expression in SW-620 (n=3). **Panel C:** IL-8 gene expression in Caco-2 (n=2). **Panel D:** IL-8 gene expression in T-84 (n=2). * = $p < 0.05$, ** = $p < 0.01$, and *** = $p < 0.001$.

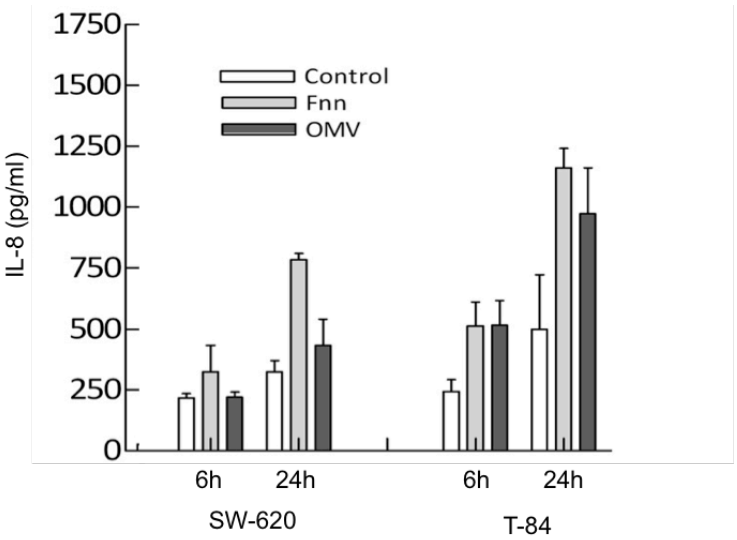

Figure 4.8: Effect of *F. nucleatum* and OMV on IL-8 secretion by SW-620, and T-84 cells. The cells were treated with *F. nucleatum* (MOI 200:1) and OMV (5 µg/ml) over 6 and 24 h. Secreted IL-8 was quantified by ELISA following treatment of cells (n=2).

In order to determine the mechanisms involved in the production of IL-8, candidate signalling pathways in the production of IL-8 and the contribution of LPS to IL-8 production were examined using specific pathway inhibitors.

4.3.2 Pathways involved in *F. nucleatum* and OMV induced IL-8 secretion

In order to determine the signalling pathways involved in IL-8 production, a number of pathway inhibitors were used including PD0325901, a selective inhibitor of extracellular signal-regulated kinase (ERK) (Park et al., 2014b); GSK690693, a pan-Akt inhibitor (Rhodes et al., 2008); LY294002, a selective inhibitor of phosphatidylinositol 3 (PI3) kinase and PI3 kinase-dependent Akt (protein kinase B) (Vlahos et al., 1994); and SP600125, a potent c-jun inhibitor (Bennett et al., 2001).

Pre-treatment of SW-480 cells with GSK690693 and LY294002 resulted in significant attenuation of IL-8 secretion (41 %, and 19 % inhibition, respectively) induced by *F. nucleatum* ($p < 0.01$ and $p < 0.05$, respectively), which indicates the involvement of the PI3k/AKT pathway. Interestingly, no significant inhibition due to pre-treatment with GSK690693 and LY294002 was observed in cells treated with OMV although the pattern of inhibition was similar to that observed with *F. nucleatum* induced IL-8 secretion. Pre-treatment with PD98059, and SP600125 also induced a significant inhibition of IL-8 secretion (83 % and 64 %, respectively) in cells treated with *F. nucleatum* ($p < 0.01$ and $p < 0.001$, respectively) (**Figure 4.9** and **Table 4.2**). Similarly, PD98059 and SP600125 inhibitors induced a significant inhibition of IL-8 secretion (65 % and 37 %, respectively) in cells treated with OMV ($p < 0.05$) (**Figure 4.9** and **Table 4.2**). This suggests that *F. nucleatum* and OMV activate the MAPK/ERK/JNK pathways and are involved in IL-8 secretion.

It is notable that there is a big difference between the amount of IL-8 induced by *F. nucleatum* and OMV (**Figure 4.4, 4.6, and 4.9**). This difference could be resulted from the different estimated amount of the microorganisms or due to the effect of freeze and thawing of OMV (especially at the early stage of my experiments, **Figure 4.4**). However, it is clearly indicated in these experiments that *F. nucleatum* and OMV are potent inducer of IL-8 production.

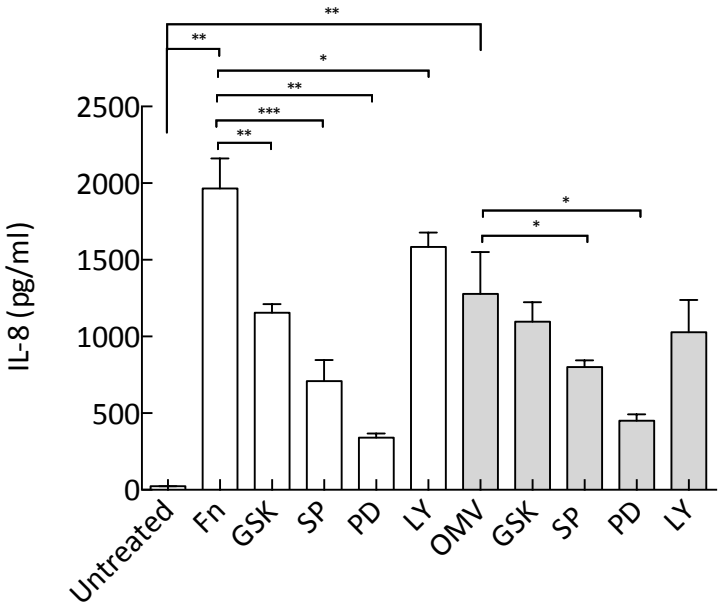

Figure 4.9: Effect of inhibitors on IL-8 production in SW-480 cells. SW-480 cells were grown in a 48-well plate until 80 % confluent before treatment with different inhibitors, GSK (10 µM), SP (50 µM), PD (1 µM), and LY (50 µM) over 2 h prior to the addition of *F. nucleatum* (MOI 150:1) and OMV (10 µg/ml) and the incubation continued for an additional 4 h (n=3). * = $p < 0.05$, ** = $p < 0.01$, and *** = $p < 0.001$.

Table 4.2: Effect of selected inhibitors on *F. nucleatum* and OMV-induced IL-8 secretion by SW-480 cells.

inhibitors	Inhibition of IL-8 secretion induced by *F. nucleatum* (%).	Inhibition of IL-8 secretion induced by OMV (%).
GSK690693	41 %	14 %
SP600125	64 %	37 %
PD98059	83 %	65 %
LY294002	19 %	19 %

4.3.3 The contribution of *F. nucleatum* OMV LPS to IL-8 production.

LPS is an important virulence factor that contributes to the immunogenicity and pro-inflammatory effects of gram-negative bacteria. LPS was purified from *F. vincentii* and *F. nucleatum* (**Figure 4.10, A**) as described in the Methods section. In order to examine the contribution of *F. nucleatum* LPS on the induction of IL-8 gene expression in SW-480 cells, the cells were incubated with *F. nucleatum* and *F. vincentii* OMV, *E. coli* LPS, and *F. nucleatum* and *F. vincentii* LPS in the absence and presence of polymyxin B (PMB). As expected *F. nucleatum* and *F. vincentii* OMV induced a significant increase in IL-8 gene expression in SW-480 cells ($p < 0.0001$). Likewise, purified *F. nucleatum*, *F. vincentii*, and *E.*

coli LPS induced a significant increase in IL-8 gene expression (20-fold, 142-fold, and 107-fold, respectively). PMB significantly reduced *IL-8* expression, induced by purified *F. nucleatum* LPS, to control levels. There was a significant but incomplete reduction in *F. vincentii* and *E.coli* LPS induced *IL-8* expression also in the presence of PMB (**Figure 4.10, B**). Notably, there was only a minor reduction in the OMV (*F. nucleatum* and *F. vincentii*) induced *IL-8* expression in the presence of PMB, which suggests that factors in OMV other than LPS were responsible for the majority of *IL-8* expression.

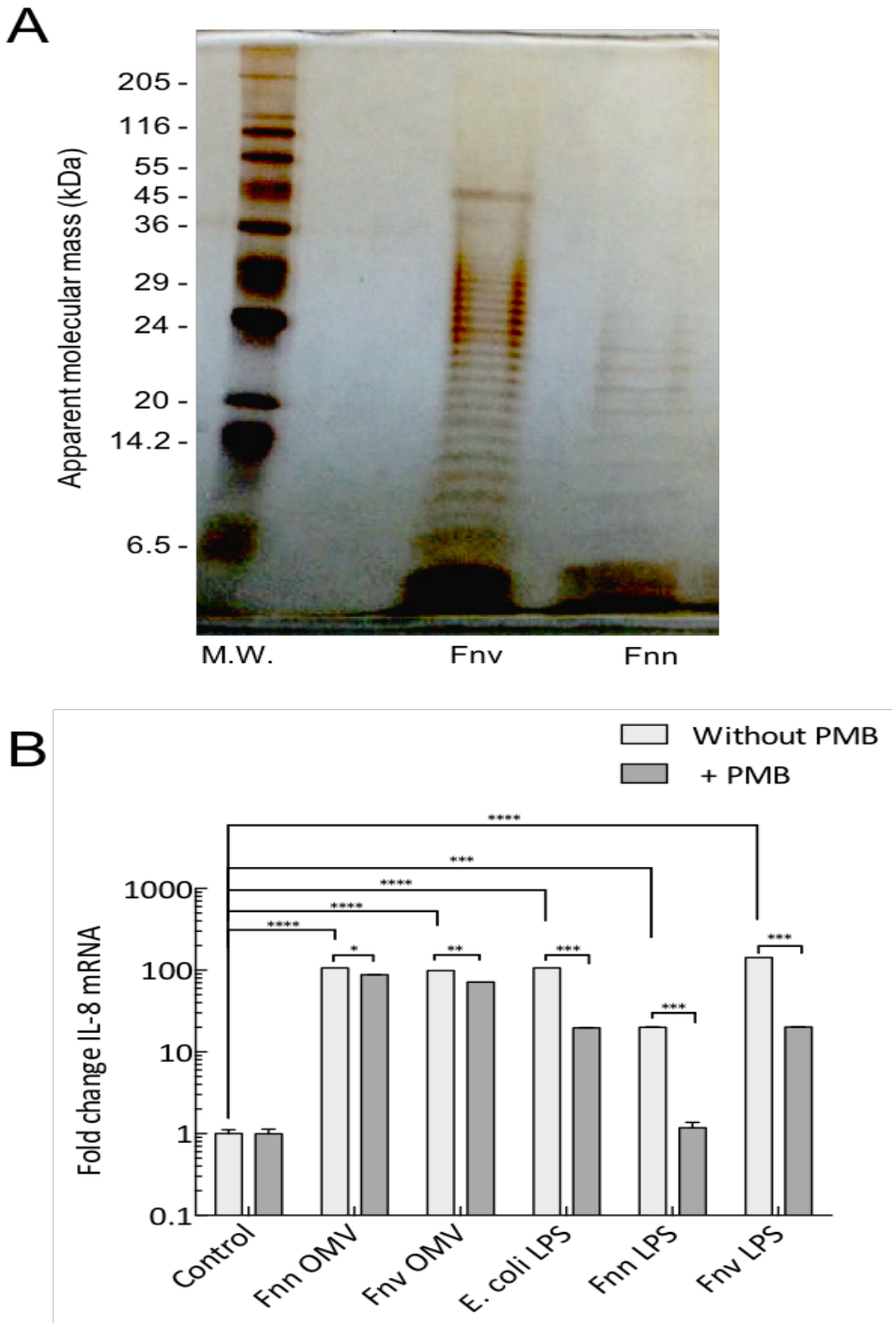

Figure 4.10: The contribution of *F. nucleatum* OMV LPS to IL-8 gene expression in SW-480 cells. Panel A: Illustrates a silver stained 12.5 % SDS-PAGE gel of *F. vincentii* (Fnv) and *F. nucleatum* (Fnn) LPS (40 μg/ml of each). The molecular mass markers are shown on the left-hand side. **Panel B:** Illustrates the effect of polymyxin B on IL-8 gene expression induced in SW-480 cells treated with *F. nucleatum* OMV, *F. vincentii* OMV, *E. coli* LPS, *F. nucleatum* LPS and *F. vincentii* LPS (10 μg/ml) for 4 h. The results are represented as the mean ± SEM (n=3). * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, and **** = $p < 0.0001$.

4.3.4 *F. nucleatum* and OMV induce pro-inflammatory cytokine secretion/expression

In order to examine the ability of *F. nucleatum* and OMV to induce the expression of other chemokines/cytokines from colonic epithelial cells, different cell lines (SW-480, SW-620, Caco-2, and T-84) were treated with *F. nucleatum* and OMV. A time course study was performed to examine the effects of *F. nucleatum* and OMV on GRO-α (CXCL-1) gene expression. *F. nucleatum* and OMV induced GRO-α gene expression in all colonic epithelial cells tested. Treatment of SW-480 cells with *F. nucleatum* and OMV resulted in sustained GRO-α gene expression over 96 h with maximum expression (236-fold) observed at 6 h. By 96 h, the expression level had decreased to 28-fold and 16-fold in the presence of OMV and *F. nucleatum*, respectively (**Figure 4.11, A**). In the case of SW-620 GRO-α gene expression started to increase slowly with time until it reached a peak of 3-fold at 48 h (**Figure 4.11, B**), whereas the T-84 cells expression was sustained over the time course studied (**Figure 4.11, C**).

Likewise, *F. nucleatum* and OMV induced the secretion of GRO-α protein from all cell lines tested (**Figure 4.12**). The highest amount of GRO-α was secreted by SW-480 cells followed by SW-620 cells, and with the least by T-84 cells.

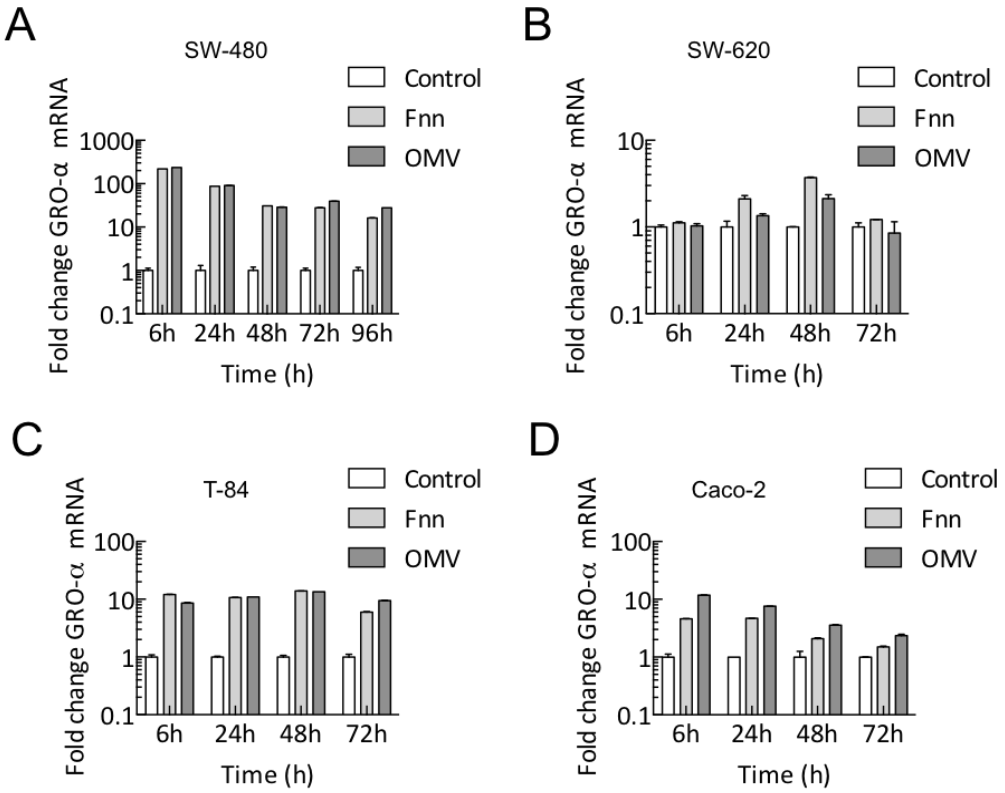

Figure 4.11: Time course of the effect of *F. nucleatum* and OMV on GRO-α gene expression in SW-480, SW-620, Caco-2, and T-84 cell lines. The cells lines were treated with *F. nucleatum* (MOI 500:1) and OMV (30 µg/ml) for the times indicated (n=2). **Panel A:** GRO-α gene expression in SW-480. **Panel B:** GRO-α gene expression in SW-620. **Panel C:** GRO-α gene expression in T-84. **Panel D:** GRO-α gene expression in Caco-2.

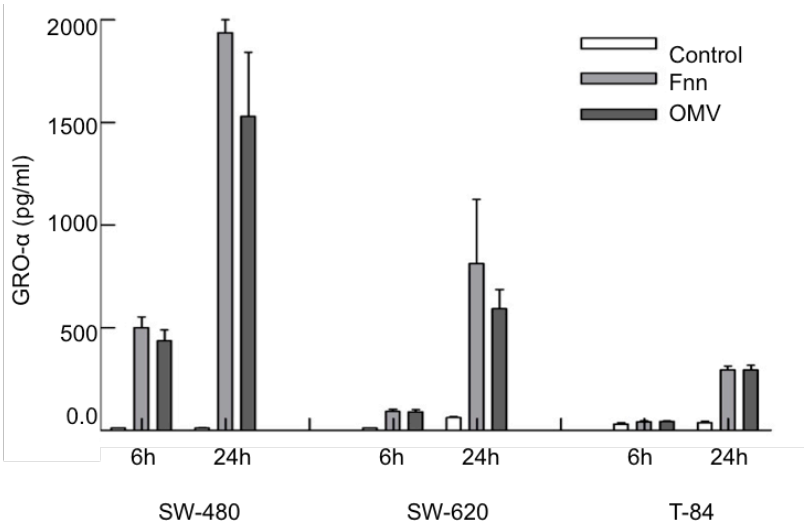

Figure 4.12: Effect of *F. nucleatum* and OMV on GRO-α (CXCL-1) secretion in SW-480, SW-620, and T-84 cells. The cells were treated with *F. nucleatum* (MOI 200:1) and OMV (5 µg/ml) over a 6 and 24 h period. Secreted GRO-α was detected by ELISA following treatment of cells (n=2).

Similarly, *F. nucleatum* and OMV induced TNF-α gene expression in all colonic epithelial cells tested. RT-PCR time course experiments revealed that the treated SW-480 cells produced the highest level of TNF-α expression when compared with other cell lines followed by T-84 cells, and SW-620, respectively (**Figure 4.13, A, B, and C**). Once again, similar to the GRO-α study, Caco-2 cells were the least responsive when exposed to *F. nucleatum* and OMV (data not shown).

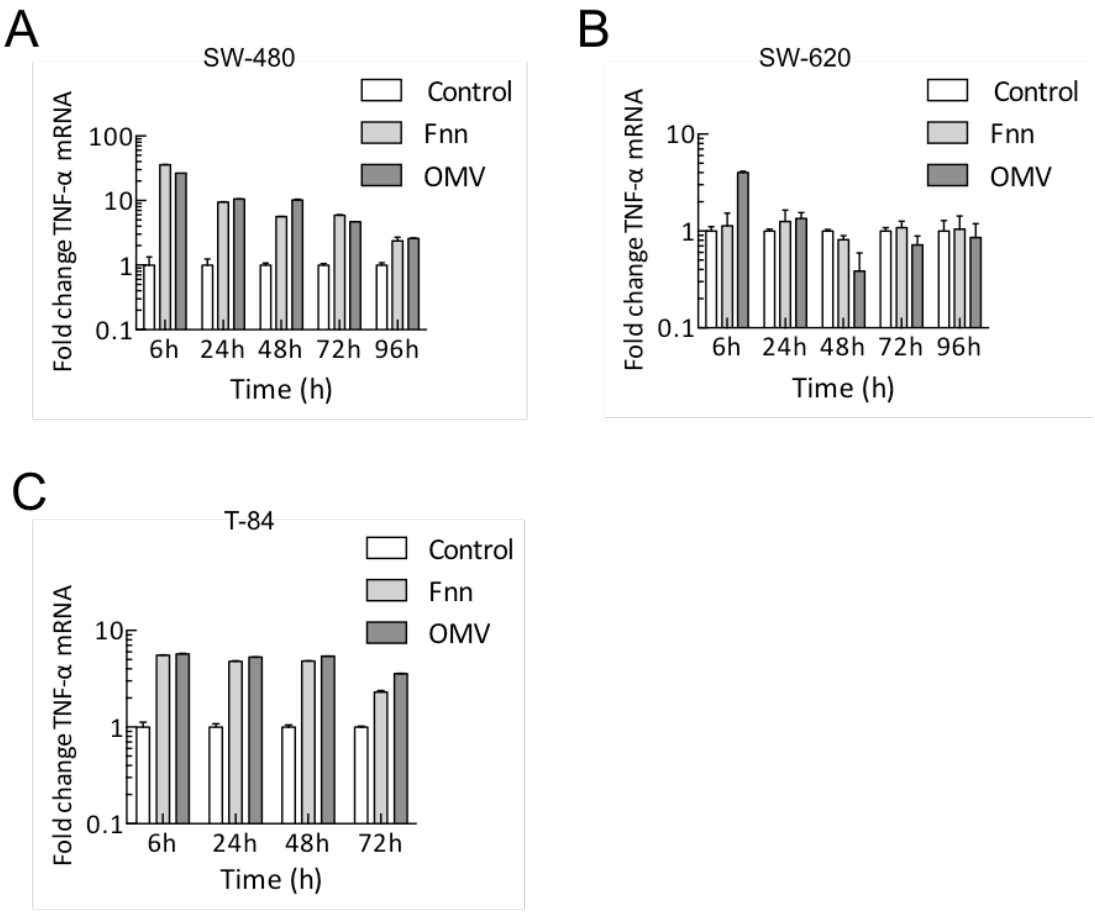

Figure 4.13: Time course of the effect of *F. nucleatum* and OMV on TNF-α gene expression in SW-480, SW-620, and T-84 cell lines. The cells were treated with *F. nucleatum* (MOI 500:1) and OMV (30 μg/ml) for the times indicated (n=2). **Panel A:** TNF-α gene expression in SW-480. **Panel B:** TNF-α gene expression in SW-620. **Panel C:** TNF-α gene expression in T-84.

In contrast to GRO-α and TNF-α, *F. nucleatum* and OMV treatments only induced expression of the IL-1 β gene at 6 h with levels returning largely to baseline in SW-480 cells thereafter (**Figure 4.14, A**), whereas there was a pronounced expression of macrophage inflammatory protein-3 (MIP3-α) also known as CCL-20 in response to *F. nucleatum* treatment (576-fold), which decreased over time. Likewise, OMV induced a robust

expression of MIP3-α (240-fold) within 6 h, peaking at 826-fold at 24 h then decreasing to 27-fold within 96 h (**Figure 4.14, B**).

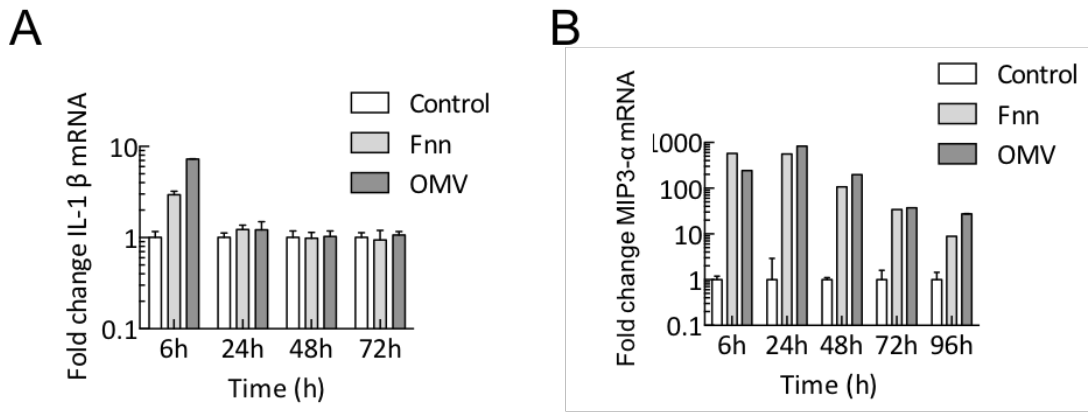

Figure 4.14: Effect of *F. nucleatum* and OMV on IL-1 β, and MIP3-α gene expression in SW-480 cells. SW-480 cells were treated with *F. nucleatum* (MOI 500:1) and OMV (30 μg/ml) for the times indicated (n=2). Where no error bar is shown, the error is smaller than the representation of the point. **Panel A:** expression of IL-1 β. **Panel B:** expression of MIP3-α.

IL-6 is one of the major cytokines in the tumour microenvironment that promotes tumorigenesis (Kumari et al., 2016). A transient expression of IL-6 gene expression was observed in response to treatment with *F. nucleatum* and OMV. *F. nucleatum* induced the expression level to 20-fold within 6 h, which decreased to 6-fold within 24 h and maintained the expression level of 2-fold at 96 h whereas OMV induced IL-6 expression to 6-fold by 6 h which decreased to control levels within 48 h (**Figure 4.15**).

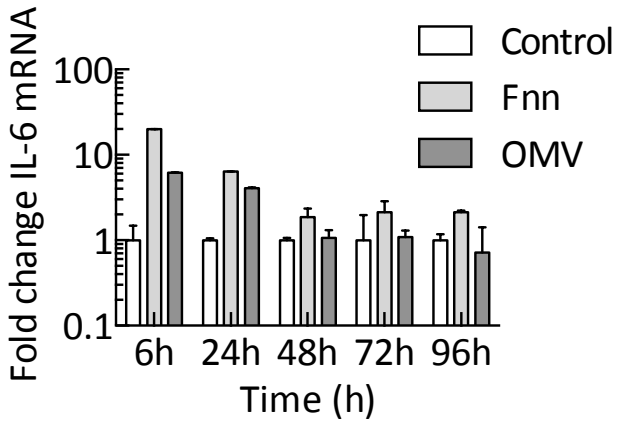

Figure 4.15: Effect of treatment with *F. nucleatum* and OMV on IL-6 gene expression in SW-480 cells. SW-480 cells were treated with *F. nucleatum* (MOI 500:1) and OMV (30 μg/ml) for the times indicated (n=2).

TGF-β plays a crucial role in vascular development, angiogenesis, and activation of TGF-β/Smad signaling, which is associated with CRC (Stanilov et al., 2016). Thus, it was of interest to examine the effect of *F. nucleatum* and OMV on TGF-β gene expression in SW-480 cells. Interestingly, *F. nucleatum* and OMV had no significant effect on TGF-β gene expression in SW-480 cells (**Figure 4.16**), at least under the conditions tested. This suggests that *F. nucleatum* and OMV do not activate the TGF-β/Smad signaling pathway in colonic epithelial cells over the time course investigated.

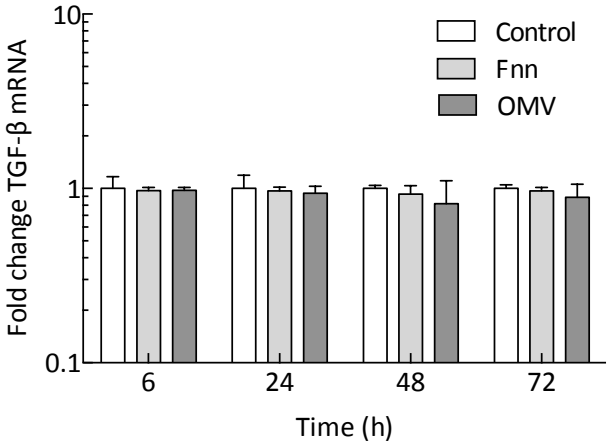

Figure 4.16: Effect of *F. nucleatum* and OMV on the induction of TGF-β gene expression in SW-480. SW-480 cells were treated with *F. nucleatum* (MOI 500:1) and OMV (30μg/ml) for the times indicated (n=3) and TGF-β mRNA was measured by RT-PCR.

Other pro-inflammatory gene signatures associated with CRC were measured also, including suppressor of cytokine signalling-3 (SOCS-3), prostaglandin-endoperoxide

synthase-2 (PTGS-2) also known as cyclooxygenase-2 (COX-2), and sphingosine kinase-1 (SPHK-1), which are associated with *F. nucleatum* abundance and over-expressed in human CRC (Kostic et al., 2013b, Long et al., 2015). *F. nucleatum* induced approximately a 6-fold increase in SOCS-3 gene expression in SW-480 cells compared with a 3-fold in response to OMV at 4 h. Also, *F. nucleatum* and OMV induced SPHK-1, and COX-2 gene expression (**Figure 4.17**).

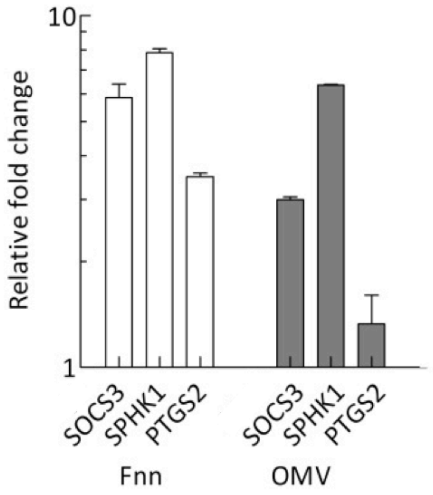

Figure 4.17: Effect of *F. nucleatum* and OMV on SOCS-3, SPHK-1, and PTGS-2 (COX-2) gene expression in SW-480. SW-480 cells were treated with *F. nucleatum* (MOI 100:1) and OMV (10 μg/ml) for 4 h (n=2).

4.3.5 *F. nucleatum* and OMV induce MMP gene expression

MMPs are associated with chronic inflammation (IBD) and cancer, which are linked to bacterial infection in lung and gastric cells (Corbel et al., 2002, Yeh et al., 2010). MMP overexpression is a key modulator of the risk of CRC progression and metastasis (Zucker and Vacirca, 2004, Banday et al., 2016). RT-PCR analysis showed that *F. nucleatum* induced a significant increase in MMP-1 gene expression ($p < 0.01$) that reached 4-fold within 24 h then dropped to 2-fold by 48 h. While OMV induced a significant increase in MMP-1 gene expression of 2-fold by 4 h, increased to 4-fold ($p < 0.01$) by 24 h, finally dropping to control levels of expression by 48 h (**Figure 4.18, A**). In the case of MMP-2, the time course analysis indicated that *F. nucleatum* induced a significant reduction ($p < 0.01$) in gene expression by 4 h, with MMP-2 gene expression increasing to 2-fold over 24 h period and finally dropped to 1.2-fold by 48 h. Interestingly, OMV had no effect on MMP-2 expression until 48 h when

significant repression was seen (**Figure 4.18, B**). Also, *F. nucleatum* induced a significant increase in MMP-9 gene expression ($p < 0.01$) that reached 4-fold within 4 h then it started to drop until it reached 0.6-fold over 48 h. Whereas OMV induced a significant increase in MMP-9 gene expression that reached 4-fold over 4 h and continued to increase with time until it reached a peak of 7-fold by 48 h (**Figure 4.18, C**). Also, *F. nucleatum* induced an increase in the expression of MMP-10 and MMP-13 whereas OMV caused an increase in the expression of MMP-10 only (**Figure 4.19**).

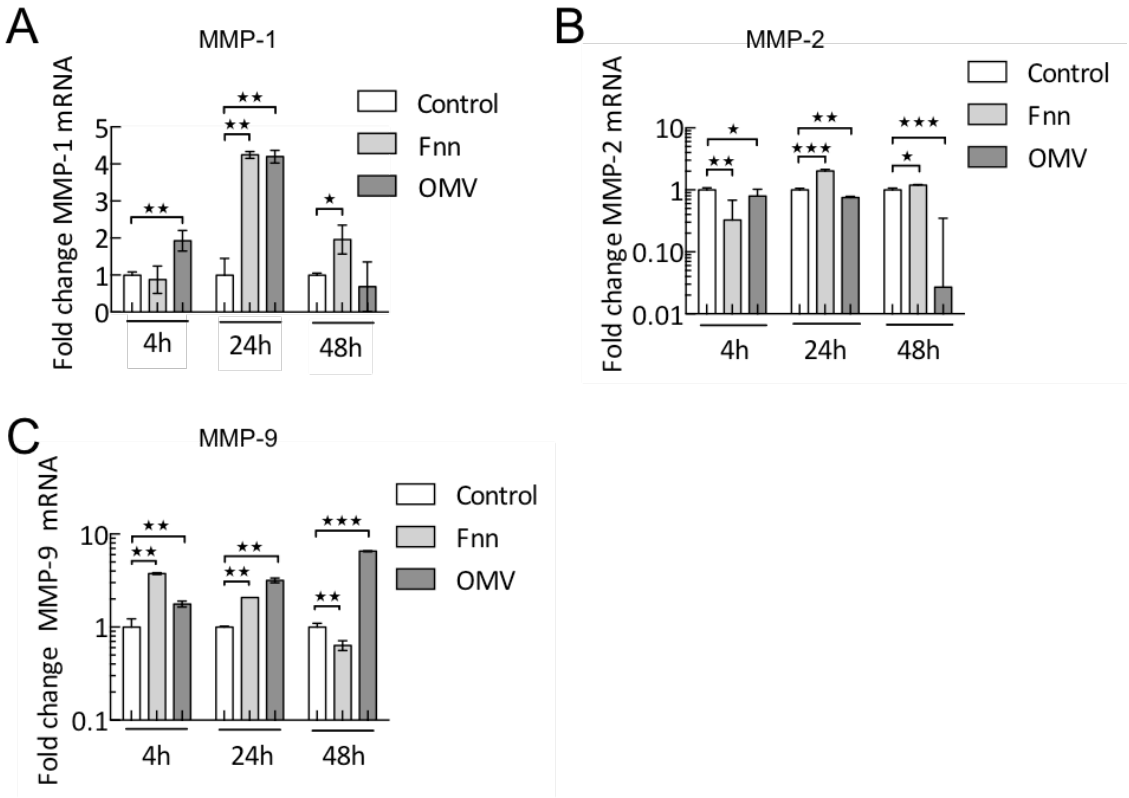

Figure 4.18: A time course analysis of the effect of *F. nucleatum* and OMV on MMP-1, MMP-2 and MMP-9 gene expression in SW-480 cells. SW-480 were treated with *F. nucleatum* (MOI 100:1) and OMV (10 μg/ml) for the time indicated. The results are presented as the mean ± SEM (n=3). * = $p < 0.05$, ** = $p < 0.01$, and *** = $p < 0.001$.

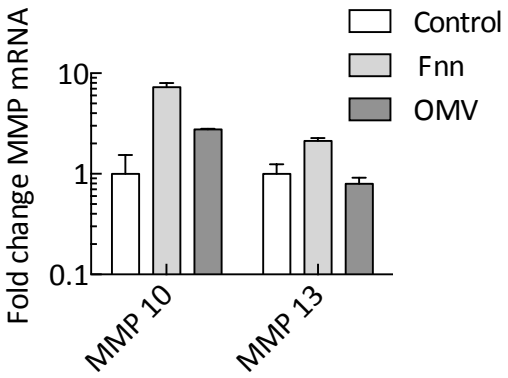

Figure 4.19: Effect of *F. nucleatum* and OMV on MMP-10, and MMP-13 gene expression in SW-480 cells. SW-480 cells were co-cultured with *F. nucleatum* (MOI 500:1), and OMV (30 µg/ml) for 48 h (n=2).

4.4 Discussion

The aim of this study was to examine the ability of *F. nucleatum* and OMV to induce pro-inflammatory cytokine/chemokine expression in colonic epithelial cells in vitro. In this study, it has been found that *F. nucleatum* and OMV were able to induce a strong inflammatory response, which was evident by the induction of pro-inflammatory cytokines/chemokines in the colonic epithelial cell lines tested. Furthermore, multiple pathways appeared to be involved in the production of IL-8 including PI3K/AKT and MAPK/ERK/JNK pathways. *F. nucleatum* and OMV induced the expression of other molecules that are associated with tumour growth and development including MMPs, SOCS-3, SPHK-1, and COX-2. The ability of *F. nucleatum* and OMV to alter the pro-inflammatory microenvironment may provide the driving force of many changes including EMT that are involved in cancer initiation and development.

Chronic inflammation is the key contributor to tumour development and is a major risk factor for CRC (Song et al., 2013). Any change in homeostasis or infection status results in inflammation as a protective response to tissue damage (Garrett et al., 2010). These changes participate in the induction of tissue damage, which recruit immune cells and induce the production of various inflammatory mediators (cytokines, chemokines, and MMPs). Inflammatory mediators are factors that directly affect tumour cells, and are produced by epithelial cells and infiltrating cells in the microenvironment (Ma et al., 2016). The production of inflammatory mediators amplify the cell damage (Medzhitov, 2008) and alter the microenvironment to become more oxidative, thus enhancing DNA mutation (Coussens and Werb, 2002, Ohnishi et al., 2013). DNA modification could result in mutation in APC, and TP53 or mutation in MAPK pathways (KRAS, NRAS, and BRAF), which are involved in CRC formation (van den Broek et al., 2016). In addition, inflammation has a great impact on supporting tumour growth via increased malignant cell survival, proliferation, angiogenesis and metastasis (Mantovani et al., 2008). Crohn's and ulcerative colitis are disorders of the gastrointestinal tract that result in IBD (Laroui et al., 2012). IBD is characterised by intestinal inflammation that leads to mucosal disruption and finally ulceration (Uddin et al., 2013). IBD is one of the risk factors that leads to the development of CRC (Itzkowitz and Yio, 2004).

NF-κβ and STAT-3 are transcription factors that are considered as the controlling centre between malignant cells and the inflammatory microenvironment (Grivennikov and Karin, 2010). Various pro-tumorigenic signals are involved in NF-κβ and STAT-3 activation within immune and inflammatory cells (Yu et al., 2007, Karin, 2006a). Overexpression of TLR-4 is stimulated by colonic bacterial infection which induces inflammation that is associated with NF-κβ activation and CRC development (Semlali et al., 2016). It has been reported that TLR-4 and NF-κβ activation in *H. pylori* increased the risk of gastric cancer development (Hold et al., 2007). In a CRC mouse model, NF-κβ and STAT-3 activation is associated with colonic lesions, which promote CRC cell growth (De Simone et al., 2015). Likewise, activation of MAPK/ERK/NF-κβ pathway is associated with cancer progression and metastasis including human colonic cancer (Simoes et al., 2015). STAT-3 activation is also involved in IBD and CRC via IL-6 (Waldner et al., 2012). Furthermore, IL-6 and TNF-α synergistically activate upon NF-κβ and STAT-3, which promote CRC growth (De Simone et al., 2015). NF-κβ and STAT-3 activation are also associated with other cancers including pancreatic cancer (Hsieh et al., 2016), prostatic cancer (Sun et al., 2010, Singh et al., 2012), lung cancer (Bassères et al., 2010, Sun et al., 2015), breast cancer (Neil and Schiemann, 2008, Zhu et al., 2016), and multiple myeloma (Demchenko et al., 2010, Zheng et al., 2015).

Inflammation drives cancer development via activation of NF-κβ, which is downstream of the TLR-Myd88 pathway (Karin, 2006b). NF-κβ promotes tumour development by regulation of cyclin D1, apoptosis, and pro-inflammatory cytokines/chemokines protein expression (Karin, 2006b). In this thesis, it has been demonstrated that *F. nucleatum* and OMV were able to induce an increase in NF-κβ (NF-κβ 1 and NF-κβ 2) gene expression in colonic epithelial cells. An increase in the activation of NF-κβ correlated with *F. nucleatum* abundance and CRC (Kostic et al., 2013b). NF-κβ is known to modulate the expression of over 400 genes involved in mutable events in the cells. These include cell proliferation, transformation, anti-apoptosis, survival, inflammation, angiogenesis, and invasion metastasis via controlling pro-inflammatory cytokines and chemokines (IL-1 β, TNF-α, and IL-8) (Serasanambati and Chilakapati, 2016), which are identified as major factors in cancer initiation and progression (Hoesel and Schmid, 2013). Upon activation, IKK kinases activation results in the degradation of IKB-α (NF-κβ inhibitor), which in turn activates NF-

κβ. This then translocates into the nucleus to be part of the gene expression regulation process (Israël, 2010). NF-κβ is composed of five subunits REL-A (p65), REL-B, cytoplasmic REL-C, p50; p105 (NF-κβ1) and p52; p100 (NF-κβ2), which is activated by bacterial infection and/or cytokine activation (Liang et al., 2004). With the induction of TNF-α, IL-1 α causes the p65 subunit of NF-κβ to translocate into the nucleus and bind to the NF-κβ repressor factor (NRF) that removes the negative regulatory element (NRE) (Stein and Baldwin, 1993, Karin and Greten, 2005). As a consequence, the repression of the IL-8 promoter is relieved and AP-1 and NF-κβ proteins become phosphorylated. *F. nucleatum* activates the NF-κβ signalling pathway via TLR-2 (Toussi et al., 2012b) and TLR-4 (Yang et al., 2016) upon infection, resulting in sustained NF-κβ activation. Other bacterial cells and their OMV also are able to activate NF-κβ and pro-inflammatory cytokines via TLR-2 and TLR-4 including *B. fragilis* (Patrick et al., 2011), *Salmonella* (Huang, 2012), *H. pylori* (Smith et al., 2003), *T. forsythia* and *P. gingivalis* (Cecil et al., 2016).

Also, it has been found in this study that *F. nucleatum* and OMV increased STAT-3 phosphorylation (activation). Similarly, it has been indicated in one study that the involvement of *F. nucleatum* and *P. gingivalis* in tumour progression occurs via the activation of IL-6-STAT-3 signalling in oral epithelial cells (Gallimidi et al., 2015). Likewise, enterotoxigenic *B. fragilis* (ETBF) toxin (Hajishengallis et al., 2012) and *H. pylori* CagA are capable of activation of STAT-3 signalling (Lee et al., 2012b). In addition, *H. pylori* OMV were shown to be able to activate STAT-3 also (Mullaney et al., 2009). STAT-3 activation is associated with increased tumour survival, cell proliferation, and invasion, in addition to its ability to suppress anti-tumour immunity and promote inflammation via NFκβ and IL-6 (Yu et al., 2009a). Bacterial biofilms enhance STAT-3 phosphorylation and IL-6 activation in colonic epithelial cells (Bacteroidetes, Lachnospiraceae, Fusobacteria, Enterobacteriaceae, *B. fragilis* were represented within the biofilms) (Dejea et al., 2014). STAT signalling pathways are an important component in the progression of CRC (Erkasap et al., 2016). It has been reported that *F. nucleatum* promotes colorectal tumorigenesis via modelling tumour microenvironments through STAT-3 phosphorylation and ERK pathway activation (in mice), and are accompanied with an increase in other opportunistic pathogens such as *Verrucomicrobia* and *Tenericutes* (Yu et al., 2015). It has been reported that *P. gingivalis* and *F. nucleatum* induce STAT-3 activation in oral cancers that associated with

tumorigenesis (Gallimidi et al., 2015). It has been shown that IL-6 serum levels were elevated in colon carcinoma patients, which correlates with CRC development (Chung and Chang, 2003). Also, it has been reported that IL-6 promote the growth of colonic epithelial cells in culture (in vitro) (Schneider et al., 2000). STAT activation is commonly mediated by IL-6 via JAK pathways (Hirano et al., 2000) as well as infection via interferons signalling (Bollrath et al., 2009). Following on from phosphorylation, STAT-3 becomes activated for transcriptional activity and is able to bind to DNA (Wen et al., 1995). STAT-3 activation also affects the activity of NF-κβ (Lee et al., 2009) and regulates cellular growth (Bollrath et al., 2009), angiogenesis, apoptosis, migration and cellular invasion (Yu et al., 2009a) that is involved in the activation of human tumours. Furthermore, STAT-3 overexpression and activation induce an increase in N-cadherin, and vimentin and a decrease in E-cadherin expression (Xiong et al., 2012). At the same time, STAT-3 is also involved in the regulation of ZEB-1, which is directly associated with EMT and CRC metastasis (Xiong et al., 2012).

Interestingly, both *F. nucleatum* and OMV were found to be capable of upregulating the production of pro-inflammatory cytokines (protein and gene level) from all cell lines tested in a dose- and in a time-dependent manner. Furthermore, the amount of IL-8 secreted correlated with IL-8 gene expression that was induced by *F. nucleatum* and OMV. Other cancer causing bacteria stimulate IL-8 expression such as *H. pylori* (Crabtree et al., 1995). Also, it has been reported that OMV is a potent inducer of IL-8 expression, as in *H. pylori* OMV from gastric epithelial cells (Ismail et al., 2003), *E. coli* OMV from IECs (Kunsmann et al., 2015), *H. influenzae* OMV from human pharyngeal cells (Sharpe et al., 2011), *P. gingivalis* OMV from endothelial cells (Ho et al., 2016), *V. cholerae* OMV from colonic epithelial cells (Mondal et al., 2016), *C. sakazakii* OMV (Alzahrani et al., 2015), *P. aeruginosa* OMV (Bauman and Kuehn, 2006), and *A. baumannii* OMV in murine cells (Jun et al., 2013). This indicates that OMV are potent pro-inflammatory agents.

In the current study, it has been shown that *F. nucleatum* and OMV are potent inducers of IL-8 gene expression (stimulate SW-480 cells to induce 1600 pg/ml of IL-8 within a 24 h). Besides, approximately one *F. nucleatum* cell (MOI 1:1) was able to induced a significant 10-fold increase in *IL-8* expression ($p < 0.001$) and a small amount of OMV (0.01 μg/ml) was able to induce a significant 4-fold increase in *IL-8* expression ($p < 0.01$). Also, *V. cholerae*

OMV induced IL-8 compared with the bacterial cells from intestinal epithelial cells (Mondal et al., 2016). Similarly, *A. baumannii* OMV are potent inducers of the innate immune response compared with parental bacterial cells in mice. Membrane proteins in OMVs play a critical role in provoking the immune response (Jun et al., 2013). This suggests that antigenicity in OMV could result from concentrated immune-dominant determinants compared with those present on the parental bacterial cell surface (Ho et al., 2016). This study indicates that *F. nucleatum* OMVs are potent stimuli for pro-inflammatory cytokines, and are likely essential for infection progression and tissue damage.

All Fusobacterium species tested (*F. nucleatum*, *F. vincentii*, and *F. polymorphum*) were able to induce IL-8 secretion from SW-480 cells. *F. vincentii* and *F. polymorphum* induced more IL-8 from SW-480 cells, yet *F. nucleatum* is considered as a potent inducer to IL-8 secretion and inflammation when compared to *P. gingivalis* in periodontal infection (Gursoy et al., 2008, Han et al., 2000). IL-8 is belongs to the CXC chemokine family and is associated with neutrophil chemotaxis and degranulation and it has both proangiogenic and proliferative effects on endothelial cells. It is also considered as a driver for migration of cancer cells and plays a significant role in metastasis (Waugh and Wilson, 2008). IL-8 is involved in other types of malignancies including pancreatic cancer (Olszewski and Hamilton, 2009), and hepatocellular carcinoma (Yu et al., 2013).

In order to understand how *F. nucleatum* and OMV interact with colonic epithelial cells it was of interest in this study to explore the different pathways involved in the IL-8 expression that were induced by *F. nucleatum* and OMV. The results demonstrated that IL-8 production was controlled by multiple pathways to various degrees, in which PD0325901 (targeting MEK), and SP600125 (targeting MAPK, c-Jun kinase) inhibitors indicated that *F. nucleatum* and OMV activated the MAPK/ERK/JNK pathways. At the same time GSK690693 and LY294002 (targeting PI3K/Akt) inhibitors showed that PI3K/Akt pathways exerted a minor effect on IL-8 production by *F. nucleatum* and OMV. This suggests that multiple TLRs could be involved in the production of pro-inflammatory cytokines in response to OMV, as in case of *F. nucleatum* (Park et al., 2014b).

Various kinases are involved in cancer progression (MAPK and p38 pathways) including CRC (Grossi et al., 2014). MAPK is associated with cell proliferation, growth, apoptosis, and

inflammatory response via activation of RAS and RAF, which trigger transcription factors and leads to up regulation of genes (Dhillon et al., 2007). Also, the activation of Akt/PI3K crosstalks with MAPK pathway and involved in the proliferation, apoptosis, and activation of bacterial effector proteins (involved in bacterial survival) (Kuijl et al., 2007).

IL-8 production is controlled by three mechanisms, which involve the derepression of the gene promoter, NF-κβ activation via jun-N-terminal protein kinase pathways (JNK), and by p38-MAPK pathway via stabilisation of mRNA (Hoffmann et al., 2002). *F. nucleatum* induces pro-inflammatory cytokines via the activation of NF-κβ signalling and all MAPKs (p38, ERK, and JNK) in macrophages (Park et al., 2014b). Similarly, MAPK/ERK/JNK signalling is involved in the regulation of IL-8 expression by *H. pylori* (Boughan et al., 2006), *S. aureus* (Venza et al., 2013), *P. aeruginosa* (Venza et al., 2009), and *Salmonella* (Huang, 2012). Furthermore, it has been reported that *F. nucleatum* invasion induces IL-8 independent of Myd88 signalling, TLRs, NOD-1, NOD-2, and NF-κβ, which is mediated by p38-MAPK signalling in embryonic kidney cells (HEK293T) (Quah et al., 2014). However, *E. coli* isolated from Crohn's disease and colon cancer was able to significantly induce IL-8 production from HT29 cells via MAPK/ERK pathways (Subramanian et al., 2008).

In this study, the contribution of *F. nucleatum* LPS to induce IL-8 was evaluated using PMB, an antibiotic used to neutralize endotoxin contamination (Cardoso et al., 2007). Co-incubation of SW-480 cells with PMB and purified LPS from *E. coli*, *F. nucleatum*, and *F. vincentii* resulted in significantly decreased IL-8 expression compared to control cells (87-fold, 20-fold, and 122-fold, respectively). Only IL-8 induced by purified *F. nucleatum* LPS was completely attenuated by PMB to control cell levels, which indicates that PMB treatment is efficient, although, it has been shown in immune cells that PMB treatment can be incomplete (Tynan et al., 2012). Interestingly, PMB treatment of SW-480 cells co-incubated with *F. nucleatum* and *F. vincentii* OMV resulted in only partial inhibition of IL-8 expression (17 % and 27 %, respectively), suggesting that LPS from OMV made only a minor contribution to IL-8 expression.

Thus, it is likely that molecules in *F. nucleatum* OMV other than LPS play a major role in the induction of pro-inflammatory cytokine production. Potential candidates include FomA

(Toussi et al., 2012b), the heat shock protein GroEL (Lee, 2012), NapA (Choli-Papadopoulou et al., 2011), and peptidoglycan (Okugawa et al., 2010) that have been identified in *F. nucleatum* OMV. It has been reported that GroEL and DnaK in *S. epidermidis* and *C. sakazakii* OMV induce pro-inflammatory responses and induce IL-8 via their interaction with TLR-4 (Alzahrani et al., 2015).

Likewise, it has been reported that OMV from *P. aeruginosa* induce innate immune responses more potently than those induced by its LPS (Ellis et al., 2010). This could explain why the knockdown of TLR-2 had no impact on IL-8 production by intact *F. nucleatum* in oral epithelial cells (Ji et al., 2009). In contrast, LPS plays a major role in IL-8 induction by *E. coli* (Kunsmann et al., 2015), and *N. meningitidis* OMV (Mirlashari et al., 2001). Furthermore, proteases in OMV contribute to pro-inflammatory cytokine production and induce inflammation as in *V. cholerae* (Mondal et al., 2016).

Other studies indicate the interaction of the *F. nucleatum* cell wall with TLR-2 is required to provoke IL-8 expression (Peyret-Lacombe et al., 2009). However, it has been shown that blocking TLR-2 and TLR-4 with antibodies have no impact on NF-κβ activation and IL-8 production induced by live *F. nucleatum*, at least in oral epithelial cells (Zhang et al., 2011). In such cases, it is believed that recognition of *F. nucleatum* LPS occurs in the cytoplasm due to the presence of intracellular TLR-4 (Guillot et al., 2004, Hornef et al., 2003). This appears to be related to *F. nucleatum* behaviour since its invasiveness is commonly associated with strong induction of IL-8 (Huang et al., 2004).

In this study, it has been found that *F. nucleatum* and OMV induce TNF-α, IL-1 β, IL-6, CXCL-1 (GRO-α), and CCL-20 (MIP3-α) expression in colonic epithelial cells. Others have shown that *F. nucleatum* can stimulate various pro-inflammatory cytokines including IL-8, IL-1 β, IL-6, and TNF-α (Martinho et al., 2016), which contribute to tumorigenesis and cancer development (Wang et al., 2014). Also, tumour associated macrophages (TAMs) are associated with the progression and metastases of cancer (Qian and Pollard, 2010). In addition, *F. nucleatum* has been shown to induce gene expression that correlate with TAM e.g. IL-1 β, IL-6, IL-8, and TNF-α, which are also associated with a CRC gene expression signature in humans (Kostic et al., 2013b).

Similar to *F. nucleatum* OMV, it appears now to be feature of OMV that they induce pro-inflammatory responses. For example, *A. baumannii* OMV, are potent inducers of pro-inflammatory gene expression in murine cells (HEp-2 cells) (Jun et al., 2013); *S. enterica* OMV are potent simulators of pro-inflammatory cytokines (Alaniz et al., 2007); *P. aeruginosa* OMV induce pro-inflammatory cytokines from bronchial epithelial cells (Bauman and Kuehn, 2006); *C. jejuni* OMV from T-84 cells (Elmi et al., 2016); *V. cholerae* OMV simulate intestinal epithelial cells and DCs to express pro-inflammatory cytokines (Chatterjee and Chaudhuri, 2013), also pro-inflammatory responses have been observed with *N. meningitidis* (Durand et al., 2009), and *Vibrio anguillarum* OMV (Hong et al., 2009), which resulted in chronic inflammation.

In this study, it has been found that *F. nucleatum* and OMV induce GRO-α protein and gene expression in all colonic cell lines tested in both a dose and time-dependent manner. GRO-α belongs to the CXC chemokine family, similar to IL-8. CCL-20 is also induced by *F. nucleatum* and OMV in colonic epithelial cells. CCL-20 belongs to the CC subfamily that exhibits chemoattractant properties to leukocytes (Schutyser et al., 2003). GRO-α and CCL-20 are involved the induction of inflammation and metastasis (Migita et al., 2009, Bechara et al., 2007). GRO-α and CCL-20 are upregulated in intestinal tumours and are involved in CRC development (Frick et al., 2013, Baier et al., 2005). GRO-α and CCL-20 promote tumour progression and poor survival in patients with CRC (le Rolle et al., 2015, Cheng et al., 2014) and are involved in CRC cell proliferation and migration. In oesophageal cancer, CCL-20 is associated with *F. nucleatum* infection, which is linked to poor patient survival and aggressive tumour behaviour (Yamamura et al., 2016). It has been reported that CCR-6 is the receptor of CCL-20 and is associated with metastasis and the expression of CCR-6 increases in various types of cancers including prostate cancer (Ghadjar et al., 2008), liver cancer (Liu et al., 2014a), and CRC (Frick et al., 2013).

As mentioned earlier, IBD is one of the risk factors associated with CRC (Triantafillidis et al., 2009). An increase in the level of TNF-α, IL-1 β, and IL-6 have been reported in inflammation including IBD in which it contributes to the disease pathogenesis (Stankovic et al., 2015). In cancer, it has been suggested that TNF-α and IL-6 promote CRC cell growth (De Simone et al., 2015) and also they are associated with the production of a tumour supportive

microenvironment (Terzić et al., 2010). TNF-α, IL-1 β, and IL-6 are upregulated in CRC and correlate with CRC progression and development (Chang et al., 2016). It has been documented that elevated levels of TNF-α, IL-1 β, and IL-6 are associated with poor survival rate, tumour stage, and metastasis in CRC patients (Jedinak et al., 2010). IL-1 is considered as a central inflammatory mediator that involves innate and adaptive immune responses (Garlanda et al., 2013). The pro-inflammatory cascades are initiated by IL-1 β, which facilitates the spread of tumours (Apte et al., 2006). This event leads to the activation of the transcription factor NF-κβ via TNF-α signalling, which promotes the expression of the downstream inflammatory mediators that activate cell proliferation and differentiation (Long and Raufman, 2011). Also, IL-6 is an important inflammation mediator that is associated with CRC risk, advancement in tumour development, an increase in tumour occurrence and linked to poor patient prognosis (Knüpfer and Preiss, 2010). IL-1 and IL-6 are key pro-inflammatory cytokines involved in cancer development via the regulation of pro-oncogenic transcription factors such as NK-κβ and STAT-3 (Dmitrieva et al., 2016). Furthermore, TNF-α is involved in the regulation of EMT and migration in colon adenocarcinoma, which is an important mechanism in cancer progression and malignancy including CRC (Bhat et al., 2016). In addition, TNF-α affect TJ proteins in intestinal cells, decreases TER in apoptosis independent mechanisms (Bruewer et al., 2003).

A correlation has been identified between *Fusobacterium* transcript in human colon tumours and a specific gene signature (Kostic et al., 2013b). In this study, it has been found that *F. nucleatum* and OMV induced an increase in SOCS-3, SPHK-1, and PTGS-2 gene expression.

SOCS-3 has an important role in the support of cancer growth and development. Decreases in the expression of SOCS-3 has been reported to be associated with CRC, also SOCS-3 expression has been found to be linked with ulcerative colitis pathogenesis (Zhang et al., 2014b, Li et al., 2010). IL-6/p-STAT-3 have a significant impact on SOCS-3 regulation and an imbalance between the expression of SOCS-3 and IL-6/p-STAT-3 signalling leads to inflammation (Mitsuyama et al., 2006).

SPHK-1 is an oncogenic enzyme that acts as an important regulator for intercellular signalling, which promotes proliferation, transformation, anti-apoptosis, and survival of many human tumour cells (Vadas et al., 2008). It has been reported that SPHK-1 overexpression is significantly increased in CRC (Long et al., 2015) and contributes to malignant phenotypes and tumour progression in colon cancer (Liu et al., 2013). Also, SPHK-1 overexpression have been demonstrated to be associated with other human cancers including gastric cancer, breast cancer, prostate cancer, ovarian cancer, lung cancer, and myeloid leukaemia (Vadas et al., 2008). Furthermore, SPHK-1 promotes the production of MMP-2 and MMP-9 by tumour cells (Liu et al., 2012), which are involved in tumour development and progression via an enhancement of cell migration and angiogenesis.

COX-2 expression is associated with an increased risk of developing CRC (Li et al., 2015a). Overexpression of COX-2 is involved in the dysregulation of inflammation (Simmons et al., 2004), proliferation, angiogenesis (Romano and Claria, 2003), inhibition of apoptosis, and promotes cancer cell invasion including colon (Eberhart et al., 1994), gastric (Ristimäki et al., 1997), and oesophageal cancer (Liu et al., 2008). In mice, COX-2 has been shown to be associated with CRC and liver cancer formation and metastases (Wang et al., 2015a). Furthermore, an increase in COX-2 expression is associated with EMT in gastric cancer (Xian et al., 2016). It has been reported that inhibition of COX-2 improved patient outcomes and survival, which reduced CRC incidence (Tougeron et al., 2014).

Finally, it has been reported that MMPs are associated with an increased risk for the development of CRC (Banday et al., 2016). For example, MMP-9 is elevated in colon adenocarcinoma compared with normal mucosa (Illemann et al., 2006, Radziwon-Balicka et al., 2013). MMPs participate in the initial stages of metastasis by regulation of ECM remodelling that involves the degradation of ECM components, which forms a protumorigenic ECM that facilitates the invasion of tumour cells into the matrix (Ungefroren et al., 2011). Furthermore, MMPs induce EMT that promote intestinal tumour metastases (Ding et al., 2016).

In this study *F. nucleatum* and OMV induced matrix metalloprotease expression including MMP-1, MMP-9, MMP-10, and MMP-13 (only *F. nucleatum*) in colonic epithelial cells and

the downregulation of MMP-2 mRNA (differential expression between MMP-9 and MMP-2 has been observed), which could facilitate migration and morphological changes in the cells. The proteolytic activity of MMPs contribute to the regulation of cell-cell and ECM-cell interaction that promote cancer cells migration, invasion and progression (Koshikawa et al., 2000, Xu et al., 2001). It has been reported that *F. nucleatum* is a potent inducer of MMP-13, which facilitates cell migration (Uitto et al., 2005) as well as IL-8 secretion (Krisanaprakornkit et al., 2000). Also, *F. nucleatum* induces the expression of MMP-9 and to a lesser extent MMP-2 from epithelial cells (Gursoy et al., 2008, Signat et al., 2011). Whereas *H. pylori* induce MMP-1, MMP-2, MMP-9 and MMP10 in gastric cells (Jiang et al., 2014, Bergin et al., 2004).

Additionally, MMP-1 and MMP-7 are involved in the epithelial cell morphological transition that accompanies EMT by contributing to cleavage of E-cadherin (Noë et al., 2001). Overexpression of MMPs (MMP-1, MMP-2, MMP-7, MMP-9, and MMP-13) is associated with cell proliferation, invasion and EMT in various types of cancer including CRC (Xu et al., 2015, Zucker and Vacirca, 2004). Furthermore, MMP overexpression in tumour cells can be induced by pro-inflammatory cytokines and growth factors (Franco et al., 2017). MMPs are involved in the pathogenesis of inflammatory disorders, cell migration, and cancer progression (Zucker and Vacirca, 2004, Nissinen and Kähäri, 2014).

Based on the above, it is clear that both *F. nucleatum* and its OMV are potent inducers of inflammation, and this is in line with other findings in which *F. nucleatum* is recognised as a potent pro-inflammatory bacterium (Liu et al., 2007, Swidsinski et al., 2011). Fusobacteria generate a pro-inflammatory microenvironment in colorectal carcinoma tissue (McCoy et al., 2013, Kostic et al., 2013b). The result indicates that OMV LPS has a minor role in the initiation of inflammation at least with respect to IL-8 production. *F. nucleatum* and OMV contain several other proteins that have been shown to provoke a strong immune response and induce inflammation in colonic epithelial cells in other studies. Such molecules contribute to the alteration of a normal microenvironment and they are able to overcome anti-tumorigenic pressures. The inflammation, by itself, is a potent tumour promoter in which cancer cells undergo many changes that are involved in tumour metastasis, including EMT and acquisition of cancer stem cells properties (Zhou et al., 2012). In that regard,

research has been conducted with the aim of bridging inflammation and cancer, in which the former is considered as the main player in the induction of EMT and cancer progression (López-Novoa and Nieto, 2009). For example, IL-8 signalling is essential for the maintenance and acquisition of tumour EMT through direct and indirect mechanisms (Long et al., 2016). IL-8 promotes tumour cell proliferation, migration, survival through the autocrine loop that induces gene expression and regulates transcription factor activity (Waugh and Wilson, 2008). It was of interest, therefore, to investigate the contribution of *F. nucleatum* and OMV to the induction of EMT in colonic epithelial cells and the various mechanisms and changes that are involved in the process.

CHAPTER FIVE

The Contribution of *F. nucleatum* and OMV to the Induction of EMT-like Behaviour

5.1 Introduction

Bacterial infection is a potent inducer of inflammation that alters the inflammatory microenvironment and homeostasis, which are associated with elevation of the oxidative state and the induction of DNA mutation (Ma et al., 2016, Tang et al., 2016). The induction of inflammatory mediators such as cytokines and chemokines contribute in the initiation of EMT, which is the major pathway for tumour metastasis (Ma et al., 2016). EMT is the fundamental morphogenic event in which epithelial cells acquire a migratory and invasive phenotype during cancer progression (Nieto, 2011). EMT for more than 10 years has been demonstrated to be closely related to cancer progression (Savagner et al., 1997) in a process that resembles wound healing (Thiery, 2002). One of the initial observation was the ability of the transcription factor Snail to inhibit E-cadherin expression (Batlle et al., 2000, Cano et al., 2000), which has been reinforced over the years with increasing numbers of EMT-inducing transcription factors (Nieto and Cano, 2012). Recently, research has focused on the role of pathogens in EMT. For example, the internalisation of CagA and CagE from *H. pylori* induces and enhances the expression of mesenchymal markers in gastric cancer (Sougleri et al., 2016, Chang et al., 2015). Other bacteria associated with the induction of mesenchymal-like behaviour include *E. coli* (Cane et al., 2010), *Mycobacterium tuberculosis* in lung cancer (Gupta et al., 2016), and *P. aeruginosa* in bronchial cells (Borthwick et al., 2011). Given the association of *F. nucleatum* with CRC, and the ability of the bacterium to promote a potent pro-inflammatory environment as shown in Chapter 4, it was of interest to determine if *F. nucleatum* and OMV could influence the process of EMT in colonic epithelial cells. This study investigated the involvement of *F. nucleatum* and OMV

in the induction of cell proliferation, and morphological changes in addition to gene expression associated with EMT.

5.2 Hypothesis

In this work, it has been hypothesised that *F. nucleatum* and OMV are able to induce EMT-like behaviour in colonic epithelial cells.

Aims of the study:

To examine the ability of *F. nucleatum* and OMV to induce EMT-like behaviour

- Induction of cell proliferation.
- Induction of morphological changes in colonic epithelial cells.
- Induction of markers of EMT.

5.3 Results

The hypothesis that *F. nucleatum* and OMV induce EMT in colonic epithelial cells was explored by examining the ability of *F. nucleatum* and OMV to induce cell proliferation, morphological changes in colonic epithelial cells, and increase the expression of prototypical mesenchymal markers.

5.3.1 *F. nucleatum* and OMV induce colonic epithelial cells to proliferate

F. nucleatum induced cell proliferation when co-cultured with colonic epithelial cells (SW-480 and SW-620). A number of methods were used to evaluate the effect of *F. nucleatum* and its OMV on cell proliferation including cell counting, protein assays, wound closure assays and viable cell dyes and examples of the data are shown below. Cells number were enumerated by counting using a Haemocytometer at two time points (24 h and 48 h). *F. nucleatum* induced significant proliferation of SW-480 cells at 24 h ($p < 0.05$) and 48 h ($p < 0.01$) (**Figure 5.1, A**). In the case of SW-620 cells a significant increase in cell proliferation was observed at 48 h ($p < 0.05$) (**Figure 5.1,** B).

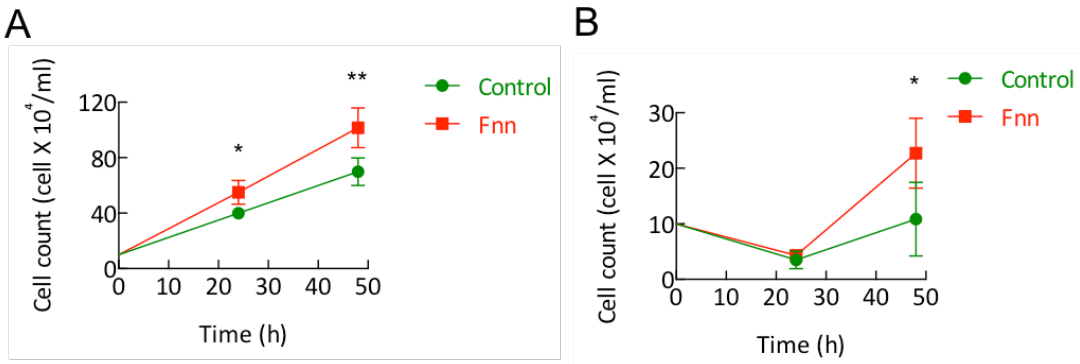

Figure 5.1: *F. nucleatum* induced proliferation of colonic epithelial cell lines (SW-480, and SW-620). Colonic epithelial cells were co-cultured with *F. nucleatum* (MOI 500:1) in 24-well plates and incubated for 24 h, and 48 h. The adherent cells were washed free of bacteria, trypsinised, and counted using a haemocytometer. Results are shown as mean ± SEM (n=3). **Panel A:** SW-480. **Panel B:** SW-620. * = $p < 0.05$, ** = $p < 0.01$.

Similarly, treatment of T-84 cells with *F. nucleatum* and OMV induced significant proliferation in T-84 cells over 48 h and OMV induced cell proliferation in a dose-dependent

manner (**Figure 5.2**). The increase in cell growth was estimated by quantitating the amount of protein using the BCA protein assay.

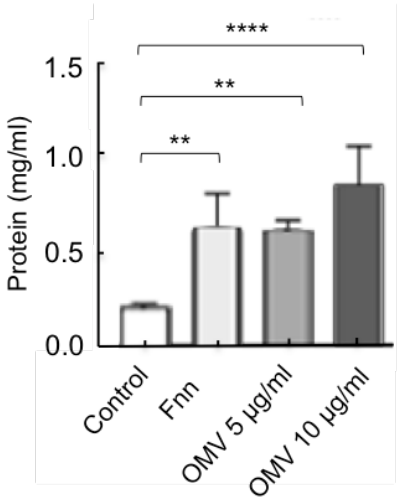

Figure 5.2: *F. nucleatum* and OMV induce proliferation in T-84. T-84 Cells were treated with *F. nucleatum* (MOI 1000:1) and OMV (5-10 µg/ml) for 48 h. The protein content of washed harvested cells was determined using the BCA protein assay (n=6). ** = $p < 0.01$, **** = $p < 0.0001$.

In order to demonstrate the ability of OMV to induce cell proliferation by an alternative test, scratch wound assays were also used. T-84 cells were treated with increasing amounts of OMV; cell growth was indicated by closure of the wound in a dose and time-dependent manner. Further, a sonicated preparation of the OMV was also capable of inducing this effect (**Figure 5.3, A**). Analysis of the rate of wound healing (**Figure 5.3, B**) indicated that these effects were significant. In another preparation, heat treatment resulted in the inhibition of the OMV-induced promoting effect and wound closure was no longer observed (data not shown) whereas sonicated preparation of OMV induced an increase in wound closure rate, suggesting that a proteinaceous component(s) was responsible for inducing proliferation. Taken together, these data demonstrate that *F. nucleatum* and OMV could induce proliferation of colonic epithelial cells.

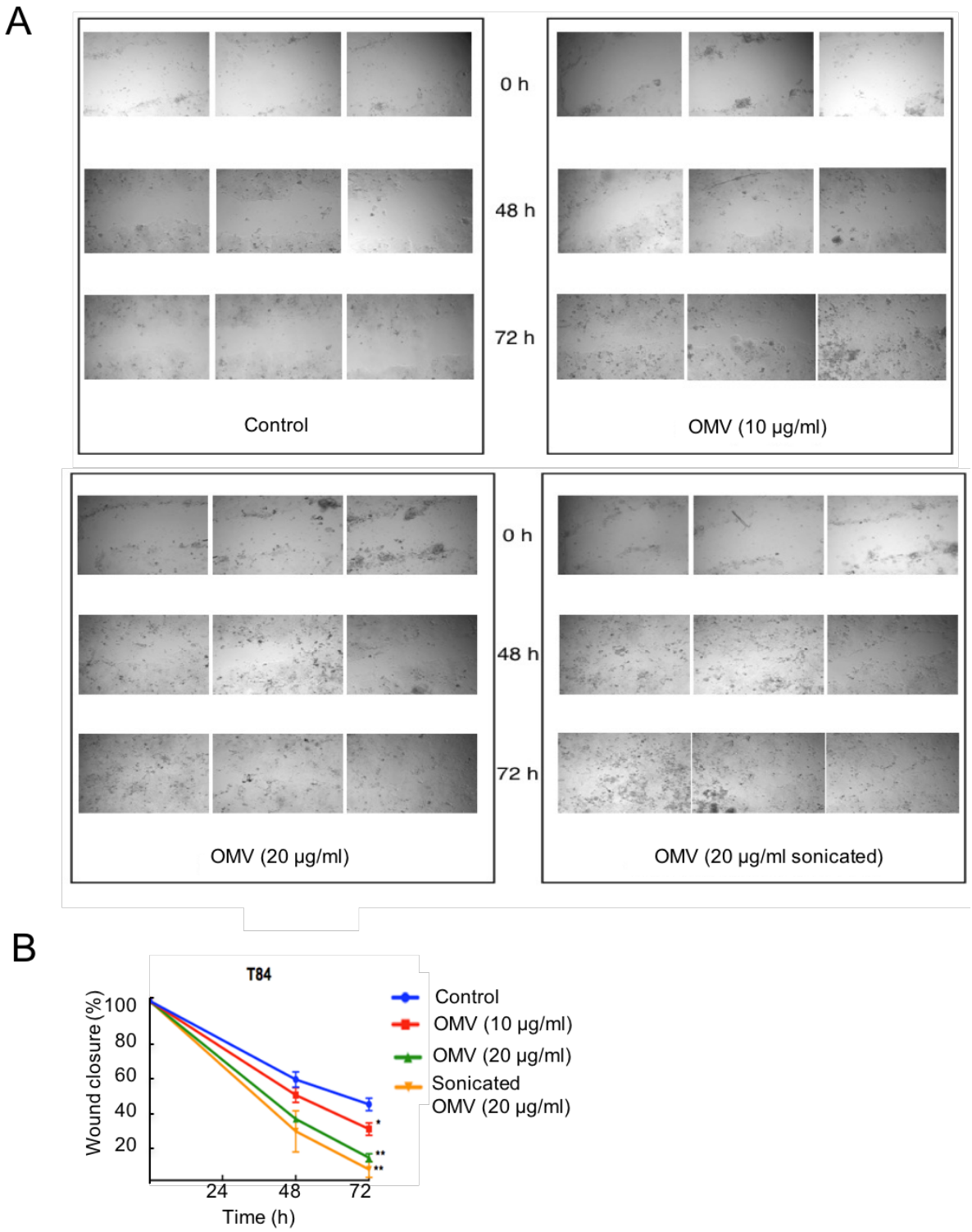

Figure 5.3: Effect of *F. nucleatum* OMV on scratch wound closure using T-84 cells. Panel A: phase contrast images of T-84 cells scratch wounds (magnification 10 X) followed over time after the indicated treatments. **Panel B:** Percentage wound closure following co-incubation of T-84 cells with OMV (10-20 µg/ml) or a sonicated preparation of OMV (20 µg/ml). * = $p < 0.05$, and ** = $p < 0.01$.

5.3.2 *F. nucleatum* and OMV-induced IL-8 exhibits an autocrine growth factor effect on colonic epithelial cells

Given the demonstrated ability of *F. nucleatum* and OMV to induce colonic epithelial cells to proliferate combined with their ability to induce significant expression of IL-8, a chemokine known to possess autocrine growth factor effects (Brew et al., 2000), the possibility that IL-8 could promote colonic cell proliferation was investigated. In order to examine the contribution of IL-8 autocrine growth factor activity serial dilutions of anti-IL-8 antibody (0.031, 0.063, 0.125, 0.25, 0.5, and 1.0 μg/ml) were added to SW-480 cells to examine the effect of different amount of anti-IL-8 on SW-480 cell proliferation (**Figure 5.4, A**). Anti-IL-8 antibody had on effect on cell viability when added to untreated cells, regardless of the amount added. According to the manufacturer's specification, in order to neutralise 20 μg IL-8/ml approximately 0.08 – 0.4 μg of anti-IL-8/ml is required. It has been estimated that SW-480 cells simulated by *F. nucleatum* secrete approximately 1.6 ng of IL-8/ml (**Chapter 4, Figure 4.6**). SW-480 cells were treated with *F. nucleatum* and OMV for 24 h and 48 h. To neutralise secreted IL-8 activity an excess of anti-IL-8 monoclonal antibody was added (0.24 μg/ml) where appropriate at the same time as the *F. nucleatum* and OMV. No significant change in proliferation of treated or untreated cells occurred at 24 h (**Figure 5.4, B**), but by 48 h, a significant difference in the proliferation of SW-480 cells was observed between treated and untreated cells (**Figure 5.4, C**). Anti-IL-8 monoclonal antibody induced a significant inhibition of cell proliferation compared to untreated cells ($p < 0.001$), and significant inhibition ($p < 0.01$) between cells treated with anti-IL-8 and OMV compared to cells treated with OMV alone. This suggests that IL-8 promotes cell proliferation in an autocrine like manner. Anti-tubulin antibody was used as an isotype matched control.

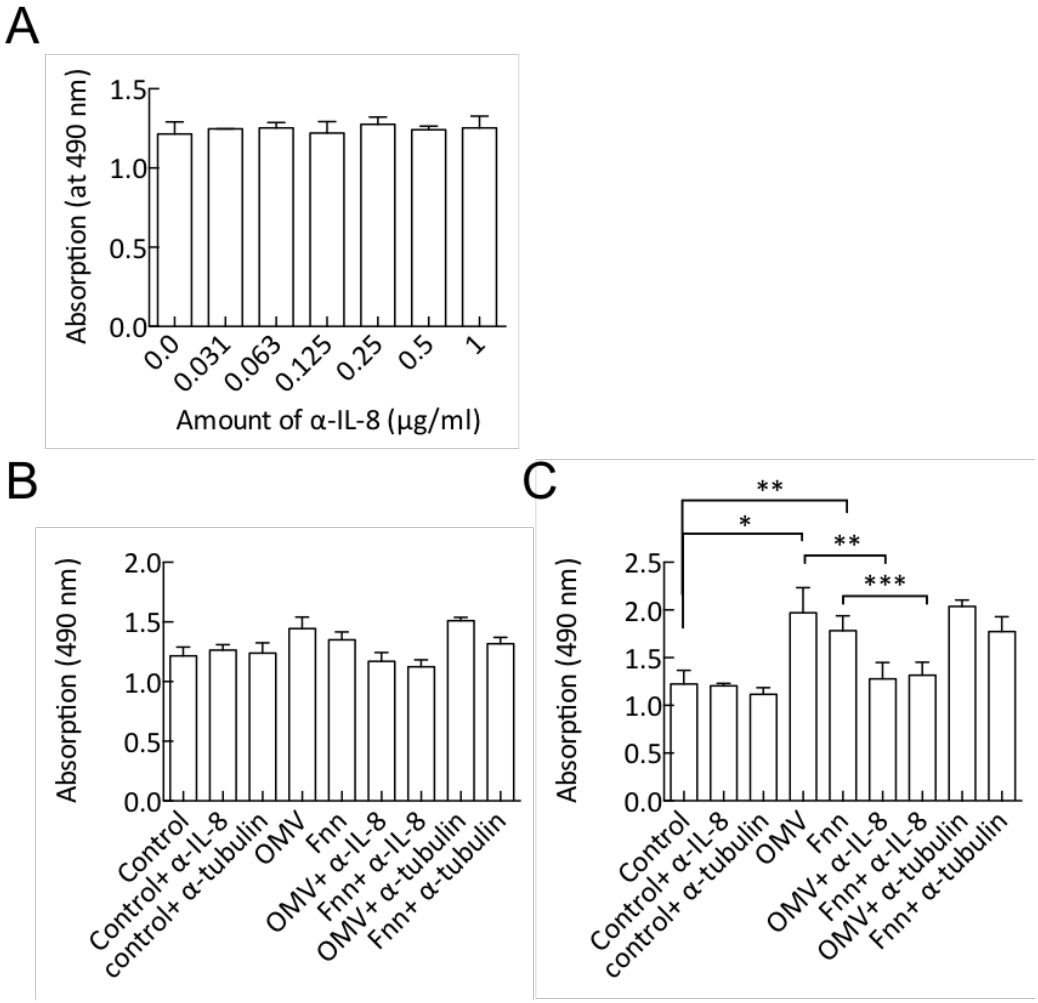

Figure 5.4: Effect of anti-IL-8 antibody on cell proliferation induced by co-culture with *F. nucleatum* and OMV in SW-480 cells. SW-480 cells were seeded in 96-well plates. **Panel A:** Show the effect of increasing amount of anti-IL-8 on the proliferation of SW-480 cells, the cells were incubated with increasing amount of anti-IL-8 (0.031, 0.063, 0.125, 0.25, 0.5, 1.0 μg/ml) over 48 h. In panel B and C the SW-480 cells were co-cultured with *F. nucleatum* (MOI 250:1), and OMV (50 μg/ml) and incubated for 24 and 48 h. Monoclonal anti-IL-8 antibody (0.24 μg/ml) and monoclonal anti-tubulin-α antibody (0.24 μg/ml) were added at the same time as the bacteria and OMV. Cell proliferation was estimated by Promega cell titer cell proliferation assay. **Panel B:** Showing the degree of cell proliferation after 24 h. **Panel C:** Showing the degree of cell proliferation after 48 h (n=4). Panel * = $p < 0.05$, ** = $p < 0.01$, and *** = $p < 0.001$.

5.3.3 *F. nucleatum* induces morphological changes in colonic epithelial cells.

Whilst growing SW-480 cells in co-culture with *F. nucleatum* it was noted that the cells appeared to undergo considerable morphological changes over time. In order to evaluate those morphological changes, the SW-480 cell line was chosen for further study, as it

appeared to undergo the most obvious morphological changes routinely. In a typical experiment, SW-480 cells were exposed to *F. nucleatum* for 48 h; they were then fixed with paraformaldehyde and stained with phalloidin-TRITC to visualise the actin cytoskeleton. The SW-480 cell line is derived from a primary adenocarcinoma, are non-metastatic and possesses an epithelial appearance as shown by a symmetrical and regular planar appearance with apparent cell-cell contacts (**Figure 5.5, A and B**). Typically, SW-480 cells form clusters of polygonal epithelia-like cells when sub-confluent (Leibovitz et al., 1976). In contrast, cells exposed to *F. nucleatum* appear distorted and irregular with a notable increase in the number of elongated cells in addition to apparent separation and loss of cell-to-cell contact (**Figure 5.5, A and B**). Similarly, cells exposed to OMV appear distorted too (**Figure 5.5, B**). Other colonic cell lines (SW-620 and Lovo) were similarly examined for morphological changes but were more difficult to work with compared with the SW-480. Both SW-620 and Lovo are not typically epithelial in appearance and more readily detached from the substratum, particularly after treatment with bacteria. Additionally, both cell lines frequently clumped and it proved difficult to capture images of sufficiently good or clear quality (data not shown). Taken together, these observations indicated that *F. nucleatum* and OMV may have the ability to initiate EMT in colonic cells. To explore this possibility the expression levels of several gene/proteins known to be associated with EMT were examined in the first instance.

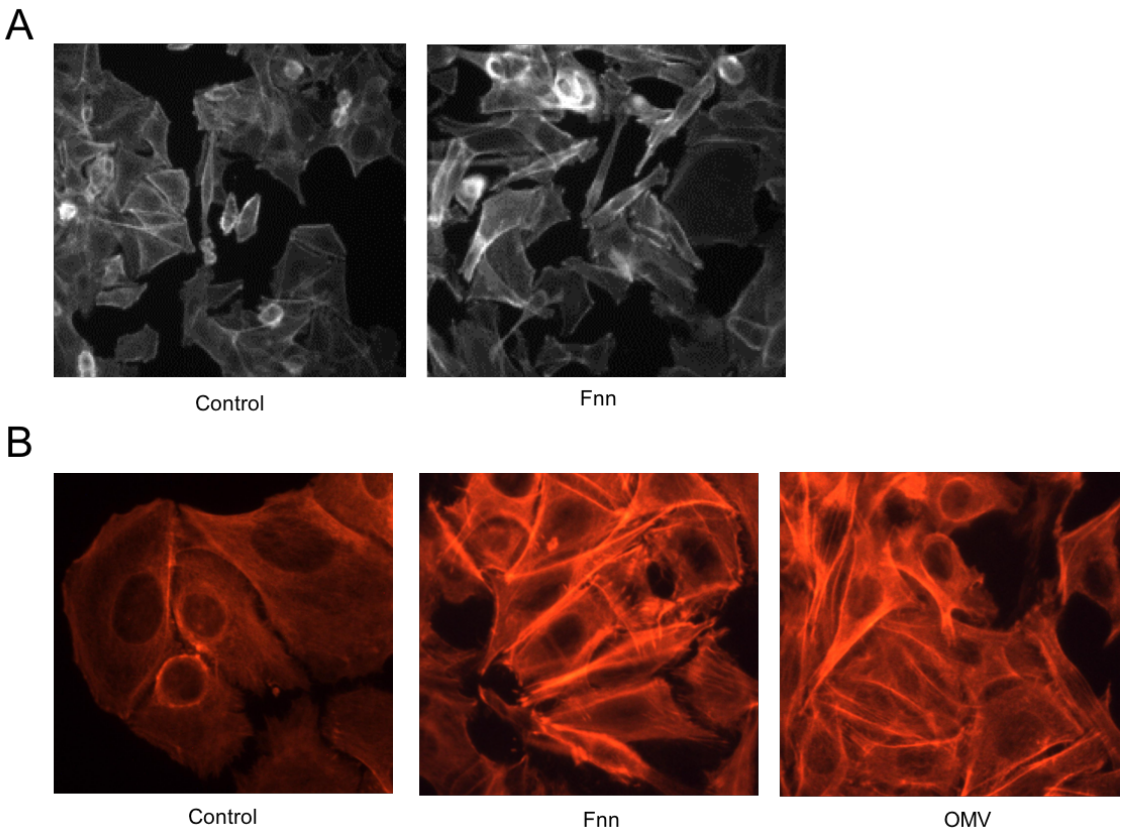

Figure 5.5: Fluorescence microscopy images showing Phalloidin-TRITC stained SW-480 cells. Panel A: SW-480 cells were exposed to *F. nucleatum* (MOI 1000:1) for 48 h (Magnification 40 X). **Panel B:** SW-480 cells were exposed to *F. nucleatum* (MOI 100:1) and OMV (50 μg/ml) for 48 h (Magnification 40 X).

5.3.4 The effect of *F. nucleatum* and OMV on Wnt and Myc gene expression

Wnt and Myc and their downstream effectors regulate various processes with important roles in cancer initiation, growth, and progression. In order to examine the effect of *F. nucleatum* and OMV on Wnt and Myc gene expression in SW-480 cells, the cells were incubated with *F. nucleatum* and OMV for 4 h. Both *F. nucleatum* and OMV induced expression of Wnt-7 α, Wnt-7 β, and Wnt-9 α in SW-480 to similar extents with the highest levels of expression seen for Wnt-9 α in both cases (**Figure 5.6**). Also, *F. nucleatum* and OMV induced Myc gene expression to similar levels (**Figure 5.6**).

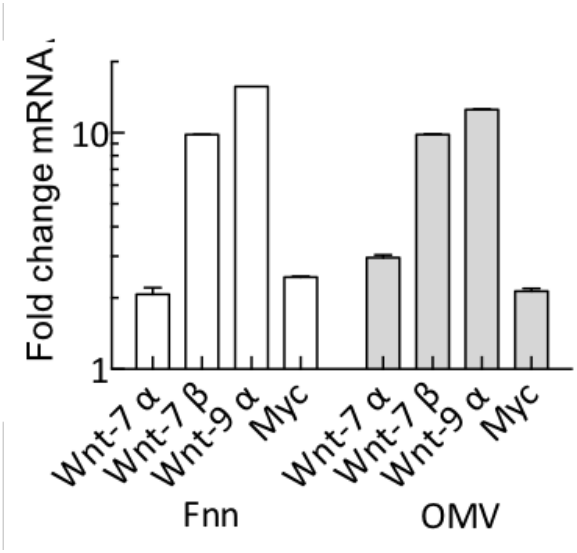

Figure 5.6: Effect of *F. nucleatum* and OMV on Wnt-7 α, Wnt-7 β, Wnt-9 α, and Myc gene expression in SW-480 cells. SW-480 were treated with *F. nucleatum* (MOI 100:1) and OMV (10 μg/ml) for 4 h (n=2).

5.3.5 *F. nucleatum* and OMV induce EMT marker expression

Given the observation that *F. nucleatum* appeared to be capable of inducing morphological changes to colonic cells over time, the issue of whether or not this process was related to transformation of the cells was addressed. Initially, several genes whose expression is associated with EMT such as Bone Morphogenetic Proteins (BMPs), Integrin-α5 (ITGA-5), CDH-2 (N-cadherin), and Fibronectin (FN) were tested in SW-480 cells. Both *F. nucleatum* and OMV induced an increase in BMP, ITGA-5, CDH-2, FN-1 (**Figure 5.7**).

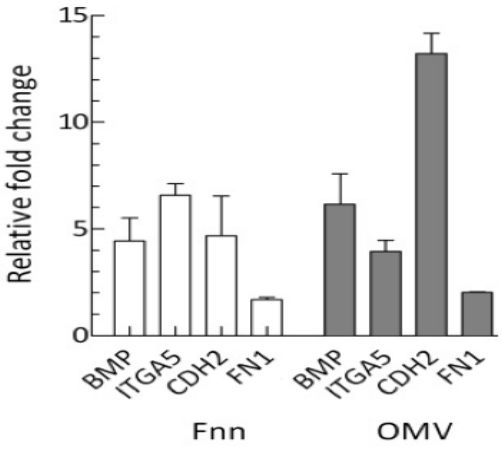

Figure 5.7: Effect of *F. nucleatum* and OMV on gene expression associated with EMT (BMP, ITGA-5, CDH-2, and Fn-1) in SW-480 cells. SW-480 were treated with *F. nucleatum* (MOI 100:1) and OMV (10 μg/ml) for 4 h (n=2).

Furthermore, *F. nucleatum* and OMV induced an increase in Snail gene expression. Snail is a transcriptional regulator that is implicated in EMT initiation (Scanlon et al., 2013). Upregulation of Snail expression is associated with induction of EMT in colon cancer cells (Wang et al., 2016a). *F. nucleatum* induced an increase in Snail-1, Snail-2, and Snail-3 gene expression of 6, 4, and 18-fold, respectively, whereas OMV induced around 4, 5, and 8-fold, respectively (**Figure 5.8**).

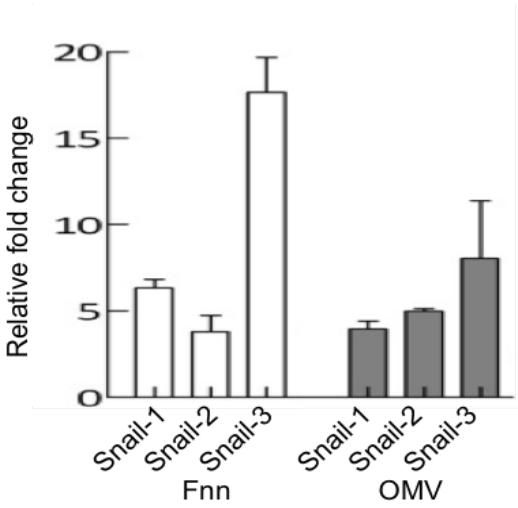

Figure 5.8: Effect of *F. nucleatum* and OMV on Snail-1, Snail-2, and Snail-3 gene expression in SW-480 cells. SW-480 were treated with *F. nucleatum* (MOI 100:1) and OMV (10 μg/ml) for 4 h (n=2).

Similar to Snail, TWIST is also a transcriptional regulator linked to EMT (Scanlon et al., 2013). TWIST is an important inducer of EMT, which is associated with the development and

progression of CRC (He et al., 2016). *F. nucleatum* and OMV induced a significant increase in TWIST gene expression ($p < 0.01$) at 4 h, with gene expression then returning to control levels by 24 h (**Figure 5.9**).

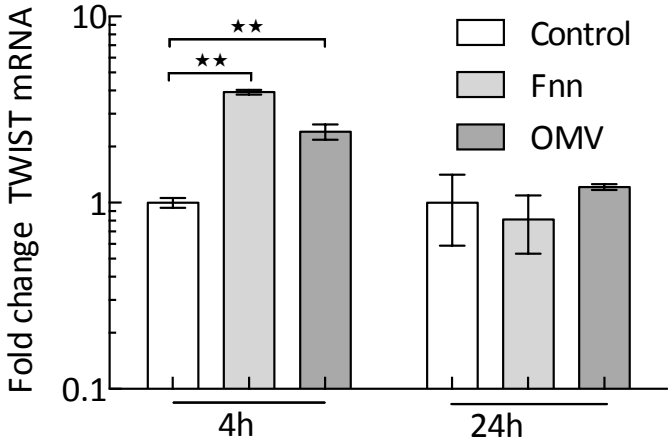

Figure 5.9: Effect of *F. nucleatum* and OMV on TWIST gene expression in SW-480 cells. SW-480 were treated with *F. nucleatum* (MOI 100:1) and OMV (10 μg/ml) for the times indicated. The results are presented as the mean ± SEM (n=3). ** = $p < 0.01$.

A number of additional markers (i.e. ZEB-1, E-cadherin, β-catenin, and vimentin) commonly associated with EMT were examined by immunofluorescence, Western blotting and RT-PCR to determine what effect co-incubation of colonic cell lines with *F. nucleatum* and OMV had on their expression and/or subcellular distribution.

Both *F. nucleatum*, and OMV induced ZEB-1 expression in the nucleus of SW-480 cells (**Figure 5.10 and Figure 5.11, A**) and also in SW-620, Lovo and Caco-2 cells (**Figure 5.11, B, C, and D**).

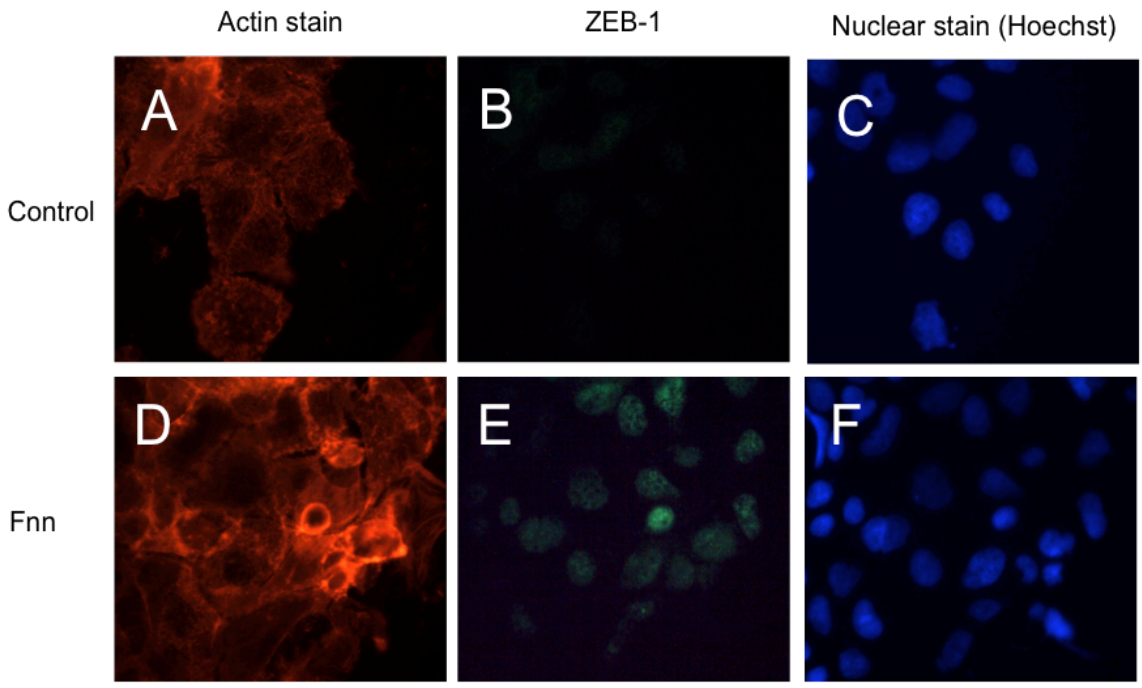

Figure 5.10: ZEB-1 expression in SW-480 cells. Panel A-C: SW-480 control, and **Panel D-F:** SW-480 cells were infected with *F. nucleatum* (MOI 100:1) for 48 h. **Panel A/D:** Actin stain. **Panel B/E:** ZEB-1 expression (stained with anti-ZEB-1 antibody (NBP1-05987)). **Panel C/F:** Nuclear stain. Magnification 40 X.

Interestingly, the elevated amount of *F. nucleatum* (MOI 1000:1) induced less ZEB-1 expression in SW-480 cells compared to the lower dose of *F. nucleatum* (MOI 100:1). However, the reason for this decrease is not known.

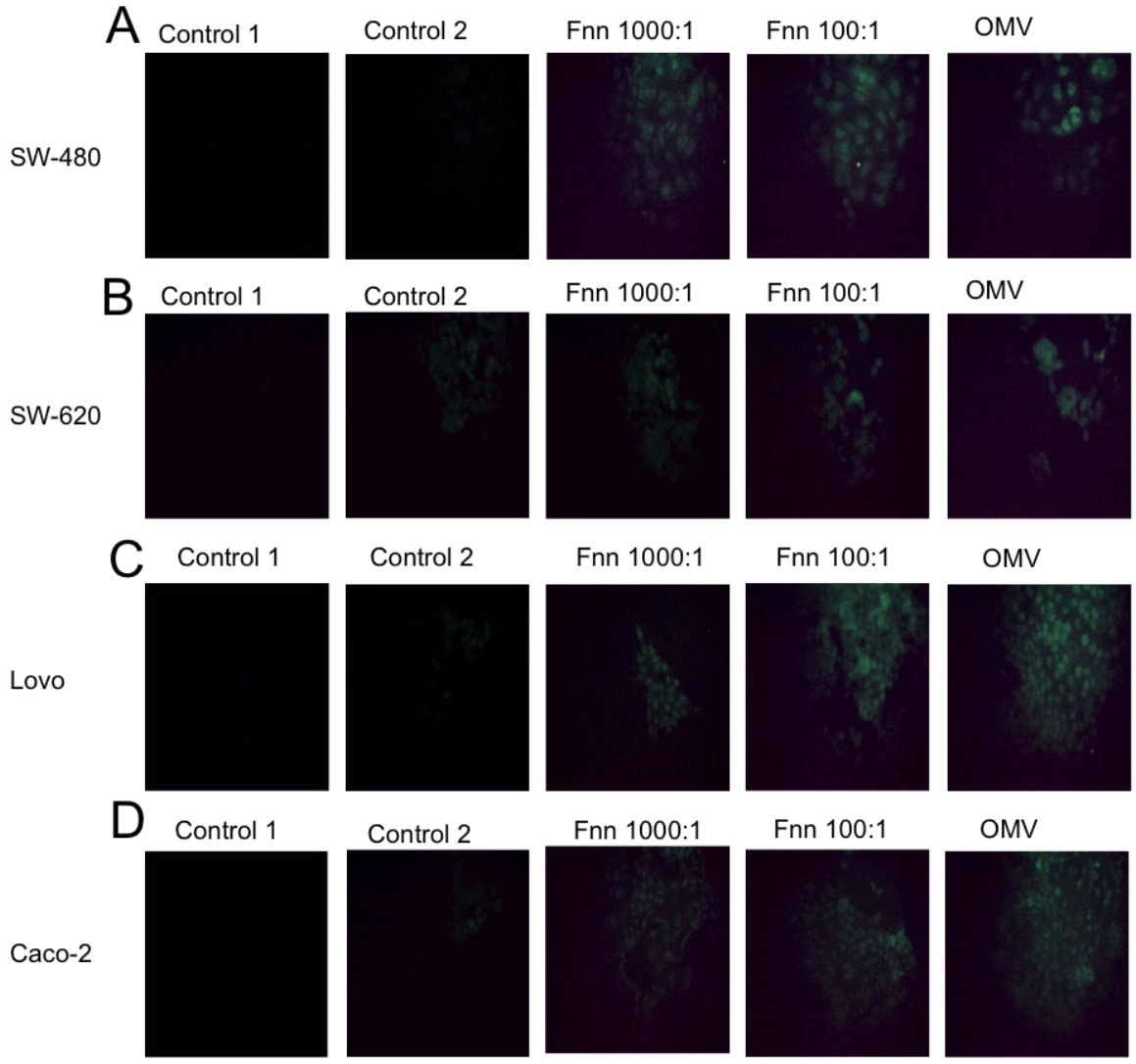

Figure 5.11: Immunofluorescence analysis on ZEB-1 expression in different cell lines. SW-480, SW-620, Lovo, and Caco-2 cells were co-cultured with *F. nucleatum* (MOI 1000:1 and 100:1) and OMV (50 µg/ml) for 48h. Cells were stained with rabbit anti-ZEB-1 antibody (NBP1-05987) as described in the methods. Control 1: cells were incubated with secondary antibody and control 2: incubated with primary and secondary antibody. The cells were observed using a fluorescence microscope. **Panel A:** SW-480. **Panel B:** SW-620. **Panel C:** Lovo. **Panel D:** Caco-2.

Assessment of the *F. nucleatum* and OMV-dependent kinetics of ZEB-1 nuclear expression in SW-480 cells indicated that this occurs rapidly (1 h) and is sustained for at least 48 h. By 72 h ZEB-1 nuclear expression became apparent in the control untreated cells (**Figure 5.12**).

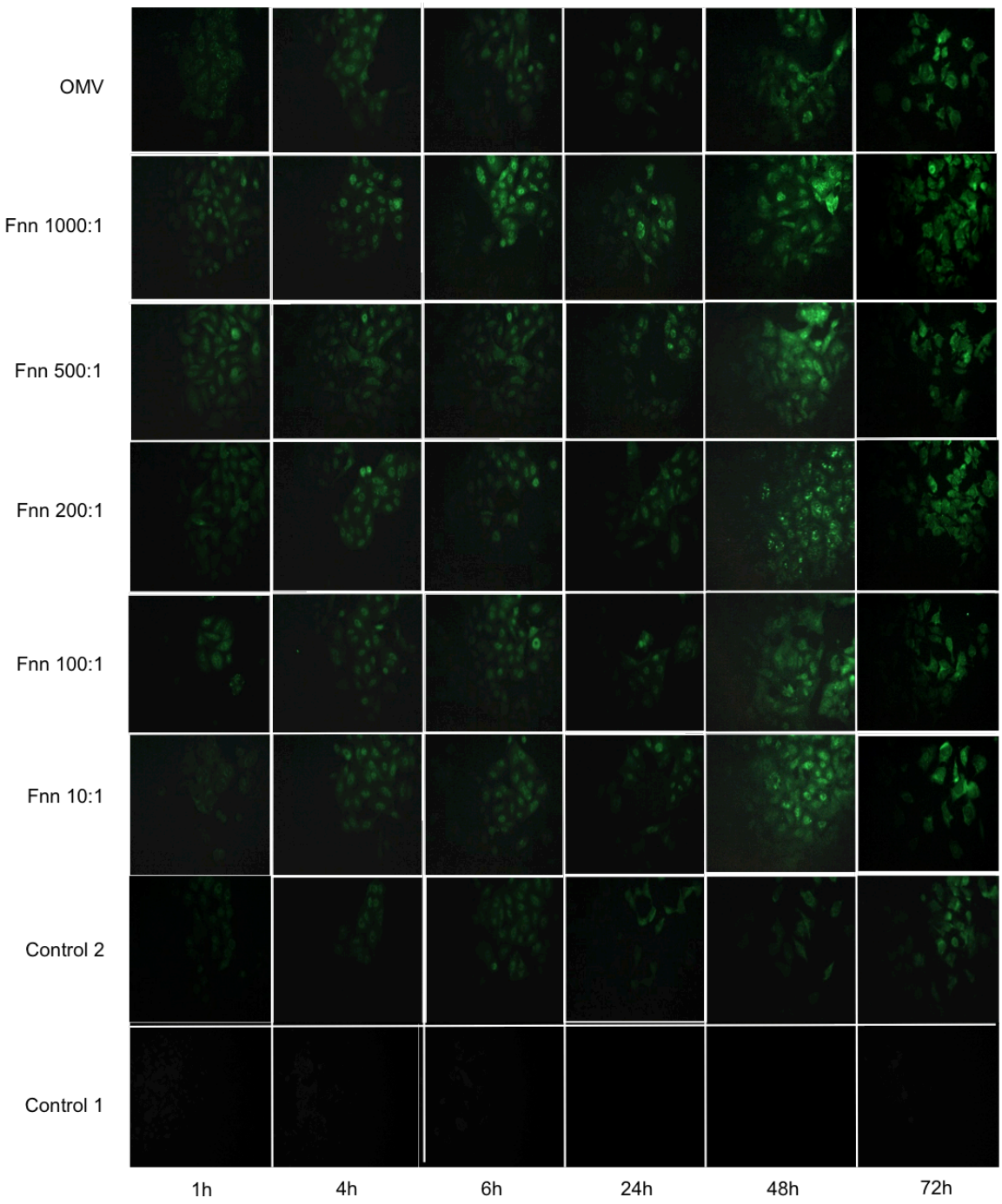

Figure 5.12: Time course of ZEB-1 expression in SW-480 cells. SW-480 cells were treated with *F. nucleatum* (MOI 10:1, 100:1, 200:1, 500:1, and 1000:1) and OMV (50 µg/ml) for the times indicated. Control 1: cells were incubated with secondary antibody only. Control 2: incubated with primary and secondary antibody. Cells were observed using a fluorescence microscope.

Nuclear expression of ZEB-1 in *F. nucleatum* and OMV-treated colonic cells was confirmed by Western blot also (**Figure 5.13, A**). Normalisation of ZEB-1 expression against the loading control PCNA (**Figure 5.13, B and C**) indicates that *F. nucleatum* and OMV were able to induce ZEB-1 expression in the nucleus of SW-480 and SW-620 cells. Interestingly, there is

a reduction in ZEB-1 expression in SW-480 treated with *F. nucleatum* at MOI 1000:1 when compared to those treated with 100:1. A similar observation was made using immunofluorescence.

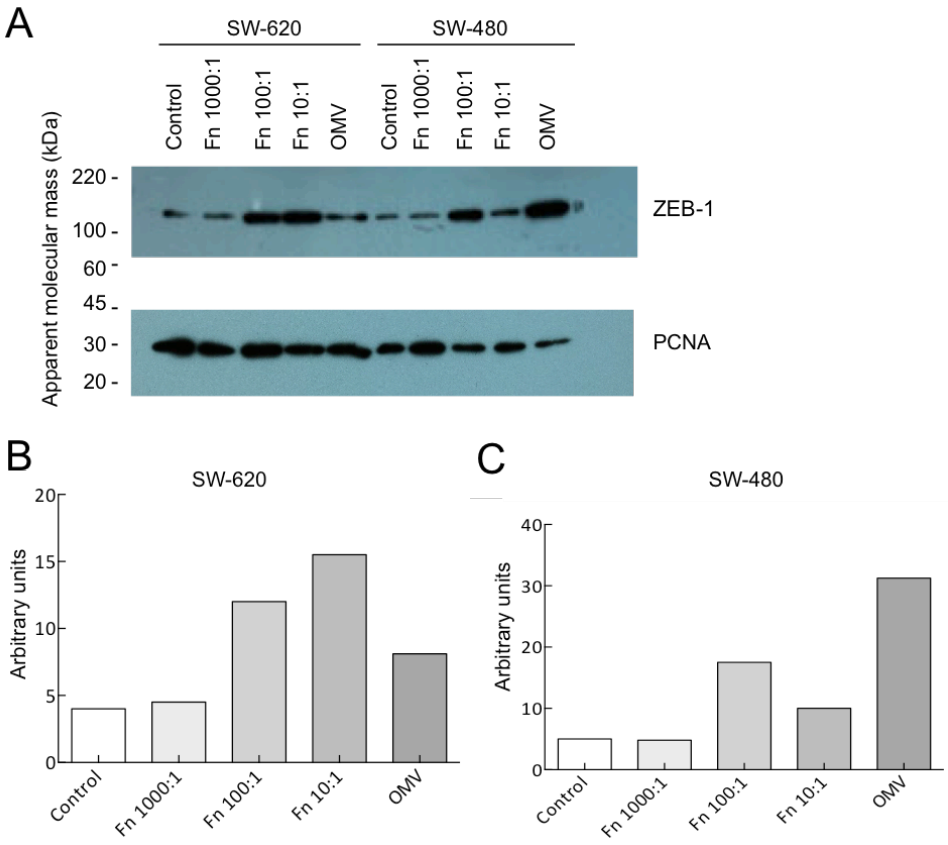

Figure 5.13: Western blot showing ZEB-1 nuclear expression in SW-480 and SW-620 cells. Cells were incubated with *F. nucleatum* (MOI 1000:1, 100:1, and 10:1) and OMV (50 µg/ml) for 48 h. **Panel A:** Western blot showing ZEB-1 expression in a nuclear extract of SW-480 and SW-620 cells. PCNA was used as the nuclear protein loading control. **Panel B:** normalization of ZEB-1 expression in SW-620 cells. **Panel C:** normalization of ZEB-1 expression in SW-480 cells. Similar results were obtained in repeated experiments.

Similarly, ZEB-1 was expressed in the nuclear fractions of Lovo and Caco-2 cells in response to *F. nucleatum* and OMV (**Figure 5.14, A**). Normalisation of ZEB-1 expression showed that *F. nucleatum* and OMV induced an increase in ZEB-1 expression when compared to control cells (**Figure 5.14, B**).

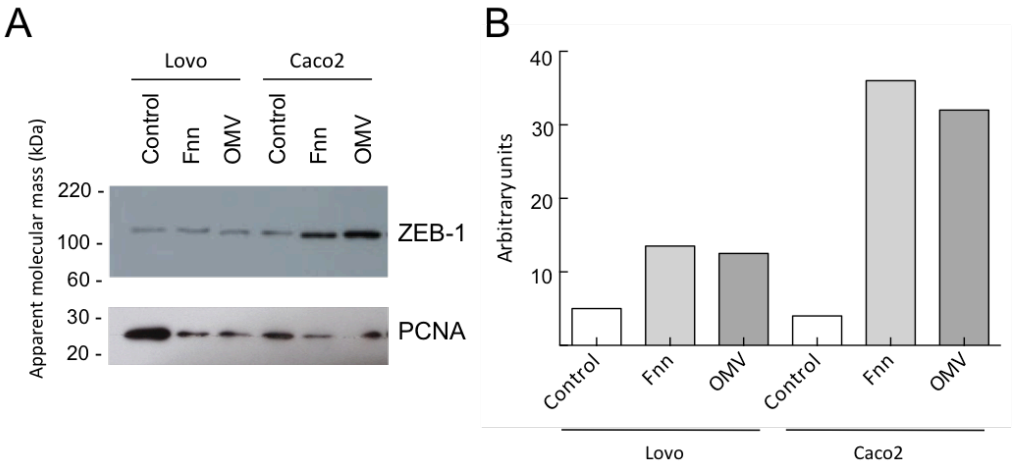

Figure 5.14: Western blot showing ZEB-1 expression in Lovo and Caco-2 cell lines. Cells were incubated with *F. nucleatum* (MOI 100:1) and OMV (50 µg/ml) for 48 h. **Panel A:** Western blot showing ZEB-1 expression in a nuclear extract of Lovo and Caco-2 cells. PCNA was used as a nuclear protein loading control. **Panel B:** Normalization of ZEB-1 expression in Lovo and Caco-2 cells.

RT-PCR analysis of ZEB-1 gene expression in SW-480 cells following treatment with *F. nucleatum* and OMV revealed a significant 9-fold increase ($p < 0.01$) in ZEB-1 gene expression over 4 h, which returned to control levels by 24 h (**Figure 5.15**).

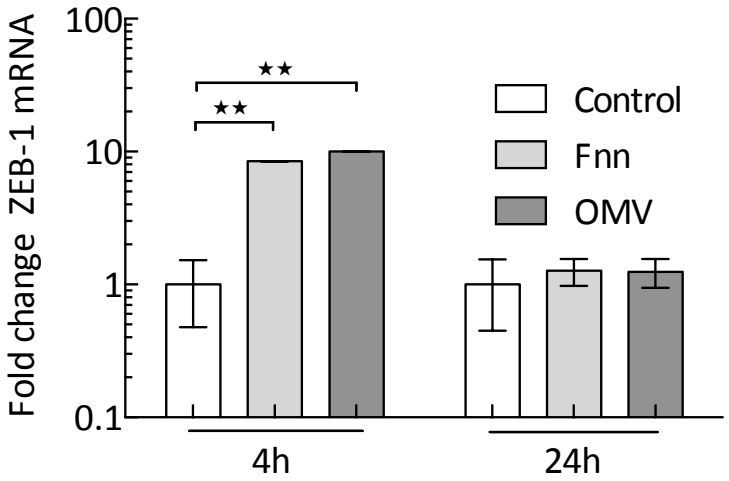

Figure 5.15: Effect of *F. nucleatum* and OMV on ZEB-1 gene expression in SW-480 cells. SW-480 cells were treated with *F. nucleatum* (MOI 100:1) and OMV (10 µg/ml) for the times indicated. ZEB-1 expression was determined by RT-PCR. The results are presented as the mean ± SEM (n=3). **= p< 0.01.

In Chapter 3 it was shown that *F. nucleatum* and OMV could degrade E-cadherin and modulate cell barrier function. Immunofluorescence analysis also demonstrated a reduction in E-cadherin expression in colonic cell lines following treatment with *F.*

nucleatum and OMV. To further examine the effect of *F. nucleatum* and OMV on colonic epithelial cells, a Western blot was performed on lysates prepared from SW-480 cells treated with *F. nucleatum* and OMV. Normalisation of the expression of E-cadherin revealed a reduction in the expression of E-cadherin in cells treated with *F. nucleatum* and OMV (**Figure 5.16, A**). E-cadherin gene expression was possibly reduced in SW-480 cells treated with *F. nucleatum* and OMV (**Figure 5.16, B**).

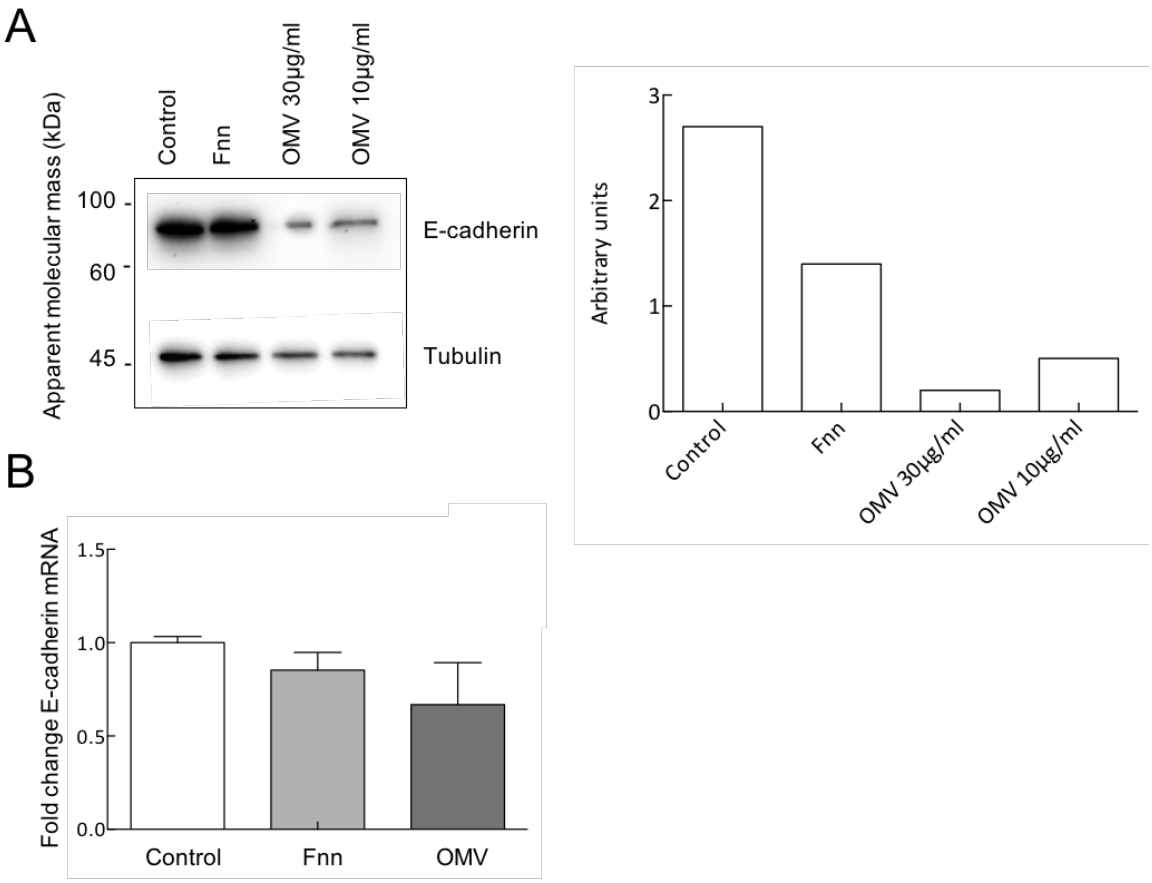

Figure 5.16: E-cadherin expression in SW-480 cells. Panel A: Western blot showing reduced expression of E-cadherin following treatment with *F. nucleatum* (MOI 100:1), and OMV (10, and 30 μg/ml) for 24 h (left side). Normalization of E-cadherin expression in SW-480 cells (right side). **Panel B:** reduced E-cadherin gene expression in SW-480 cells treated with *F. nucleatum* (MOI 500:1) and OMV (30 μg/ml) for 48 h (n=2).

As β-catenin is a component of the E-cadherin complex it was of interest to determine if OMV could modulated its subcellular location given that E-cadherin expression is downregulated. In addition, β-catenin has a role in EMT thus its subcellular distribution was investigated. To determine the expression of β-catenin, Western blotting was performed on nuclear fractions of SW-480 cells which had been treated with *F. nucleatum*. β-catenin

expression was evident in the nuclear fractions (**Figure 5.17, A**). Normalization of β-catenin expression demonstrated that *F. nucleatum* induced translocation of β-catenin into the nucleus. Immunofluorescence analysis of SW-480 and SW-620 cells support the Western blot data indicating *F. nucleatum* induced accumulation of β-catenin in the nucleus. Immunofluorescence showed that co-culture of cell lines with *F. nucleatum* and OMV induced an increase in β-catenin expression (**Figure 5.17, B**). Also, it was observed that *F. nucleatum* at MOI 100:1 induced more intense expression of β-catenin than an MOI 1000:1, but this was not observed in the Western blot result.

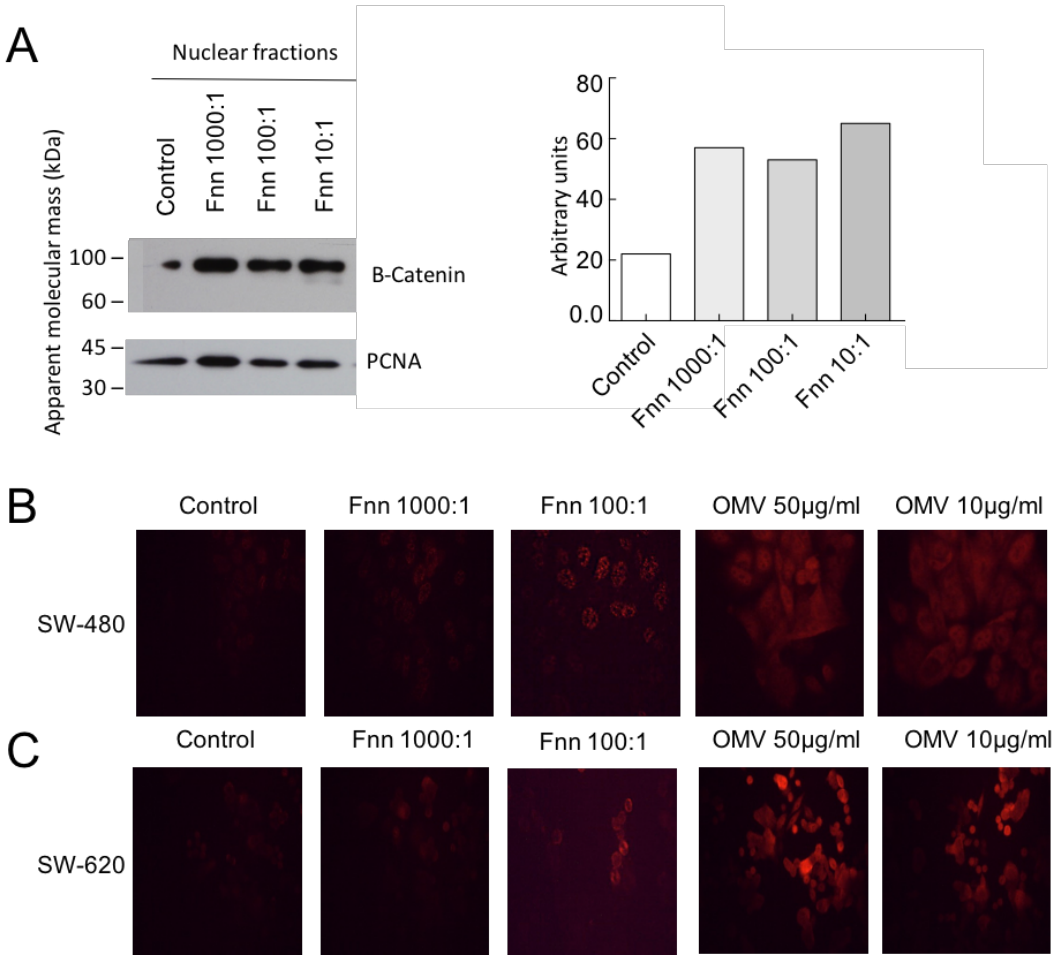

Figure 5.17: Western blot of β-catenin in SW-480 cells and Immunofluorescence analyses of β-catenin expression in SW-480, and SW-620. In the Western blot, SW-480 cells were co-cultured with *F. nucleatum* (MOI 1000, 100, and 10) and OMV (50 µg/ml) for 48 h. PCNA was used as a nuclear protein loading control. **Panel A:** Western blot of SW-480 showing β-catenin expression in nuclear and cytoplasmic fractions. In the immunofluorescence images (B-C) SW-480 and SW-620 cells were co-cultured with *F. nucleatum* (MOI 1000:1 and 100:1) and OMV (10 and 50 µg/ml) for 24 h. **Panel B:** SW-480 (40X magnification). **Panel C:** SW-620 (20X magnification).

Upregulation of vimentin is an important mesenchymal marker that is responsible for maintaining cell shape (involved in stabilising cytoskeletal interactions) (Liu et al., 2015a). The expression level of vimentin in *F. nucleatum* and OMV-treated and untreated cells was analysed by immunofluorescence. The immunofluorescence analysis of SW-480 cells showed an increase in the expression of vimentin following treatment with *F. nucleatum* and OMV (**Figure 5.18, A and B**). The vimentin expression can be observed around the nucleus and in the cytoplasm of the control cells and vimentin expression increased following the exposure to *F. nucleatum* and OMV. Cells treatment with *F. nucleatum* at an MOI of 100:1 induces stronger vimentin expression when compared with the treatment

with an MOI of 1000:1 (**Figure 5.18, B**). RT-PCR analysis of vimentin gene expression demonstrated that *F. nucleatum* and OMV induced vimentin gene expression in SW-480 cells. Following 4 h *F. nucleatum* and OMV induced a significant increase in vimentin gene expression ($p < 0.001$ and $p < 0.05$, respectively) (**Figure 5.19**).

Attempts to examine the effect of *F. nucleatum* and OMV on the expression of vimentin in other cell lines (SW-620, Lovo, and Caco-2) was not successful and produced unclear images (data not shown). Also, attempts were made to detect vimentin expression in SW-480 cells by Western blot, but two separate batches of anti-vimentin antibody failed to produce a clear result (data not shown).

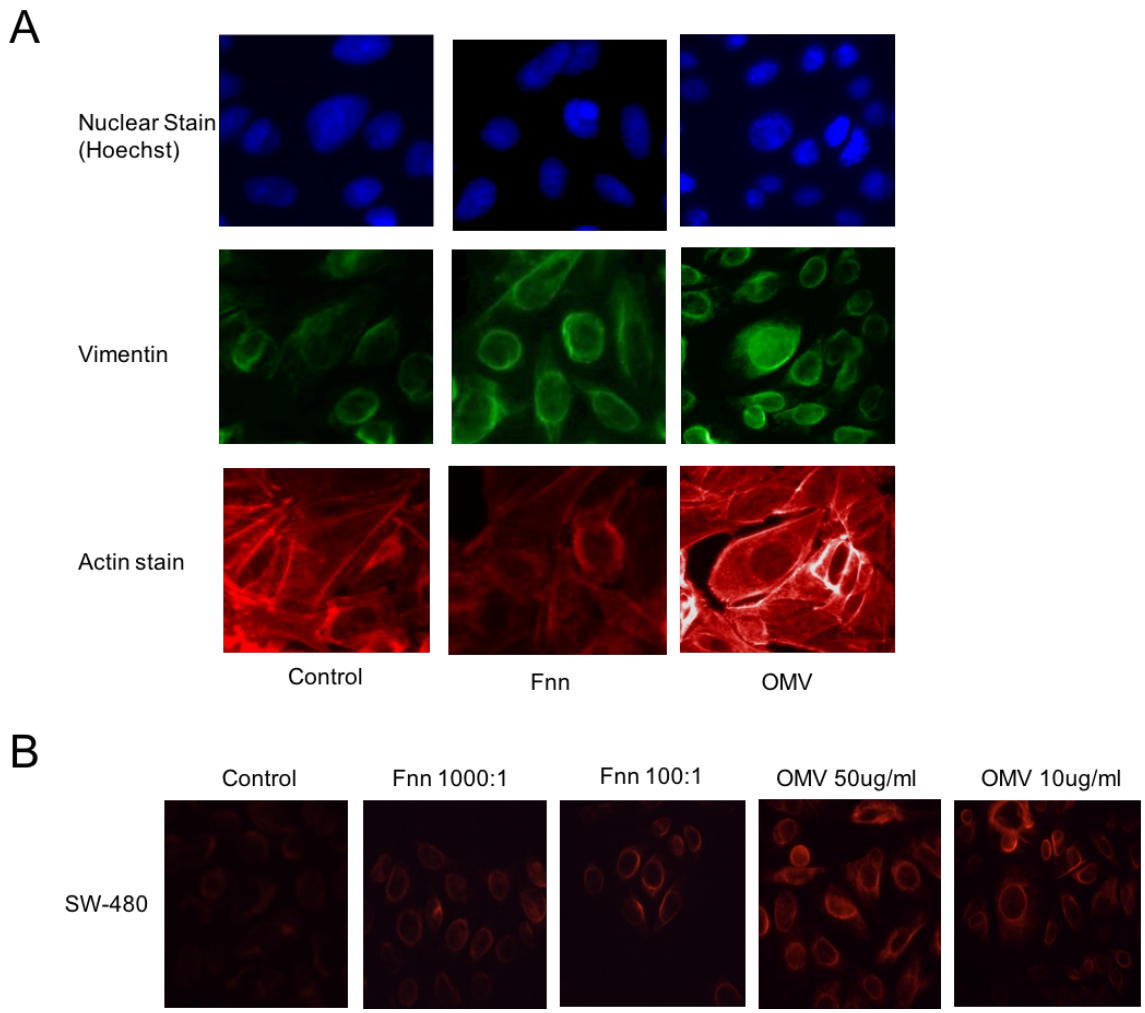

Figure 5.18: Vimentin expression in SW-480 cells. Panel A: SW-480 co-cultured with *F. nucleatum* (MOI 100:1) and OMV (50 µg/ml) for 24 h and probed for vimentin (green). Also shown are the nuclear stain (blue) and actin (red). **Panel B:** SW-480 cells were co-cultured with *F. nucleatum* (1000:1 and 100:1) and OMV (10 and 50 µg/ml) for 24 h.

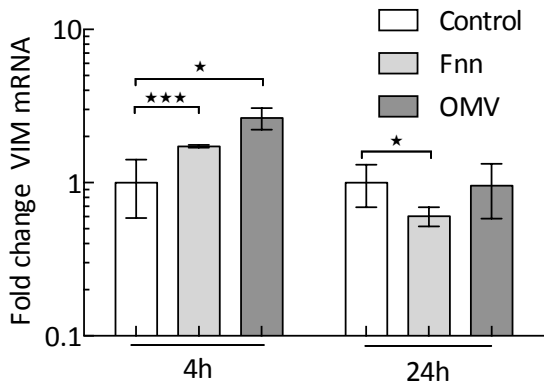

Figure 5.19: Effect of *F. nucleatum* and OMV of vimentin gene expression in SW-480 cells. SW-480 were treated with *F. nucleatum* (MOI 100:1) and OMV (10 µg/ml). The results are presented as the mean ± SEM (n=3). * = $p < 0.05$, and *** = $p < 0.001$.

Given that the *F. nucleatum* OMV-treated cells were adopting features consistent with mesenchymal cells as evidence by the appearance of mesenchymal markers and the disappearance of epithelial markers, the resistance of the treated cells in the presence of the anti-cancer drug 5-fluorouracil (5-Fu) to apoptosis was examined. SW-480 cells were treated with increasing doses of 5-Fu in the presence of *F. nucleatum* and OMV over 48 h. Cell number was assessed using the cellTiter proliferation assay. Both *F. nucleatum* and OMV increased colonic cell resistance to 5-Fu as indicated by the significant increases survival of the treated cells compared to controls (**Figure 5.20**).

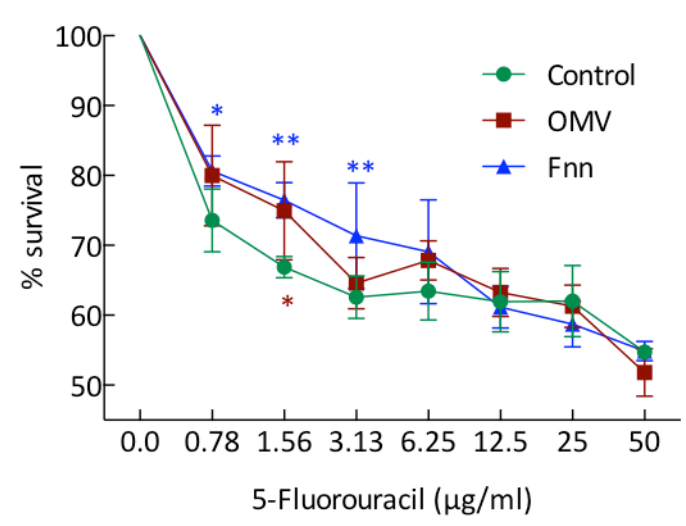

Figure 5.20: Effect of 5-Fu on SW-480 cell number induced by *F. nucleatum* and OMV. SW-480 cells were treated with increasing amounts of 5-Fu (0.78, 1.56, 3.13, 6.25, 12.5, 25, and 50 µg/ml) in the presence of *F. nucleatum* (MOI 200:1) and OMV (50 µg/ml) for 48 h. Cells **number** was estimated using the cellTiter proliferation assay. The statisticaly significant changes were calculated using two-way ANOVA. * = $p < 0.05$, ** = $p < 0.01$.

5.4 Discussion

The finding of enrichment of *F. nucleatum* in colorectal adenomas has raised the question of the role of *F. nucleatum* in colorectal carcinogenesis. Since EMT is a stage that precedes carcinogenesis, it was of interest to examine the ability of *F. nucleatum* to influence EMT to drive inflammation and malignant cell transformation that enable neoplastic progression. The aim of this work was to explore the carcinogenic potential of *F. nucleatum* and OMV to induce EMT-like behaviour and inflammation. This response was investigated by infecting colonic epithelial cells with *F. nucleatum* and OMV, which revealed the ability of *F. nucleatum* and OMV to induce EMT phenotypes, including morphological changes in colonic epithelial cells, and expression of prototypical mesenchymal markers.

Recently, EMT has gained much attention concerning metastatic dissemination. EMT is considered an early event in metastasis, which participates in the migration and invasion of tumour cells from the primary tumour. It is associated with modification of gene expression involved in the repression of the epithelial phenotype and activation of the mesenchymal phenotype (Lamouille et al., 2013). During this process, epithelial tumour cells passes through various changes that include increased resistance to apoptosis and increased cell proliferation, which contribute to the survival of tumour cells (Thiery et al., 2009). It has been reported that the genetic signature of EMT is highly correlated with intrinsic gene expression in colon cancer (Loboda et al., 2011). Also, EMT induces cell proliferation that mimics the progression of invasive colon carcinoma, which correlates with CRC progression in humans (Bates and Mercurio, 2005). Such processes can be observed in other cancer including gastric cancer (Zong et al., 2016), breast cancer (Moyret-Lalle et al., 2014), and cholangiocarcinoma (Vaquero et al., 2016). In addition, deregulation of tumour suppressor, epigenetic reprograming, and the activation of stem cell associated pathways induced by bacteria, are possible cause of cancer development and progression in epithelial cells (Hofman and Vouret-Craviari, 2012).

In this work, various methods were utilised to examine the effect of *F. nucleatum* and OMV on epithelial cell proliferation which revealed the ability of *F. nucleatum* and OMV to promote cell proliferation in colonic epithelial cells. This observation links *F. nucleatum* and

OMV ability to induce EMT-like behaviour via promoting the proliferation of colonic epithelial cells. Other bacteria are associated with the induction of EMT-like behaviour such as *P. gingivalis* (Sztukowska et al., 2016), *H. pylori* (Sougleri et al., 2016), *K. pneumoniae* (Leone et al., 2016), and *Mycobacterium tuberculosis* (Gupta et al., 2016).

In addition, this study indicates that *F. nucleatum*- and OMV-induced IL-8 can behave as an autocrine growth factor for colonic cells as neutralisation of secreted IL-8 by anti-IL-8 monoclonal antibody resulted in attenuation of the growth of SW-480 cells treated with *F. nucleatum* and OMV. This suggests that IL-8 may act indirectly to support tumour growth (Brew et al., 2000). Also, IL-8 signalling is involved in the maintenance of EMT through direct and indirect mechanisms in addition to autocrine growth factor activity between IL-8 and tumour cells via EMT (Long et al., 2016).

IL-8 and its receptor CXCR-2 are the most prominent upregulated chemokine and receptor pair in colon cancer (Chen et al., 2004). IL-8 and CXCR-2 expression in the tumour microenvironment play an essential role cancer growth and metastasis (Waugh and Wilson, 2008), including colon cancer (Lee et al., 2012c). IL-8 and CXCR-2 mediate autocrine growth properties in tumour cells to evade apoptosis (Maxwell et al., 2007) and IL-8/CXCR-2 autocrine behaviour was been shown to participate in tumour growth, invasion, survival, metastases, angiogenesis, and resistance (Lee et al., 2012c). Other cytokines could be involved in the activation of IL-8 production such as GRO-α that is also associated with colon cancer development and progression (Baier et al., 2005). GRO-α (Li et al., 2005a), TNF-α (Kuno et al., 2005, Al-Lamki et al., 2010), and IL-6 (Miki et al., 1989) also exert autocrine growth factor activity similar to IL-8 and may thus enhance the metastatic activity of tumour cells.

Many studies have focused on the molecular signals controlling EMT initiation. EMT can result from growth factor-induced signalling pathways, which mainly effect downstream transcriptional regulators and cell integrity. Commonly this signalling targets the expression of the adherens junction proteins (e.g. E-cadherin). In this study, it has been found that both *F. nucleatum* and OMV increase the expression of Myc and Wnt genes. Interestingly, some of EMT signalling pathways could be upregulated by bacterial infections including *F.*

nucleatum infection (Hofman and Vouret-Craviari, 2012) and *Salmonella typhimurium* (Tahoun et al., 2012). It has been reported that *F. nucleatum* promotes colorectal carcinogenesis through Wnt pathway modulation (Rubinstein et al., 2013). Also, *F. nucleatum* is involved in the inhibition of T-cell responses against colorectal tumours (Kostic et al., 2013b, Gur et al., 2015). Typically, the activation of transforming growth factor β (TGF-β) activates the SMAD family of transcription, which control cell differentiation, proliferation, and migration (Massagué, 1998). However, in the case of *F. nucleatum* and OMV, nether is capable of activation of TGF-β at least at the time points tested (**Chapter 4, Figure 4.16**).

Additionally, it has been reported that Myc regulatory gene overexpression has been associated with stress response, growth, proliferation, as well as cancer supported Ras and MAPK pathway. Also, it is linked to EMT via regulation of N-cadherin, Snail, and TWIST (Vazquez-Martin et al., 2016). The Wnt-β-catenin signalling pathway is an important characteristic feature of CRC development (Haase et al., 2016). Around 90 % of CRC involved aberrant Wnt signalling as an early progression event (Fodde et al., 2001). This event commonly occurs via mutation of APC and to a less often of CTNNB-1 (gene encoding β-catenin) (Fodde et al., 2001). The other important role of β-catenin is in the linkage between E-cadherin transmembrane adhesion and the actin cytoskeleton (Heuberger and Birchmeier, 2010); also, β-catenin is a major transducer of Wnt signalling, which involves the activation of DNA-binding proteins T-cell factor (TCF)/lymphoid enhancer factor (LEF) and the translocation of the phosphorylated β-catenin from the cytoplasm into the nucleus (Behrens et al., 1996, Haase et al., 2016). In the absence of Wnt, β-catenin is degraded by a protein complex containing APC, glycogen synthase kinase 3β, axin and casein kinase (Polakis, 2012, Chiurillo, 2015). This prevents binding between Wnt and the frizzled receptor, and the translocation of β-catenin into the nucleus. Whereas the activation of the Wnt signalling results in inhibition of β-catenin degradation, which then accumulates in the cytoplasm and the nucleus (Conacci-Sorrell et al., 2002). This results in the interaction of β-catenin with TCF/LEF, which drives gene transcription that plays a role in cell proliferation and transformation or apoptosis (Polakis, 2012, Chiurillo, 2015). Aberrant activation of ZEB-1 and Wnt-β-catenin signalling could involve stabilising mutation in APC

and β-catenin gene in the majority of colon cancer patients, which is considered a hallmark of CRC development (Sanchez-Tillo et al., 2015).

Various genes representing mesenchymal markers were screened in this work, which revealed an increased in their expression following *F. nucleatum* and OMV treatment of SW-480 cell including BMP, ITGA-5, FN-1, N-cadherin, Snail (1, 2, and 3), and TWIST.

BMP is associated with EMT (Hamada et al., 2007) and BMP signalling induces the maturation of secretory cell lineage of the small intestine in vivo (Auclair et al., 2007) as well as apoptosis in mature colonic epithelial cells (Hardwick et al., 2004). Many studies have reported the involvement of BMPs in CRC (Loh et al., 2008, Hardwick et al., 2008). However, the definitive association between BMPs and the promotion of metastasis and tumorigenesis is not defined yet.

Also, ITGA-5 gene expression was induced by both *F. nucleatum* and OMV. The α-5 chain of integrins belongs to a family of adhesion receptors whose primary ligand is the filament fibronectin (Bates and Mercurio, 2005). Increase in the expression of ITGA-5 has been demonstrated to regulate migration of epithelial cells and is associated with EMT in renal epithelial cells (White et al., 2007). Additionally, ITGA-5 β is related to increased invasiveness of cancer cells in various epithelial cancer including colon, ovarian (Schaffner et al., 2013), prostate, and breast (Jia et al., 2004). Also, upregulation of ITGA-5 gene expression in colon cancer is influenced by ZEB (Nam et al., 2012), which results in enhancing cell migration during EMT. It has been reported also that IL-8 and TNF-α are involved in the regulation of ITGA-5 expression in endothelial cells (Kim et al., 2000).

Fibronectin (FN) is a glycoprotein found in the ECM of connective tissues and is involved in cell adhesion, growth, migration, proliferation, and wound healing (Schwarzbauer, 1991). The cellular FN can from fibers that are involved in the interaction with cell surface integrin α-5 and actin (Singh et al., 2010). The multiple isoforms of FN result from alternative splicing of mRNA (Schwarzbauer, 1991). It has been observed that FN expression is increased in breast cancer (Matsumoto et al., 1999), liver cancer (Oyama et al., 1993), lung cancer (Sun et al., 2013), head and neck cancers (Birchler et al., 2003) and CRC (Pujuguet et al., 1996). Also, it has been reported that FN could be involved in promotion of colorectal

carcinomas via the induction of EMT (Ou et al., 2014). FN expression is associated with proliferation and malignancy with poor prognosis in CRC (Yi et al., 2016). This suggests that FN expression is involved in tumour progression (Kumra and Reinhardt, 2016).

The genetic expression of EMT induction was found to be subject to temporal variation and correlated with the transitory repression of epithelial markers and promotion of mesenchymal markers. The process of cadherin switching appeared to be sustained in which induction of N-cadherin was accompanied by suppression of E-cadherin expression. Elevation of N-cadherin is associated with cancer progression and promote cell adhesion (Blaschuk, 2015). The activation of the mesenchymal phenotype involves several changes, which take place at the adherens junctions involving the deregulation of the E-cadherin/β-catenin complex, which plays an essential role in the initiation of EMT in cancer progression (Tian et al., 2011). Loss of apical-basal polarity results from disruption of tight junction components. As part of this process, epithelial cells undergo morphological and phenotypic changes including differential expression of marker proteins. For example, typical epithelial markers such as E-cadherin is downregulated by EMT-inducing transcription factors Including ZEB-1, TWIST, and Snail, which are involved in the regulation of E-cadherin and N-cadherin expression (Puisieux et al., 2014, Montserrat et al., 2012) and up-regulation of the expression of prototypical mesenchymal marker proteins including vimentin (Mendez et al., 2010), and MMPs (Nisticò et al., 2012), and fibronectin (Nisticò et al., 2012), that enhance cell motility and adhesion properties in addition to morphological changes to the cells. Thus, the loss of cell contacts due to redistribution of E-cadherin could both promote β-catenin release and transfer to the nucleus to facilitate EMT and result in disruption of barrier function. The data presented herein indicate that both *F. nucleatum* and OMV elicit cellular responses commensurate with the initiation of EMT and most notably by the increased nuclear localisation of the transcription factor ZEB-1 in addition to the reduction in E-cadherin expression. The gastric pathogen *H. pylori* CagPAI^{+} strain induces similar morphological changes to gastric epithelial cells (Azuma et al., 2004, Sougleri et al., 2016). *H. pylori* induces a reduction in the expression of E-cadherin and induces elevation in the expression of Snail, TWIST, Slug, and vimentin (Choi et al., 2015b). Similarly, other bacteria are capable of inducing EMT-like behaviour in different cell types such as *Mycoplasma hyorhinis* (Duan et al., 2014), *E. coli* (Cane et al., 2010, Berger et al., 2004), *C. trachomatis*

(Igietseme et al., 2015), *K. pneumoniae* (Leone et al., 2016), and *P. aeruginosa* (Borthwick et al., 2011).

The initiation of these changes resembles that of EMT in which, losing cell-cell contacts and acquiring a spindle shape appearance (Thiery et al., 2009, Brabletz and Brabletz, 2010). Many transcription factors play a role in EMT activation including ZEB-1 and ZEB-2 (Wellner et al., 2009) and β-catenin. Others have described the *F. nucleatum*-induced modulation of β-catenin induced signalling (Rubinstein et al., 2013). The ZEB-1 has been linked to tumour progression and are considered essential activators of EMT in many human cancers including colon, prostate, and pancreatic (Graham et al., 2008, Spaderna et al., 2006, Wellner et al., 2009), as they are associated with the reduction in basement membrane component expression (Spaderna et al., 2006) and cell polarity factors (Aigner et al., 2007).

Binding of *F. nucleatum* FadA, a surface adhesin, to E-cadherin causes E-cadherin phosphorylation and internalization as well enhanced nuclear translocation of β-catenin (Rubinstein et al., 2013). This leads to activation of β-catenin-regulated transcription factor. The activation of E-cadherin and β-catenin stimulate CRC cell growth and increase the expression of oncogenes, transcription factors, inflammation genes, and Wnt genes (Rubinstein et al., 2013). The Wnt pathway is responsible for activation and cytosolic accumulation of β-catenin in the cell (Shimizu et al., 1997). Hence, EMT and late-stage cancer progression is characterized by loss of E-cadherin expression (Peinado et al., 2004). The data presented here supports also a role for OMV in modulating E-cadherin expression in colon cell lines. In this regard, the increased expression of nuclear ZEB-1, which occurs in a bacterial and OMV-dependent manner, further supports a role for these structures in initiating the EMT process. ZEB-1 is a potent repressor of E-cadherin expression (Vandewalle et al., 2009). Phenotypically, epithelial cells undergoing EMT loose their cobblestone appearance and acquire an elongated fibroblastic morphology. Downregulation of E-cadherin (Chen et al., 2016) and β-catenin (Oh et al., 2016) occurs at the same time as translocation of β-catenin (phosphorylated form) from the cell membrane into nucleus (Sánchez-Tilló et al., 2011b). Also, these events are accompanied by upregulation of EMT markers including ZEB-1, vimentin, N-cadherin (Wang et al., 2016b, Lamouille et al., 2014), TWIST (Li and Zhou, 2011), and Snail (Wang et al., 2013) and were

also observed in the current study to be upregulated in response to infection with *F. nucleatum* and OMV. Overall, these alterations permitted the disassembly of cell-cell junction, reorganization of actin cytoskeleton and induction of contractile proteins, which contributes in the conversion of non-motile cells into motile, individual, invasive mesenchymal phenotypic cells (Nieto, 2011). Impairment of E-cadherin expression is the primary indication of EMT that could be a trigger for activation of transforming growth factor-β (TGF-β) and Wnt signaling (Thiery and Sleeman, 2006, Lamouille et al., 2014).

A property of mesenchymal cells is that they become more resistant to chemotherapy. This is associated with elevated level of IL-6 in the tumour microenvironment, which is associated with angiogenesis, cell proliferation and metastasis via STAT-3/MAPK/Akt signalling (Bharti et al., 2016, Spitzner et al., 2014). Such events are commonly associated with human malignancies including CRC resistance to chemotherapy such as 5-Fu (Spitzner et al., 2014). It was hypothesised in this study that *F. nucleatum* and OMV induce cell growth and increase cellular resistance to anti-cancer drugs. Drug resistance is still the main limitation in clinical use of anti-cancer drugs. 5-Fu is widely used as an anti-cancer drug in the treatment of CRC and breast cancer (Paschall et al., 2016). 5-Fu is a cytotoxic agent, which selectively targets rapid proliferating cells (Dallas et al., 2009). In this regard, SW-480 cells were treated with *F. nucleatum* and OMV in the presence of increasing doses of 5-Fu over 48 h. When compared to control cells, *F. nucleatum* and OMV induced cell proliferation and contributed to the development of resistance to 5-Fu. In colorectal patients, chemoresistance, including resistance to 5-Fu, metastasis and tumour recurrence account for more than 90 % mortality (Longley and Johnston, 2005, Paschall et al., 2016). It has been reported that Slug could mediate 5-Fu resistance via the inhibition of miR145 that accompanies a reduction in E-cadherin in CRC patients (Findlay et al., 2014). However, the mechanisms of acquiring resistance toward 5-Fu and other anti-cancer drugs remain unclear. In CRC, it has been reported that elevation of IL-8 expression contributes in the regulation of cell proliferation, angiogenesis, progression, migration, and chemosensitivity in vivo and in vitro (Ning et al., 2011, Lee et al., 2012c). Also, polymorphisms in IL-8 and CXCR-2 genes are reported to be associated with colon cancer progression and drug resistance (Ning et al., 2011, Lurje et al., 2008).

The above, combined with the ability of both *F. nucleatum* and OMV to induce EMT-like behaviour via enhancing proliferation of colonic epithelial cells, results in the induction of colonic cell growth indirectly through increased IL-8 expression via its autocrine growth factor activity, the induction of Wnt and Myc signalling, and the induction of EMT marker expression all contribute to phenotypic changes. The elevation of IL-8 levels and other chemokines would likely have an additive effect in initiating damage at the site of infection due to an influx of neutrophils. However, it is still not clearly defined whether *F. nucleatum* and its OMV have a role in the initiation of colonic cancer or it simply involved in the progression of the disease following the initiating events (such as APC mutations). In this study, the results indicate that *F. nucleatum* OMV are potent inducers of mesenchymal marker expression compared to the intact parental bacteria. It is worth noting that *F. nucleatum* OMV could potentially induce EMT elsewhere in the intestine and not be solely restricted to the infected niche once released from the parental bacterium. Understanding the complex interactions that connect EMT and metastases to microbial infection and inflammation could have the potential to identify novel therapeutic targets for tumour progression and cancer.

CHAPTER SIX

Overall Discussion

CRC is a complex disease that results from an accumulation of somatic mutations and epigenetic alterations including a mutation in Kras, as well as loss of tumour suppressor genes like APC and p53, which contribute to the transformation of normal colonic epithelium (Fearon, 2011, Mundade et al., 2014). Infection contributes to about 20 % of all human cancers (Gagnaire et al., 2017). Bacteria and bacterial effectors can promote cancer directly by influencing proliferation-inducing signalling pathways and the induction of DNA damage. Indirect mechanisms include inducing immunosuppression, oxidative stress, inflammation, and tissue damage (Gagnaire et al., 2017). Altering bacterial diversity of the gut and microbial dysbiosis strongly influences CRC development (Keku et al., 2015). However, despite various studies comparing intestinal bacterial communities over time (Rodriguez et al., 2015) for different cancer stages (Nugent et al., 2014), little is known about the contribution of bacteria to CRC development.

As considered in the introduction, many studies have identified the association between specific species of bacteria and colon cancer induction including *E. coli* and *B. fragilis*. It has been reported that *F. nucleatum* status is associated with genetic mutations in BRAF, KRAS, TP53, CHD-8, and CHD-7 (Tahara et al., 2014) and with other genetic and epigenetic factors playing a role in CRC (Zoratto et al., 2014). The *F. nucleatum* induction of chronic inflammation links infection to tumour formation and cell proliferation. Known virulence factors induce inflammation and directly participate in the induction of tumour formation (Kostic et al., 2013b, Rubinstein et al., 2013, Park et al., 2014a). Chronic inflammation resulted in the loss of homoeostasis, which contributes in the modulation of the microenvironment during the pathogenesis of CRC (Sansone and Bromberg, 2011). *F. nucleatum* is known as a potent pro-inflammatory bacterium (Swidsinski et al., 2011, Liu et al., 2007), which is associated with IBD (Kostic et al., 2013b). McCoy et al. (2013) demonstrated a connection between *F. nucleatum* and inflammation as evident by elevation of the pro-inflammatory cytokines (IL-6, IL-8, and TNF-α) in colorectal adenoma.

This finding was also consistent with the findings of Kostic et al. (2013a) who indicated that *F. nucleatum* overexpression induces inflammation via upregulating NF-κβ in colorectal adenomas.

F. nucleatum is perhaps one of the most intensively studied bacteria given its association with several diseases states and cancer and is now considered an emerging pathogen. Recently, it has been shown that *F. nucleatum* contributes to CRC in part due to the interactions between Fusobacterial factors and the host, which includes FadA, Fap2, LPS, RadD, and FomA (**Table 6.1**).

Fusobacterial Fap2 for example interacts with Gal-GalNAc that is over expressed in CRC (Abed et al., 2016). Fusobacterial Fap2 and FadA interaction facilitate invasion and stimulate potentially oncogenic β-catenin signalling (Rubinstein et al., 2013, Holt and Cochrane, 2017). Also, Fap2 is involved in co-adherence and bridges adherence between different species and enhance diversity and stability of dental plaque (Coppenhagen-Glazer et al., 2015). Fap2 can also modulate host immunity via the binding and activation of TIGIT, an immunoregulatory signalling receptor in NK and T-cells (Yu et al., 2009b). Fap2-TIGIT interaction inhibits NK and tumour infiltrating lymphocytes cytotoxic ability toward tumour cells (Gur et al., 2015). Also, Fap2 has been reported to be associated with the induction of lymphocyte apoptosis (Kaplan et al., 2010). FomA is one of the major OMP in *F. nucleatum* that play a major role in bacterial aggregation and biofilm formation that enhance bacterial survival (Liu et al., 2010a). FomA is highly immunogenic and modulates the host immune response and induces cytokine production via TLR-2 (Toussi et al., 2012a). Also, RadD is an important OMP associated with the induction of cell death (Jurkat cells) (Kaplan et al., 2010) and biofilm formation (Lima et al., 2017). Furthermore, *F. nucleatum* LPS is highly immunogenic and a potent inducer of miRNA-21 expression via TLR-4 on T-cells that modulates the oncogenic state of the host and is associated with CRC cell proliferation (Yang et al., 2016).

Table 6.1: *F. nucleatum* key features contribute to CRC.

Factors	Function	References
FadA	Increase tumour cells proliferation via E-cadherin/β-catenin signalling. Facilitate bacterial adhesion/invasion (internalization).	Rubinstein et al. (2013)
Fap2	interact with TIGIT and inhibit NK and lymphocytes. Bind to Gal-GalNAc. Induce apoptosis in lymphocytes and NK cells.	Gur et al. (2015) Abed et al. (2016) Kaplan et al. (2010)
LPS	Elevate miRNA expression.	Yang et al. (2016)
RadD	Cell death. Biofilm formation.	Kaplan et al. (2010) Lima et al. (2017)
FomA	Induce inflammation via TLR-2. Aggregation and biofilm formation.	Yang et al. (2016) (Liu et al., 2010a)

Taken as a whole, these data suggest the association between *F. nucleatum* and CRC, further supporting the implication of *F. nucleatum* as a driver pathogen rather than just a passenger in CRC. However, there is a key question that remains unanswered. Could other factors participate in CRC? Given the multifactorial nature identified to date, analogous to what is seen for the gastric pathogen *H. pylori*, it is likely that other *F. nucleatum*-molecules contribute to CRC pathogenesis.

In the current study, TEM analysis of *F. nucleatum* OMV indicates the presence of two types of OMV, those with a bilayer structure and vesicles with a single membrane. MS analysis revealed that 52 % of the OMV-associated proteins are represented by cytoplasmic proteins, and the OMV are enriched with proteolytically active proteases, which appear to be differentially sorted and concentrated into the OMV compared with parental bacterium. It has been shown that OMV-associated proteinases can degrade host cell component such as E-cadherin and can modulate cellular functions. Other bacterial proteases can also cleave and degrade cellular E-cadherin including *H. pylori* (Hoy et al., 2010), *C. jejuni* (Elmi et al., 2016), *E. coli*, and *S. flexneri* (Hoy et al., 2012). The ability of *F. nucleatum* OMV to degrade tight junction components (E-cadherin) and decrease the electrical resistance across polarised epithelial cells, which effects the integrity of the epithelial barrier function. In this study, OMV caused a significant reduction in TER compared to intact *F. nucleatum* suggesting the effects of OMV in the disrupting of epithelial barrier function may be important. Also, it has been shown that OMV contained FadA, which possibly enables OMVs to bind to E-cadherin. This may explain the coalesce of fluorescently labelled OMV

at the cell-cell boundaries of the colonic cells where E-cadherin is located. Also, the interaction of FadA with E-cadherin could release β-catenin and activate oncogenic pathways (Rubinstein et al., 2013). This suggests that OMV possibly enhance bacterial internalisation and invasion of host cells. OMV also have the potential to invade other tissues and are not restricted to the parental bacterial infection sites. Similarly, *H. pylori* OMV are able to coalesce with and be internalised by AGS cells (Parker et al., 2010). Also, other bacteria are capable of reducing TER and modulating epithelial barrier function such as *H. pylor, E. coli, C. jejuni* (Hoy et al., 2012, Canil et al., 1993).

The current study demonstrated that *F. nucleatum* OMV are potent inducers of inflammation via pro-inflammatory cytokine/chemokine production due to increased expression of NF-κβ. Also, *F. nucleatum* OMV upregulate STAT-3 activation. NF-κβ and STAT-3 activation is involved in facilitating many of the classical hallmarks of cancer (Li et al., 2005b, Waldner et al., 2012). Many genes are expressed upon activation of NF-κβ and STAT-3 (Yu et al., 2009a, Bhatt and Ghosh, 2014) and the corresponding proteins contribute to metastasis and invasion (proteases and MMPs). Other bacterial cells and their OMV can also activate NF-κβ and pro-inflammatory cytokines via TLR-2 and TLR-4 including *B. fragilis* (Patrick et al., 2011), *Salmonella* (Huang, 2012), and *H. pylori* (Boughan et al., 2006, Ko et al., 2016). *F. nucleatum* OMVs are strong inducers of IL-8 as in *H. pylori* OMV from gastric epithelial cells (Ismail et al., 2003), *E. coli* OMV from IECs (Kunsmann et al., 2015), and *V. cholerae* OMV from colonic epithelial cells (Mondal et al., 2016). In addition, this study indicates that *F. nucleatum* OMV contain molecules other than LPS which could play a major role in the induction of pro-inflammatory cytokine/chemokine production. Potential candidates include FomA (Toussi et al., 2012b), the heat shock protein GroEL (Lee, 2012, Wick et al., 2001), NapA (Choli-Papadopoulou et al., 2011), and peptidoglycan (Okugawa et al., 2010) that were identified in *F. nucleatum* OMV. Also, in this study, it has been shown that OMV induced an increase in gene expression of cytokines including TNF-α, IL-1 β, IL-6, CXCL-1 (GRO-α), and CCL-20 (MIP3-α) in colonic epithelial cells. A similar gene expression signature is also induced by other bacteria such as *Escherichia, Streptococcus, Bacteroides*, *Propionibacterium* (Kostic et al., 2013b), and *C. jejuni* OMV from T-84 cells (Elmi et al., 2016). Also, *F. nucleatum* OMV induces GRO-α and CCL-20 induction in colonic epithelial cells. GRO-α and CCL-20 are associated with CRC (Frick et al., 2013, Baier et al., 2005) and

linked to poor patient survival (le Rolle et al., 2015, Cheng et al., 2014). Worth to note, in this study, *F. nucleatum* OMV induce the upregulation of many *Fusobacterium*-associated pro-inflammatory genes that correlate with CRC have been investigated in this and other studies (IL-1 β, IL-6, IL-8, TNF-α, MIP3-α, GRO-α) (Dharmani et al., 2011, Trinchieri, 2012) in addition to upregulation of MMPs, SOCS-3, SPHK-1, and PTGS-2 (COX-2), which are part of the 50 top ranked gene of *Fusobacterium*-associated human CRC gene signature that been identified by Kostic et al. (2013b).

Furthermore, EMT promotes cell proliferation that mimics the progression of invasive colon carcinoma, which correlates to CRC progression in human (Bates and Mercurio, 2005). It has been shown that *F. nucleatum* OMV can induce EMT-like behaviour by promoting the proliferation of colonic epithelial cells. Enhancement of cell proliferation can be observed in various cancer types including CRC as epithelial cells acquiring a mesenchymal phenotype (Loboda et al., 2011). Similarly, *H. pylori* OMV promote gastric epithelial cells to proliferate (Ismail et al., 2003). Also, OMV induces IL-8 production that act indirectly to support tumour growth (Brew et al., 2000) and cross-linked inflammation and tumour EMT (Long et al., 2016).

One of the key features of EMT activation is the reduction in E-cadherin expression in addition to the elevation of several key markers of EMT including ZEB-1, β-catenin, Vimentin. EMT is the main pathway of metastasis, which arises in part a consequence of inflammation (Ma et al., 2016, Cao et al., 2015). As mention before OMV proteinases contributes to the degradation of E-cadherin in addition to downregulation of CDH-1 expression. In which OMV induce and increase in N-cadherin, a process known as cadherin switching (Wheelock et al., 2008), which provokes cell migration in EMT and correlates with poor prognosis in CRC (Yan et al., 2015) where it is associated with tumour differentiation, size, and metastasis stage. OMV also induced the expression of various transcription factors including ZEB-1, Snail-1, Snail-2, Snail-3, and TWIST, which are involved in driving EMT and cancer metastasis (Cao et al., 2015). Similarly, *H. pylori* can induce a reduction in the expression of E-cadherin and induce an elevation in the expression of Snail, TWIST, Slug, and vimentin (Choi et al., 2015b).

Binding between these repressors and the CDH-1 promoter result in epigenetic silencing of E-cadherin expression and activation of oncogenic pathways such as Wnt/β-catenin signalling pathway, which is associated with EMT in CRC (Tulchinsky, 2017). Transcription factor such as ZEB-1 target β-catenin/T-cell factor 4 in CRC cell (Sánchez-Tilló et al., 2011a). β-catenin is a major transducer of Wnt signalling in which the phosphorylated β-catenin is translocated from the cytoplasm into the nucleus (Behrens et al., 1996, Haase et al., 2016). The interaction between β-catenin and TCF/LEF are involved in driving gene transcription that play a role in cell proliferation and transformation or apoptosis (Polakis, 2012, Chiurillo, 2015). Also, Snail forms part of a complex which remove an acetyl group from histone to increase its binding affinity to DNA, which prevents E-cadherin transcription (von Burstin et al., 2009). Additionally, Snail can upregulate ZEB-1 expression that further decreases E-cadherin expression (Guaita et al., 2002). Slug overexpression is another key factor in EMT, invasion and proliferation in CRC and is also a prediction marker of poor prognosis. Moreover, OMV is shown to be able to upregulate the production of MMPs. Upregulation of MMPs are associated with EMT progression and migration (Jang et al., 2016, Xu et al., 2015) and also linked to the enhancement of the envision ability of cancer and metastases. **Figure 6.1** and **Table 6.2** summarised the key finding of this work.

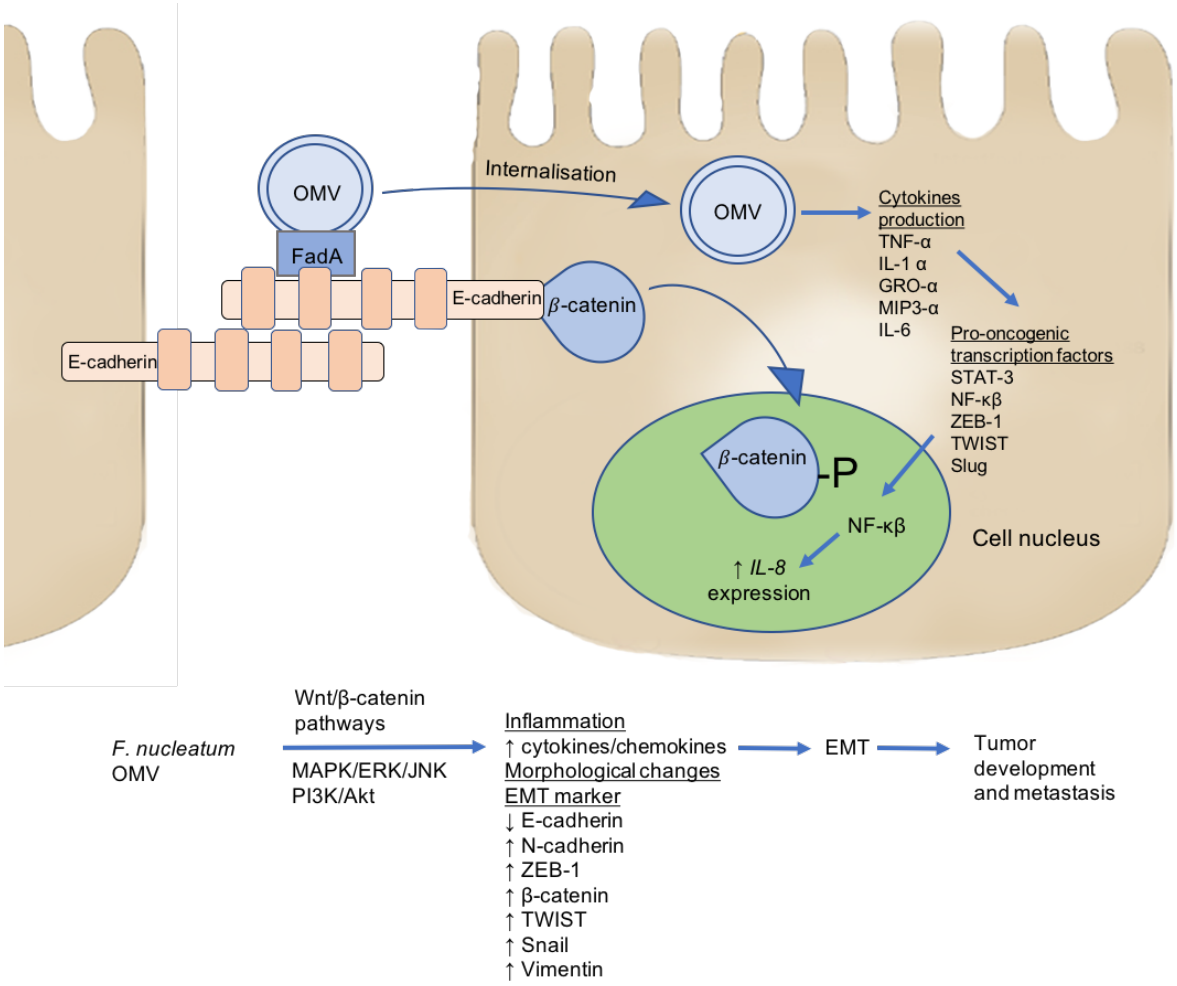

Figure 6.1: Summary of the potential mechanisms involved in the internalisation of *F. nucleatum* OMV in colonic epithelial cells and the endogenous pathway involved.

Table 6.2: Summary of the factors that contribute to CRC.

Factor	Possible function	Results
OMV proteases	Degrade ECM components (E-cadherin)	Decrease TER and disrupt epithelial barrier function.
OMV FadA	Bind to E-cadherin.	Internalization and cell proliferation.
OMV potential virulence factors.	Induce inflammation.	Cytokine/chemokine production.
OMV	Decrease E-cadherin expression, increase N-cadherin, and vimentin expression.	Induce EMT.
OMV	Increase in transcription factors (ZEB-1, Snail (1, 2, and 3), and TWIST expression.	Induce EMT.
OMV	Induce β-catenin translocation into the nucleus.	Activate Wnt/β-catenin signalling and induce EMT.

All in all, a lot is known about OMV as virulence and mediator of bacterial survival, but the mechanism of oncogenic properties of *F. nucleatum* and *F. nucleatum* OMV that associated with bacterial colonisation in the host will remain an area of considerable debate. The severity of precancerous lesion demonstrates a close relationship of the OMV with pathogenesis. Considering that the OMV can induce oncogenic pathway and they are a strong inducer of inflammation that can contribute to tissues damage and disruption of epithelial barrier function in addition to their ability to induce EMT in colonic epithelial cells. This is suggesting that *F. nucleatum* OMV is important virulence factors, which may play a major role in pathogenesis associated with *F. nucleatum* and CRC. However, much remains to be discovered in order to fully understand the true impact of those nanoparticles on other microbes and the human host.

6.1 Future perspectives

Many questions remain unanswered and further studies are required before a full understanding of the role of *F. nucleatum* and its OMV in CRC can be determined. For example, during this work, a number of interesting observations were made with regard to *F. nucleatum*'s ability to self-aggregate, particularly after transient exposure to oxygen (atmospheric). Reasoning that *F. nucleatum* must pass from the oral cavity, where it would be exposed to oxygen at least transiently in its former way to the GIT, it was of interest to determine if short-term exposure of *F. nucleatum* to oxygen could modulate any phenotypic change. Of note, oxygen exposure enhanced its ability to auto-aggregate (**Figure 6.2, A**). As this likely reflected the expression of an adhesin on the surface of *F. nucleatum*, this possibility was tested by biotinylating surface proteins followed by their separation and analysis by Western blotting (**Figure 6.2, B**). As indicated in the **Figure 6.2, B** (arrowheads) at least two additional proteins are present in the *F. nucleatum* exposed to oxygen, and these proteins may be involved in auto-aggregation. An attempt to identified those proteins by MS analysis was not successful. Furthermore, *F. nucleatum* that was exposed to oxygen demonstrated a greater ability to modulate cell barrier function (**Figure 6.2, C**). Other have shown that oxidative stress increases chaperone expression including ClipB, DnaK, and heat shock proteins (Steeves et al., 2011) suggesting that

oxidative stress may be associated with enhanced *F. nucleatum* virulence. Additional studies are required to confirm the identities of the proteins identified as being expressed on the surface of *F. nucleatum* to determine their possible role in aggregation and virulence.

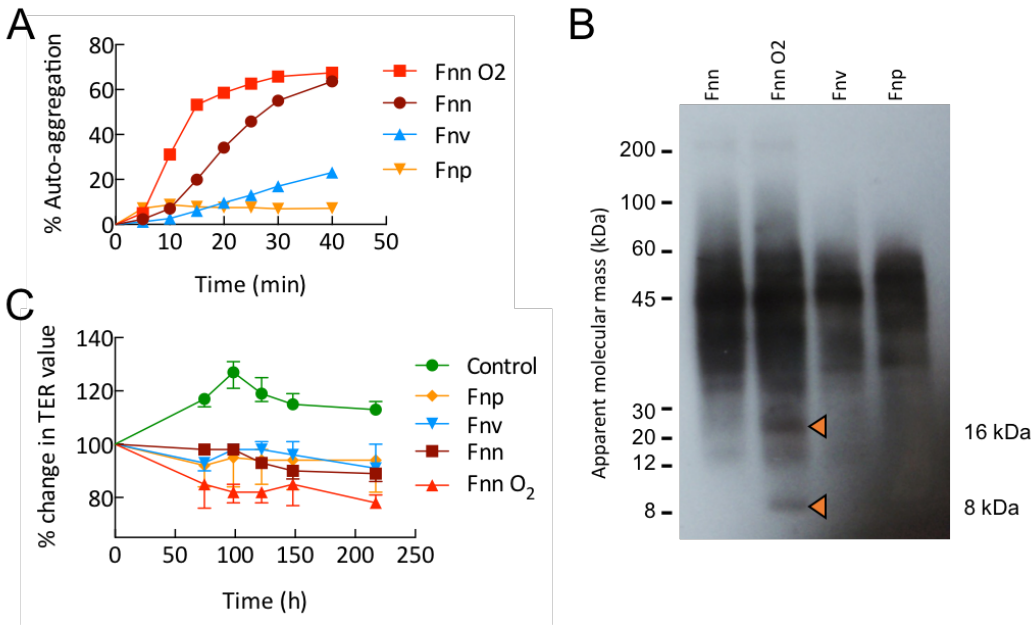

Figure 6.2: Effect of transit exposure of *F. nucleatum* to atmospheric oxygen. Panel A: shown a comparison of auto-aggregation between oxygen-adapted *F. nucleatum*, *F. nucleatum*, *F. vincentii*, and *F. polymorphum*. Fusobacteria species were suspended into PBS, and OD_{600} was used to obtain an equal bacterial concentration then OD_{600} readings were used to calculate the auto-aggregation percent that plotted against time. The decrease in the OD_{600} within time indicates bacterial autoaggregation. **Panel B:** Western blot shows an increase in the expression of oxygen-adapted *F. nucleatum* biotinylated OMP. Different *F. nucleatum* species were biotinylated using EZ-link sulfo-NHS-LC-biotin (Thermo Scientific) then all bacteria were sonicated, and the biotinylated outer membrane proteins were fractionated (5 μg/ml) using 12.5 % SDS-PAGE that probed by streptavidin-HRP. **Panel C:** Effect of different *Fusobacterium* species on Caco-2 TER. Caco-2 cells were grown until a stable TER was achieved and then subjected to serum deprivation for 24h prior to sample addition. Cells were incubated with different *F. nucleatum* species (MOI 2000:1) for 9 day. The results show the mean ± SEM (n=4). All tested Fusobacteria species significantly decreased the TER ($P < 0.01$).

It would be of interest to study the effect of OMV on other cells from non-colonic/intestinal sites since *F. nucleatum* infection occurs in various tissue types. Although, it was initially planned to evaluate the effects of *F. nucleatum* OMV on susceptible in vivo models (e.g. $APC^{min/+}$ mouse) this was not undertaken. Thus, it would be of great interest to undertake such studies in vivo to fully identify the pathogenic potential of the *F. nucleatum* OMV. Also,

the TEM analysis revealed the presence of single and bilayer structures in the OMVs it would be interesting to separate these variants and examine how the proteins composition varies in each type and evaluate how each OMV type interacts with host cells. Also, if would be worthwhile to examine various OMV-associated proteins that provoke pro-inflammatory response given that the majority of OMV-induced IL-8 occurs in an LPS independent manner.

A comprehensive assessment of substrates capable of being degraded by *F. nucleatum* and OMV-associated proteins would help identify the substrate specificity of these enzymes and help identify additional means by which the pathogens can adversely affect the host during infection. For example, components of tight junctions and adherence junctions should be tested including α-catenin, occludin, claudin, and ZO proteins. The induction of the EMT is due to the presence of proteinases or to the presence of another factor such as FadA (anti-FadA antibody could be used to block FadA receptor to exclude the effect of FadA in the initiation of EMT).

Up to this moment, the genetic trigger for OMV secretion has not yet discovered. Such mechanisms could be general for most bacterial species. Possibly the finding of the means that prevent bacterial OMV production might give a clue to how important OMVs are in bacterial survival and pathogenesis of in mediating inflammation and initiation of EMT.

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