Regulation of Enteroendocrine Networks and Mediation of Gut-Brain Communication by *Bacteroides thetaiotaomicron*

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Declaration

I certify that the work contained in this thesis submitted by me for the degree of Doctor or Philosophy is my original work except where due reference is made to other authors and has not been previously submitted by me for a degree at this or any other university.

Part of the work in this thesis has previously appeared in the publication below (Appendix A):

Modasia A, et al., Regulation of Enteroendocrine Cell Networks by the Major Human Gut Symbiont Bacteroides thetaiotaomicron, Frontiers in Microbiology (2020).

In line with the regulations for the degree of Doctor or Physiology I have submitted a thesis that has a word count, including footnotes and bibliography, but excluding appendices of 91 690 words.

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Lastly, I would like to dedicate this Thesis to my father, for it was he who encouraged and facilitated my education and early scientific career. I am forever grateful for all the sacrifices you made, and I only hope that you would be proud of what I have achieved.

Preface

Studying Biomedicine as a undergraduate at the University of East Anglia (UEA) back in 2012 was the first step towards me ultimately undertaking my PhD. During my undergraduate studies, I carried out a research project within the Watson Lab group at the Institute of Food Research, where I focused on inflammation associated with inflammatory bowel diseases. This was driven by my curisosity of disease pathogenesis, and held personal relevance as my father was at the time undergoing numerous surgeries for Ulcerative colitis. Having breifly experienced laboratory-based research for the first time, my interest and commitment to persue a career in scientific research led me to undertake a research based Masters at UEA. Here, I carried out a 12-month research project focusing on chronic and acute intestinal inflammation and started to develop the critical and problem-solving skills needed as a research scientist. Knowing that I wanted to develop further as a research scientist, I decided to undertake a PhD. My project allowed me to continue my research into gut health, combining microbiological assoications and more in-detailed cellular responses. Losing my father has made me, more so than ever, committed and motivated to continue my career as a scientific researcher, helping to further our understanding of human disease pathogensis, prevention and treatments.

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List of non-standard abbreviations

AcH	Acetylation of histone
ACHT	Adrenocorticotrophic hormone
AD	Alzheimer's disease
AHR	Aryl hydrocarbon receptor
AMP	Antimicrobial peptide
AMPK	AMP-activated protein kinase
ANS	Autonomic nervous system
APOE	Apolipoprotein E
APP	Amyloid precursor protein
APS	Acetate succinate and propionate
ASD	Autism Spectrum Disorder
ASO	Alpha-synuclein overexpressing
Aβ	Amyloid-beta
BBB	Blood brain barrier
BEV	Bacterial extracellular vesicle
BD	Bipolar disorder
BDNF	Brain derived neurotrophic factor
BDI	Beck depression inventory
BHI	Brain heart infusion
bHLH	Basic helix-loop-helix
BMEC	Brain microvascular endothelial cell
BMP	Bone morphogenetic protein
Bt	*Bacteroides thetaiotaomicron*
BV-2	Mouse microglia cell line
CA	Cortical area
Caco-2	Human epithelial cells derived from colorectal adenocarcinoma
CCK	Cholecystokinin
CD45	Lymphocyte common antigen
ChrA	Chromogranin A
CFU	Colony forming unit
CMS	Chronic mild stress

CMV	Cytoplasmic membrane vesicles
CNS	Central nervous system
ConA	Concanavalin A
CONV-R	Conventionally raised
CORT	Corticosterone
CP	Caudoputament
CRF	Corticotrophin releasing factor
CRS	Chronic restraint stress
CSF	Cerebrospinal fluid
CXCL2	Chemokine ligand 2
DA	Dopamine
DGC	Dentate granule cell
DC	Dendritic cell
DiD	1,1’-dioctadecyl-3,3,3’,3’-tetramethylindodicarbocyanine
DiO	3,3’-dioctadecyloxacarbocyanine perchlorate
DMM	Dirichlet multinomial mixtures
DOPAC	3,4-dihydroxyphenylacetic acid
DSS	Dextran sulphate sodium
DPPIV	Dipeptidyl peptidase 1
eGPF	Enhanced green fluorescent protein
EBST	Elevated body swing test
EC	Enterochromaffin cell
ECM	Extracellular matrix
EEC	Enteroendocrine cell
EH	Enterohemorrhagic
ENS	Enteric nervous system
ERK	Extracellular-signal regulated kinase
FACs	Fluorescent activated cell sorting
FFAR	Free fatty acid receptor
FFPE	Formalin fixed paraffin embedded
FMT	Faecal microbiota transplantation
FOS	Fructo-oligosaccharide
FOV	Field of view
GABA	Gamma aminobutyric acid

GF	Germfree
GFAP	Glial fibrillary acidic protein
GFBt	Germfree conventionalised with Bt
GFLr	Germfree conventionalised with Lr
GFP	Green fluorescent protein
GIP	Glucose-dependent insulinotropic polypeptide
GIT	Gastrointestinal tract
GIP-R	Glucose-dependent insulinotropic polypeptide receptor
GLP-1	Glucagon-like peptide 1
GLP-2	Glucagon-like peptide 2
GLP-1R	Glucagon-like peptide 1 receptor
GPCR	G-protein coupled receptor
GOS	Galacto-oligosaccharide
GSK-3β	Glycogen synthase kinase-3β
GTA	Glycerol triacetate
HBMEC	Human brain microvascular endothelial cell
hCMEC/D3	Human cerebral microvascular endothelial cell line
HDAC	Histone deacetylase
HMEC-1	Human microvascular endothelial cell line
HO-1	Heme oxygenase-1
HPA	Hypothalamic-pituitary-adrenal axis
HUVEC	Human umbilical vein endothelial cell
HVC	Hemi-villus crypt
IAA	Indole-3-acetic acid
IA1d	Indole-3-carboxyaldehyde
Iba-1	Ionised calcium binding adaptor molecule-1
IBS	Irritable bowel syndrome
IDE	Insulin degrading enzyme
IFN-y	Interferon gamma
IL	Interleukin
IPA	Indole 3 propionic acid
IPAN	Intrinsic primary afferent neurone
IR	Insulin receptor
IRS-1	Insulin receptor substrate 1

ISF	Interstitial fluid
JAKA	Junctional adhesion molecule A
JNK	c-Jun N-terminal kinase
LPS	Lipopolysaccharide
Lr	*Lactobacillus reuteri*
LRP-1	Low density lipoprotein receptor-related protein-1
LTP	Long-term potentiation
MAMP	Microbe-associated molecular pattern
MAPK	Mitogen-activated protein kinase
MCT	Monocarboxylate transporter
MCP-1	Monocyte chemoattractant protein-1
MDD	Major depressive disorder
MDS-UPDRS	Movement Disorder Society-Sponsored Revision of the Unified Parkinson's Disease Rating Scale
MIMP	Microintegral membrane protein
MOS	Mannose oligosaccharide
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MS	Maternal separation
NDE	Neprilysin degrading enzyme
ND1	NeuroD1
NE	Norepinephrine
NFkB	Nuclear factor kappa B
NGF	Nerve growth factor
Ngn3	Neurogenin 3
NLRP3	NLR family pyrin domain containing 3
NLR	NOD-like receptor
NMDA	N-methyl-D-aspartate
NMR	Nuclear magnetic resonance
NO	Nitric oxide
NO_2^-	Nitrite
Nrf2	Nuclear factor erythroid-derived 2
NTA	Nanoparticle tracking analysis
NTF3	Neurotrophin 3
NTS	Nucleus of the solitary tract

NVU	Neurovascular unit
OMP	Outer membrane protein
OMV	Outer membrane vesicle
OIMV	Outer inner-membrane vesicle
OLFR78	Olfactory receptor 78
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
PET	Polyethylene terephthalate
PD	Parkinson’s disease
PPARy	Peroxisome proliferator-activated receptor gamma
PRR	Pathogen recognition receptor
PSA	Polysaccharide A
PSD95	Postsynaptic density protein 95
PYY	Peptide YY
PVN	Paraventricular nucleus of the hypothalamus
RAGE	Receptor for advanced glycation end-products
RANTES	Regulated on activation normal T cell expressed and secreted
SCFA	Short chain fatty acid
SH-SY5Y	Human neuroblastoma cell line
SMCT	Sodium-coupled monocarboxylate transporter
SN	Substantia nigra
SOM	Supraoptic nucleus
SPF	Specific pathogen free
SSRI	Selective serotonin reuptake inhibitor
Sst	Somatostatin
TBI	Traumatic brain injury
TEER	Transepithelial electrical resistance
TGF-b	Transforming growth factor-beta
TH	Tyrosine hydrolase
Tph	Tryptophan hydrolase
TLR	Toll-like receptor
TNF-α	Tumour necrosis factor-α
Treg	T regulatory

TREM119	Transmembrane protein 119
TSMS	Tube shaped membraneous structure
T2D	Type 2 diabetes
ZO	Zonula occludin
5-HT	Serotonin
5-HIAA	5-hydroxyindoleacetic acid
5-HT1A	Serotonin 1A receptor
6-OHDA	6-hydroxydopamine

Abstract

Recent research has shed light onto the bi-directional relationship between the gastrointestinal microbiome and the brain, termed the gut-microbiota-brain axis, implicated in many metabolic, psychiatric, and neurological disorders. *Bacteroides thetaiotaomicron* (Bt) is one of the most abundant symbiont species of the human gastrointestinal tract (GIT) and has important roles in maintaining host homeostasis. Like other fermentative members of the gut microbiota, Bt produces an array of short-chain and organic acids, that in addition to serving as energy sources, are important signalling molecules in host communication pathways including the neuroendocrine system, through recognition by G-protein coupled receptors (GPCRs) present on host cells in the GIT. Furthermore, Bt along with other gram-negative and gram-positive bacteria, produces a diverse array of membrane vesicles, termed bacterial extracellular vesicles (BEVs) that are increasingly being recognised as long-distance mediators in communication between the gut microbiota and host tissues.

To gain further insight into the role of Bt and its mediators in communication between the host gut and brain, a multifaceted approach utilising *in vivo* animal models and *in vitro* cell culture-based systems was carried out. The results presented in this Thesis demonstrate the ability of Bt to singularly regulate intestinal enteroendocrine cell (EEC) networks *in vivo* through the production of its major fermentation products, acetate, propionate, and succinate (APS), with these findings also recently published (Modasia A *et al*., 2020). In addition to microbial fermentation products, nano-sized BEVs have begun to recieve growing interest in contributing to host physiology, including immune, neurological and metabollic functions. Using BEVs isolated from Bt cultures (Bt-BEVs) and *in vitro* cell culture systems modelling the gut-brain axis, demonstrate the ability of Bt-BEVs to cross gut epithelial and brain endothelial cell barriers. Following translocation across these cellular barriers, Bt-BEVs were shown to be acquired by central nervous system (CNS) microglia and neurones and then sequestered to the intracellular lysosomal pathway. These experiments highlight the potential of Bt to indirectly modulate host gut-brain pathways through production of metabolites and BEVs.

1. Introduction

The past couple of decades has seen the newfound appreciation of the gut microbiota as key regulators of gut-brain function and has gained some insights into the significance of these interactions in a biological and physiological setting (Dinan and Cryan 2017). The gut microbiome comprises the collective genome of trillions of microorganisms that reside in the gastrointestinal tract (GIT). Microbiota, a dominant prokaryote constituent of the gut microbiome profoundly influences many aspects of host physiology, including nutrient metabolism, development and maintenance of the immune system and neural networks as well as behavioural and cognitive function. Owing to its diverse role in host physiology, microbial dysbiosis plays an important role in manifestation of many gastrointestinal and non-gastrointestinal related diseases. Microbial signalling to the host often involves several microbial mediators, including their fermentation products, neuroactive metabolites, and membrane-derived vesicles. These can interact with the host via the enteroendocrine and immune system, neural pathways involving the enteric nervous system (ENS) and autonomic nervous system (ANS), or by crossing the intestinal epithelial barrier and entering the systemic circulation. Despite progress in this field, the mechanisms underlying microbial communication to the host via these pathways, and in particular the magnitude of influence on individual's health remain to be fully understood.

In this Chapter, the major gut-brain communication pathways and mediators involved in microbial signalling across the host gut-brain communication pathways and their implication in central nervous system (CNS) disorders, are explored.

1.1. The intestinal microbiota

The human microbiome is a complex community of microorganisms that co-inhabit the host in a body region specific manner, consisting of a diverse range of prokaryotes, archaea, eukaryotes, viruses, protozoa and their collective genomes (Turnbaugh, Ley et al. 2007, Shreiner, Kao et al. 2015). The microbiome generates a complex and adaptive ecosystem, finely attuned to the constantly changing nature of host physiology (Lloyd-Price, Abu-Ali et al. 2016).

The gastrointestinal microbiome forms the most intensively studied ecosystem, containing the largest microbial biomass than any other host habitat site of over 100 trillion different microorganisms (Sender, Fuchs et al. 2016). The most well-studied microbial constituent of the gastrointestinal microbiome are bacteria (2012, Lloyd-Price, Mahurkar et al. 2017), partly because of abundance, molecular profiling and characterisation techniques are less developed, lack of universally conserved genes/genomes and difficulties in isolation methods for other members of the microbiome (Norman, Handley et al. 2014). The microbiota form an integral prokaryote component of the host intestinal microbiome, assisting with physiological functions including the development and function of intestinal barrier (Backhed, Ley et al. 2005), immune system (Kau, Ahern et al. 2011, Shi, Li et al. 2017) and the CNS (Diaz Heijtz, Wang et al. 2011) which also includes the development of the blood brain barrier (BBB) (Engelhardt 2003). A breakdown of microbial diversity and composition are believed to be key drivers in many gastrointestinal, metabolic, immunological, and neurological disorders (Gomaa 2020). The GIT consists of bacteria mainly belonging to the phyla Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, Verrucomicrobia, Fusobacteria and Tenericutes (Gomaa 2020). Whilst the initial colonisation and shaping of diversity occurs at the early stages of life, alterations in diversity and taxonomic composition during development and adulthood depend mainly on nutrition, environmental and genetic factors.

1.2. Prokaryote colonisation of the infant microbiome

The *in utero* environment was previously been believed to be sterile (Rodríguez, Murphy et al. 2015) but this has been recently challenged by evidence of bacterial presence in the placenta, umbilical cord and amniotic fluid in healthy full-term pregnancies (DiGiulio, Romero et al. 2008, Jimenez, Marin et al. 2008, Aagaard, Ma et al. 2014). The main drivers of microbial colonisation of the infant intestine post-birth are mode of delivery and nutrition (Fig. 1.1). Infants born vaginally encounter the vaginal and faecal microbiome, and results in colonisation of the infant intestinal microbiota dominated by maternal-derived genera *Lactobacillus* and *Prevotella* (Biasucci, Rubini et al. 2010, Dominguez-Bello, Costello et al. 2010). Caesarean section (c-section) delivered infants are not exposed to the maternal vaginal or faecal microbiome but instead are exposed to environmental bacteria such as those from

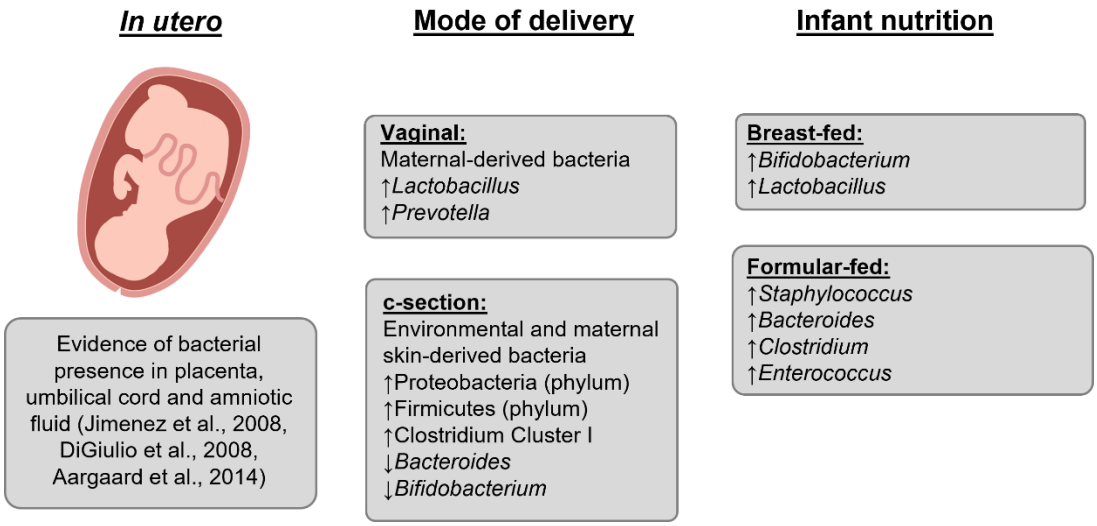

Figure 1.1 Prenatal and early environmental factors that influence prokaryote colonisation of the infant microbiome. Recent evidence suggests that colonisation of the infant GIT begins *in utero*. During post-partum development the infant microbiome continues to be influenced by environmental and host factors, including mode of delivery and infant nutrition. Arrows indicate relative abundance of bacterial genera reported in the infant gut.

maternal skin or hospital environments (Biasucci, Rubini et al. 2010, Bäckhed, Roswall et al. 2015, Rodríguez, Murphy et al. 2015, Bokulich, Chung et al. 2016). Therefore, c-section delivered infants have intestinal microbiota dominated by bacteria from the phyla Proteobacteria and Firmicutes the first few days following birth and tend to display an overall reduced microbial diversity (Del Chierico, Vernocchi et al. 2015). Specifically, lower abundances of genera *Bifidobacterium* and *Bacteroides*, and a higher abundances of *Clostridium* Cluster 1 and *Clostridium difficile* (*C. difficile*) have been reported in c-section delivered infants (Biasucci, Rubini et al. 2010, Dominguez-Bello, Costello et al. 2010, Del Chierico, Vernocchi et al. 2015, Hill, Lynch et al. 2017).

During development however, the differences between vaginal and c-section delivered infants slowly decrease (Bäckhed, Roswall et al. 2015, Martin, Makino et al. 2016), but some distinct differences do remain. Vaginally-delivered infants harbour significantly higher abundances of bacteria from the class Clostridia (Salminen, Gibson et al. 2004). There are indications of c-section delivered infants being prone to health implications later on in life, with increased risk of developing immune disorders including asthma (Thavagnanam, Fleming et al. 2008), allergy (Bager, Wohlfahrt et al. 2008), type 1 diabetes (Cardwell, Stene et al. 2008) and obesity (Pei, Heinrich et al. 2014). These findings suggest that natural vaginal birth has long-term protective effects through the transfer of microbiota from the mother, although why this is, is not fully understood.

As well as being influenced by the birthing method, the infant gut microbiota is also influenced by infant nutrition. Breastmilk contains a mix of nutrients, oligosaccharides and pro-microbial and anti-microbial agents that promote the growth and function of beneficial bacteria (Praveen, Jordan et al. 2015). This results in infants having higher abundances of *Bifidobacterium* and *Lactobacillus*, whereas, formula-fed infants have a higher microbial diversity dominated by *Staphylococcus*, *Bacteroides*, *Clostridium* and *Enterococcus* (Harmsen, Wildeboer-Veloo et al. 2000, Penders, Thijs et al. 2006, Bezirtzoglou, Tsiotsias et al. 2011, Guaraldi and Salvatori 2012, Martin, Makino et al. 2016). As a result of these differences in microbial composition, levels of microbial fermentation products are also different in faecal samples from breast-fed and formula-

fed infants (Le Huërou-Luron, Blat et al. 2010). During post-partum development, antibiotic use can cause disturbances in gut microbial composition and can have long lasting effects on the microbial composition (Mathew 2004, Jernberg, Löfmark et al. 2010). Exactly how antibiotics, nutrition, and early colonisation of the gut microbiota influence long term host health and immunity, requires further investigations.

1.3. Classification of the intestinal microbiota

Due to the intimate complexity of the human intestinal microbiome, methods to stratify the microbiota have been developed providing useful insights into analysing some of the gut microbiome datasets, facilitating their structural understanding and diagnostic value. In 2011, Arumugam and colleagues carried out analysis of faecal metagenomic samples across Europe, North America, and Asia. They proposed the existence of three defined microbial clusters driven by the indicator *taxon* (the dominant genus), identified as Enterotypes that were independent of host age, gender, cultural background and geographics (Arumugam, Raes et al. 2011).

Using this classification method, Enterotypes with *Prevotella* (Enterotype P) as the indicator were reported to be enriched in individuals with non-Western and fibre-rich diets (Yatsunenko, Rey et al. 2012, Ou, Carbonero et al. 2013, Smith, Yatsunenko et al. 2013). *Bacteroides*-rich Enterotypes (Enterotype B) on the other hand, are associated with animal protein and saturated fat rich diets (Wu, Chen et al. 2011, David, Maurice et al. 2014) and a lower overall diversity (Costea, Hildebrand et al. 2018). Enterotype F is associated with an over-representation of Firmicutes, with *Ruminococcus* as the indicator taxon (Arumugam, Raes et al. 2011, Costea, Hildebrand et al. 2018).

In healthy adults, the overall composition of the enteric microbiota remains stable over habitual long-term diets, indicating that Enterotypes themselves are stable (Caporaso, Lauber et al. 2011, Wu, Chen et al. 2011). Controlled short-term (10-day) dietary interventions can significantly impact microbial compositions and can even shift Enterotypes, but these have been shown to revert to their original state at the end of short-term dietary intervention (Wu, Chen et al. 2011). In contrast, long-term (1 year) dietary interventions have a more profound effect on the ratio between *Bacteroides*

and *Firmicutes* (Ley, Turnbaugh et al. 2006, Wu, Chen et al. 2011) that could contribute to significant shifts in Enterotypes. However, due a to lack of follow-up studies in long-term interventions and the stability of Enterotypes observed during the intervention, the effect on Enterotype resilience and stability or whether different Enterotypes vary in their recovery following an intervention cannot be fully justified.

Several studies have reported associations between certain Enterotypes and human disease phenotypes. For example, Enterotype B is associated with colorectal cancer (Sobhani, Tap et al. 2011, Ou, Carbonero et al. 2013, Zeller, Tap et al. 2014), celiac disease (De Palma, Nadal et al. 2010) and chronic inflammation (Claesson, Jeffery et al. 2012, Le Chatelier, Nielsen et al. 2013). Long-term antibiotic use (Jernberg, Löfmark et al. 2010), rheumatoid arthritis (Scher, Sczesnak et al. 2013) and type 2 diabetes (T2D) (Larsen, Vogensen et al. 2010) have associations with Enterotype P. Enterotype F on the other hand, is linked to a high microbial diversity and is associated with anti-inflammatory, health promoting statuses (Karlsson, Fåk et al. 2012). Due to the complexities and magnitude of factors that are associated with disease phenotypes, Enterotype classification alone may not accurately determine disease progression or serve as a disease biomarker but may help to identify an individual's increased risk to developing diseases (Knights, Ward et al. 2014, Costea, Hildebrand et al. 2018).

Since the publication of clustering-methods and identification of Enterotypes, there has been much discussion on whether this broad community-wide stratification captures the complexity of the human gut microbiome. Further studies carried out have supported the genus level Enterotype stratification method published by Arumugam et al., (Dethlefsen, Eckburg et al. 2006). A caveat to this method is that it doesn't consider strain and species level variation within and between individuals that can contribute to functional differences (Schloissnig, Arumugam et al. 2013, Zhu, Sunagawa et al. 2015). A study on the enteric microbiome of infants revealed that Enterotype-like composition occurs between 9 and 36 months (Yatsunenko, Rey et al. 2012). Therefore, stratification of the microbial population across different ages needs to be addressed with caution (Costea, Hildebrand et al. 2018).

Other approaches have been put forward to identify structures in the human enteric microbiome. For example, Holmes et al., developed a generative model, Dirichlet

multinominal mixture model (DMMs), to identify four groups from the original data used by Arumugam et al. Two of the groups identified by Holmes et al., were similar to Enterotype B and P. The third group identified was dominated by the genus *Ruminococcus* and other members of the Firmicutes phylum, while the fourth group had high abundances of unidentified taxa (Holmes, Harris et al. 2012). Similar findings were also reported in a later study also using DMM (Ding and Schloss 2014) and appears to be a more robust method for identifying clusters within the microbiota (Costea, Hildebrand et al. 2018). The number of different Enterotypes and their statistical power varies depending on the approach used to identify and characterise the major microbial patterns in the human gut microbiota. This can be improved by standardisation in sample processing and data analysis and using larger cohort samples, including populations from developing and lesser-developed parts of the world.

1.4. Regional variations in prokaryotic composition

The composition of the gut microbiota also changes along the length of the intestinal tract (Fig. 1.2). Oxygen, bile acid and digestive enzyme levels in the small intestine limits' bacterial density. The duodenum contains the lowest abundance of bacteria, around 10^{3-4} CFU/ml, of which Firmicutes and Proteobacteria are the most predominant phyla (Sartor 2008, El Aidy, van den Bogert et al. 2015). As oxygen levels decrease in the jejunum, the density increases to around 10^{3-7} CFU/ml, preferentially supporting the growth of gram-negative bacteria, including those from the genera *Lactobacillus*, *Enterococcus* and *Streptococcus* (El Aidy, van den Bogert et al. 2015). The pH and luminal contents of dietary fibres increase, and oxygen levels decrease along the ileum, supporting the growth of anaerobic bacterial species, such as those from the genera *Bacteroides*, and *Clostridium*. Here, bacterial density increases to around 10^{9} CFU/ml (Gorbach, Plaut et al. 1967). Bacterial density is the highest in the colon, reaching 10^{11-12} CFU/ml, and is dominated by the bacteria from the phyla Firmicutes, Bacteroidetes and Actinobacteria (Eckburg, Bik et al. 2005, Sartor 2008) including genera from *Bacteroides*, *Bifidobacterium*, *Streptococcus*, *Lactobacillus*, *Clostridium* and *Enterococcus*, which are predominantly anaerobic microbes (Hollister, Gao et al. 2014).

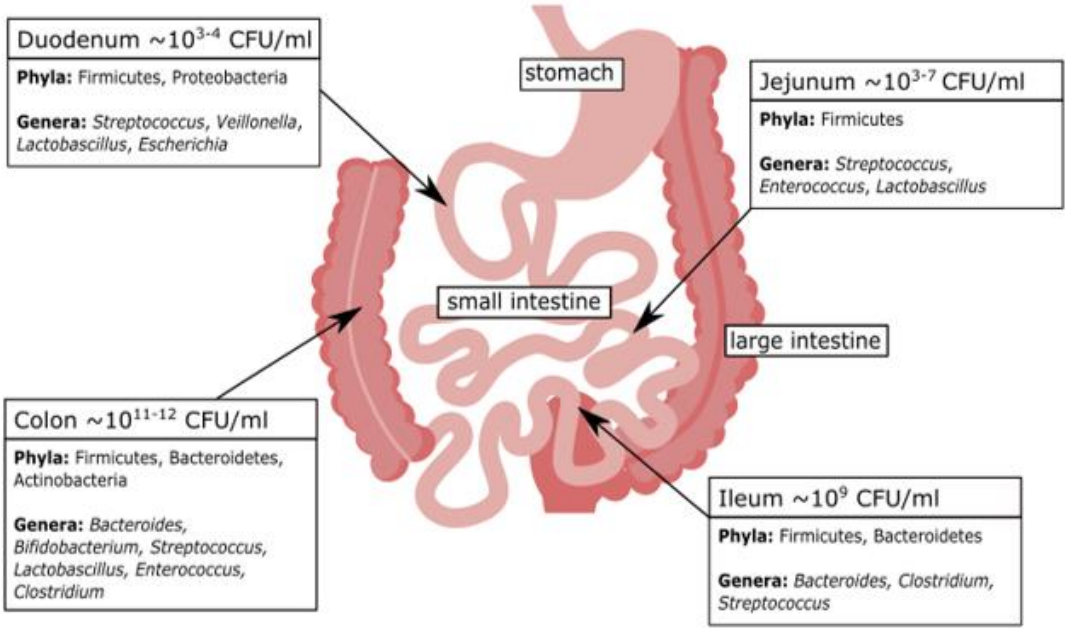

Figure 1.2 Regional variations in luminal microbial composition along the small intestine and colon. Composition and luminal concentrations of dominant microbial phyla and genera along the small and large intestine. Microbial density is lowest in the duodenum but increases further along the small intestine. Microbial density is highest in the colon, with low oxygen levels supporting the growth of anaerobic bacteria. Adapted from Sartor R (2008).

In addition to differences amongst microbial genera, there is also species level differences along the GIT. For example, species from the *Bacteroides* genus have a large metbollic genome that allows them to occupy a range of metabollic niches. B. *vulgatus*, *B. thetaiotaomicron*, *B. uniformis* and *B. caccae* are reported be more abundant in the distal region of the ileum, wherease *B. faecis* has a higher relative abundance in the colon (Donaldson, Lee et al. 2016).

There are also differences along the villus-crypt axis, between the lumen, mucus layer and intestinal epithelium (Fig. 1.3) (Li, Limenitakis et al. 2015). In the small intestine, the structure of the outer mucus layer allows the entry of some molecules and bacteria including segmented filamentous bacteria (*Helicobacter* spp.) (Ermund, Schütte et al. 2013, Donaldson, Lee et al. 2016). The high concentration of antimicrobial peptides (AMPs) close to the epithelium in the inner mucus layer, limits the presence of bacteria (Fig. 1.3A) (Johansson and Hansson 2011, Vaishnava, Yamamoto et al. 2011). Here, *Clostridium*, *Lactobacillus*, *Akkermansia* and *Enterococcus* are the most predominant mucosa and mucus associated genera (Swidsinski, Weber et al. 2005). In the colon, the outer mucus layer is dominated by mucin-degrading bacteria such as B. fragilis, A. muciniphila and B. acidifaciens. The inner mucus layer prevents direct contact of bacteria (>200 μm in humans and >50 μm in mice) with host cells and tissues and therefore habours a lower density of bacteria rescricted to species such as *B. fragilis* and *Acinetobacter spp.* (Fig. 1.3B) (Johansson, Phillipson et al. 2008, Johansson, Gustafsson et al. 2014, Donaldson, Lee et al. 2016). The composition of the gut microbiota can influence mucosal barrier properties and disruptions to this fine balance can lead to inflammation (Johansson, Gustafsson et al. 2010, Fu, Wei et al. 2011, Jakobsson, Rodríguez-Piñeiro et al. 2015). In general terms, a higher microbial diversity is associated with a well-developed, non-permeable mucus mucus layer, with increased abundances of species from the genera *Bacteroides* and *Prevotella* (Jakobsson, Rodríguez-Piñeiro et al. 2015). Whereas higher abundance in Proteobacteria is associated with a more permeable colonic mucus layer (Jakobsson, Rodríguez-Piñeiro et al. 2015) and Crohn's disease.

1.5. Microbial mediators in host signalling

The gut microbiota metabolises a range of host dietary components into an array of metabolites, including short-chain fatty acids (SCFAs), vitamins, amino acid derivatives and tryptophan metabolites that serve as mediators in signalling to modulate host metabolic, immune and neurological functions (Wikoff, Anfora et al. 2009, Tolhurst, Heffron et al. 2012, Zeng, Umar et al. 2019). Certain gut microbes can also independently or contribute to the production of several neuroactive molecules including γ-aminobutyric acid (GABA) (Barrett, Ross et al. 2012), serotonin (5-HT) (Shishov, Kirovskaia et al. 2009, Yano, Yu et al. 2015), norepinephrine (NE) and dopamine (DA) (Shishov, Kirovskaia et al. 2009, Asano, Hiramoto et al. 2012). Recent studies have also shed light onto the emerging role of bacterial extracellular vesicles (BEVs) to influence host cellular functions, believed to play an integral part of

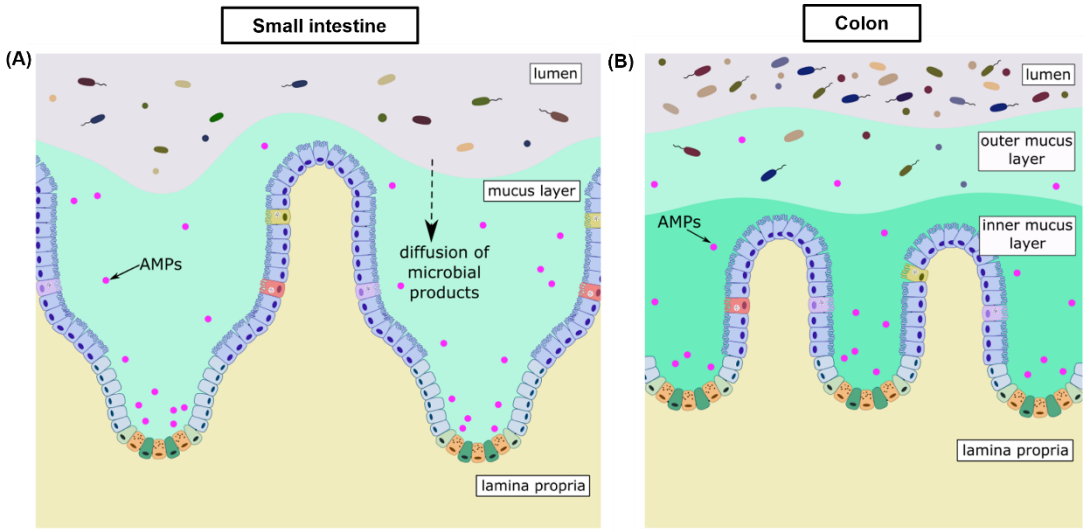

Figure 1.2 Spatial variations in microbial colonisation in the small intestine and colon. Schematic illustrating the differences in the structure of the mucus layer between the (A) small intestine and (B) colon. The structure of the mucus layer in the small intestine prevents entry of luminal bacteria but allows diffusion of microbial products such as metabolites. In the colon, the mucus layer is composed of the inner and outer layer, with the outer layer being more permeable and allowing entry of some microbes and their products, and the inner layer being impermeable to bacteria, but allows some diffusion of microbial products.

microbiota-host communications. These microbial derived metabolites, molecules and membrane vesicles have been shown to serve as mediators in signalling to influence host physiology.

1.5.1. Short chain fatty acids

SCFAs are monocarboxylic acid products of microbial fermentation of indigestible polysaccharides such as dietary plant-derived fibre and resistant starch implicated as signalling molecules in microbe-host communication (Bergman 1990, Flint, Scott et al. 2012). The concentration of SCFAs along the GIT varies in parallel with bacterial populations, with the highest SCFA concentrations found in the caecum and colon (~100 mM) where bacterial density is the highest (Cummings, Pomare et al. 1987), compared to portal blood concentrations of ~400 μM and peripheral blood concentrations ~100 μM (Cummings, Pomare et al. 1987). Other factors including type of dietary fibre consumed by the host and intestinal transit time, also influence levels of SCFAs in the GIT (Wong, de Souza et al. 2006). SCFAs can also be produced from amino acid metabolism by members of the intestinal microbiota, but this only accounts for 1% of the total SCFAs produced (Smith and Macfarlane 1998, Louis, Scott et al. 2007).

In the human colon, absorption of SCFAs is rapid, with estimated absorption rates of 5.2, 1.8 and 1.9 mumol/cm^2/h for acetate, propionate and butyrate, respectively (McNeil, Cummings et al. 1978). Approximately 75% of the total SCFA produced in the GIT being metabolised by intestinal epithelial cells, contributing to approximately 10% of the host caloric requirement (McNeil 1984, Bergman 1990). This, combined with difficulty in obtaining luminal samples from the GIT in humans means that *in vivo* luminal SCFAs concentrations are often estimated (Cummings, Pomare et al. 1987). A non-invasive method of determining SCFA concentrations in the human intestine is by using isotopically labelled carbohydrates. Once ingested, blood or expired air levels can be analysed for production of metabolites (Kien, Kepner et al. 1992, Boets, Deroover et al. 2015, Boets, Gomand et al. 2017). A disadvantage of analysing SCFAs from expired air is that it only gives a global indication of microbial fermentation. Other approaches for analysing SCFAs directly are being developed, such as the Intellicap system (Maurer, Schellekens et al. 2015). SCFAs have also been

detected in the CNS, and whilst obtaining physiological brain levels of SCFAs is difficult, they can be detected in the cerebral spinal fluid where acetate, propionate and butyrate can reach concentrations up to 171 μM, 6 μM and 2.8 μM, respectively (Silva, Bernardi et al. 2020).

The three main SCFAs, acetate, propionate, and butyrate, differ considerably in their effects on host cells and mediate their effects on host cells expressing G-protein coupled receptors (GPCRs). Acetate, the most abundant SCFA in the peripheral circulation, can be produced from pyruvate by the majority of gut bacteria (Louis, Hold et al. 2014). Much of propionate produced in the gut is taken up by the liver where it contributes to gluconeogenesis (Morrison and Preston 2016), but propionate has also been shown to regulate appetite through promoting increased secretion of colonic peptide hormones (including glucagon-like peptide 1 (GLP-1) and peptide YY (PYY)) that have roles in energy intake (Tolhurst, Heffron et al. 2012, Chambers, Viardot et al. 2015). Butyrate on the other hand, is a key energy source for colonocytes, taken up via H^+-linked monocarboxylate transporters (MCTs) and sodium-linked MCTs (SMCTs) (Vijay and Morris 2014), and therefore only a small amount of butyrate enters the peripheral circulation with concentrations up to 10 μmol/L being detected (Ktsoyan, Mkrtchyan et al. 2016). Butyrate has also been identified as a key regulator of tight junction proteins, enhancing colonic epithelial barrier integrity through the upregulation of claudin-1 and zonula occludens-1 (ZO-1) via histone deacetylase (HDAC) inhibition (Wang, Wang et al. 2012).

Unlike acetate, butyrate and propionate production by the intestinal microbiota seems to be highly conserved and substrate specific (Reichardt, Duncan et al. 2014). *Bacteroides thetaiotaomicron* (Bt) has a genome encoding over 26 hydrolases (Cantarel, Lombard et al. 2012) and are one of the major producers of acetate, propionate and succinate (Wrzosek, Miquel et al. 2013, Curtis, Hu et al. 2014, Modasia, Parker et al. 2020). Bacteria from the Firmicutes phylum, including *Clostridium butyricum* (*C. butyricum*) and *Faecalibacterium prausnitzii* (*F. prausnitzii*), are major butyrate producers (Macfarlane and Macfarlane 2003, Louis, Young et al. 2010). Some bacterial species including those from the genera *Anaenostripes* and *Eubacterium*, can also metabolise lactate and acetate to produce butyrate (Duncan, Barcenilla et al. 2002, Louis, Duncan et al. 2004). The production

of butyrate from lactate is important as it prevents the build-up of lactate, thereby stabilising the intestinal environment (Vital, Howe et al. 2014). There are also some bacterial species, such as *Akkermansia muciniphila* (*A. muciniphila*), that can utilise host intestinal mucus to generate SCFAs and reside within the intestinal mucus layer (Derrien, Vaughan et al. 2004). Co-culture experiments of *A. muciniphila* with non-mucus degrading butyrate-producing bacteria, such as *Eubacterium hallii* (*E. hallii*) haven shown that vitamin B12 production by *E. hallii* results in the production of propionate by *A. muciniphila* (Belzer, Chia et al. 2017). Tropic interactions between species have also been demonstrated in another co-culture experiment, where the production of SCFAs by *E. hallii* was shown to be altered by co-culturing with *Bifidobacterium bifidum* (*B. bifidum*) (Bunesova, Lacroix et al. 2018). These studies demonstrate the importance of syntropy between the members of the intestinal microbiota.

Several GPCRs are receptors for SCFAs and are expressed on intestinal epithelial cells (Kimura, Inoue et al. 2011). Acetate is approximately 100-fold less potent than propionate and butyrate for GPR41 (also known as free-fatty acid receptor 3 (FFAR3)), whereas all three SCFAs have a similar potency on GPR43 (FFAR2) (Brown, Goldsworthy et al. 2003, Le Poul, Loison et al. 2003). In addition, butyrate has been shown to directly stimulate vagal afferent neurones via FFAR3 (Lal, Kirkup et al. 2001) and propionate activates FFAR2/3 expressed on sympathetic neurones to stimulate NE secretion (Kimura, Inoue et al. 2011), indicating a role for SCFAs in neuroendocrine signalling. Other SCFAs receptors, including GPR109A are specifically activated by β-hydroxybutyrate and butyrate (Ahmed, Tunaru et al. 2009) and are expressed on colonic epithelial cells (Cresci, Thangaraju et al. 2010). Olfactory receptor 78 (OLFR78) are activated by acetate and propionate, but not butyrate, and are expressed in blood vessels, particularly renal vessels where it is involved in renin secretion (Pluznick, Protzko et al. 2013, Pluznick 2014).

Due to their ability of influence host physiological functions through multiple GPCRs expressed within the GIT and in peripheral tissues, it is important to understand their role under physiological conditions as microbial dysbiosis is likely to have significant impacts on microbial metabolite production and utilisation, impacting the bidirectional signalling pathways between the microbiota and host.

1.5.2. Bacterial extracellular vesicles

While previously believed to be merely a method for cellular waste disposal, BEVs produced by both gram-negative and gram-positive bacteria, are increasingly being recognised as important intercellular communication mediators in bacteria-host signalling (Deatherage and Cookson 2012, Toyofuku, Nomura et al. 2019, Haas-Neill and Forsythe 2020). Ranging from 10-400 nm in diameter, they carry and shelter a diverse array of cargo including peptidoglycans, polysaccharides, proteins, nucleic acids, metabolites, enzymes and toxins, and combined with the expression of many outer membrane proteins (OMPs), enable them to interact with host cells (Kulp and Kuehn 2010).

Due to the differences in structure and composition of the membranes in gram-negative and gram-positive bacteria, the formation routes and vesicles produced differ. Gram-negative bacterial membranes consist of an inner and outer lipid membrane, separated by the periplasmic space and peptidoglycan layer. All gram-negative bacteria produce outer membrane vesicles (OMVs) formed from the budding and pinching off the outer membrane (Beveridge 1999, Guerrero-Mandujano, Hernández-Cortez et al. 2017). They are enriched for OMPs and lipopolysaccharide (LPS) that are also found on the parent bacterium (Fig. 1.4) (Kesty, Mason et al. 2004, Bryant, Stentz et al. 2017, Haas-Neill and Forsythe 2020). The presence of cytoplasmic contents in OMVs have baffled scientists over the years, as none of the models of outer membrane blebbing could explain the presence of plasmid DNA and other cytoplasmic components in gram-negative OMVs. A new model of membrane vesicle formation was proposed, involving weakening of the peptidoglycan layer in the bacterium by autolysins causing the inner membrane to protrude out into the periplasm, thereby allowing cytoplasmic contents of the bacterium to be packed into double bilayer vesicles, termed outer-inner membrane vesicles (OIMVs) (Pérez-Cruz, Carrión et al. 2013, Toyofuku, Nomura et al. 2019). The identification of both OMV and OIMVs from *Shewanella vesiculosa* culture, indicate that bacteria can produce multiple types of BEVs (Pérez-Cruz, Carrión et al. 2013, Pérez-Cruz, Delgado et al. 2015). The presence of plasmid DNA fragments also raises another question in relation to the "health" of the parent bacterium, which could be considered suggestive of cell lysis (Zhou, Srisatjaluk et al. 1998, Renelli, Matias et al. 2004). Another possible route for the formation of BEVs

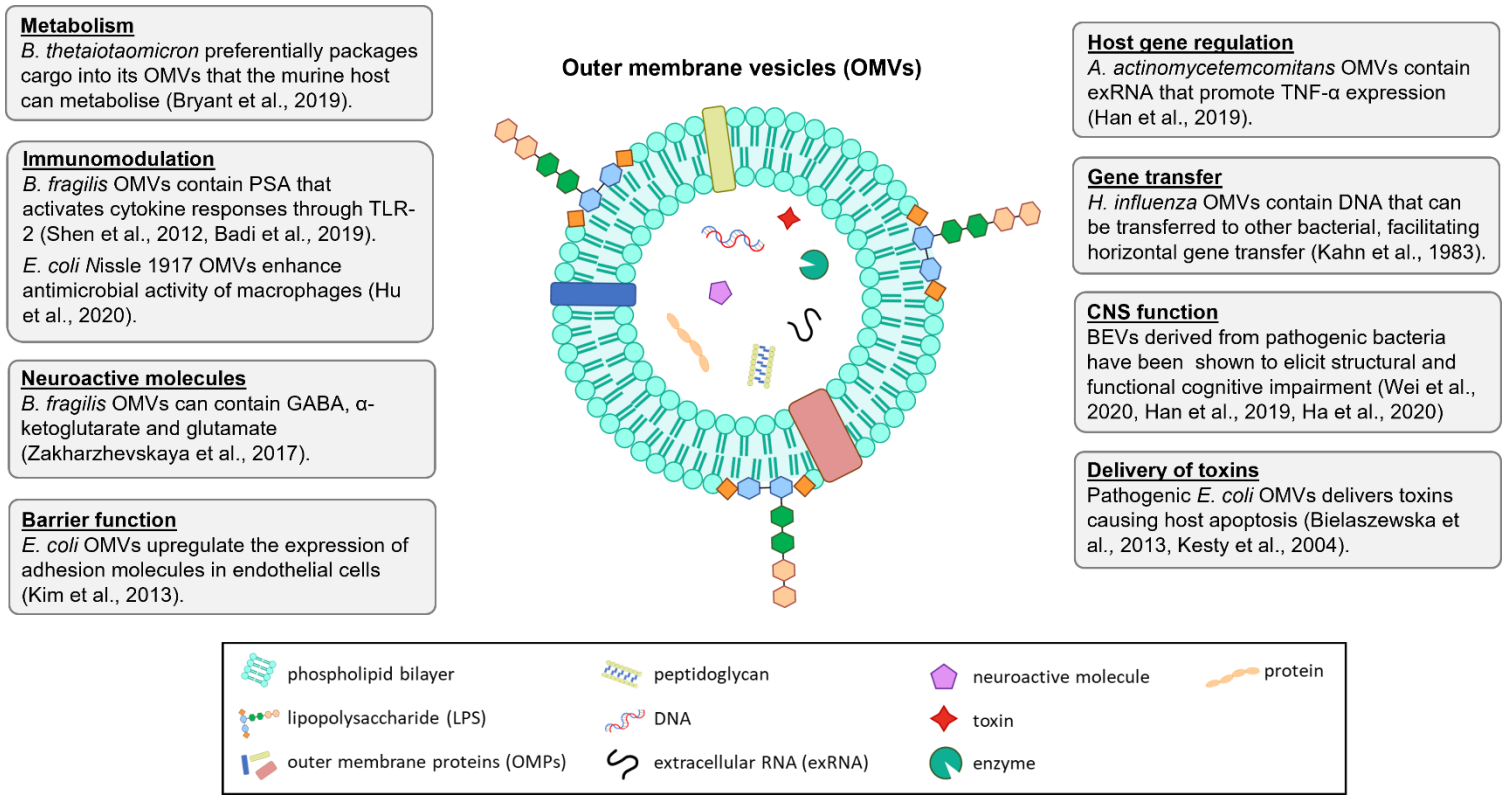

Figure 1.3 Structure and function of bacterial outer membrane vesicles in host gut-brain signalling. OMVs are produced by the budding of the outer membrane of gram-negative bacteria. They are spherical bilayer vesicles, containing various periplasmic and cytoplasmic components found within the parent bacterium. They express many proteins and LPS also expressed on the outer membrane of the parent bacterium. These features enable OMVs to interact with host cells to modulate a range of biological processes such as long-distance delivery of toxins and virulence factors and modulating the immune system, intestinal barrier function and effects within the central nervous system itself.

termed explosive cell lysis, that occurs upon DNA damage resulting in re-circulation of cell envelop fragments that encapsulate the released DNA (Kadurugamuwa and Beveridge 1997, Li, Clarke et al. 1998, Turnbull, Toyofuku et al. 2016).

Less studied are membrane vesicles derived from gram-positive bacteria, including cytoplasmic membrane vesicles (CMVs) and tube-shaped membranous structures (TSMSs) (Remis, Wei et al. 2014, Wei, Vassallo et al. 2014, Toyofuku, Cárcamo-Oyarce et al. 2017), however, their biogenies and function remain to be fully elucidated. Neither the less, their lack of LPS and periplasmic components, mean they can be easily distinguished from gram-negative OMVs (Kim, Lee et al. 2015).

The main biological relevance of BEV production for bacteria is facilitating their own survival and growth through the presence and delivery of virulence factors (OMPs and endotoxins), secretion of adhesins for cellular aggregation (Gui, Dashper et al. 2016) and the encapsulated cargo that can be transported over long distances. Due to their unique features, BEVs possess features that make them suitable for development of vaccine delivery and therapeutics, facilitating the delivery of drugs to target tissues (Arigita, Jiskoot et al. 2004, Stentz, Carvalho et al. 2018, Carvalho, Fonseca et al. 2019, Wang, Gao et al. 2019, Stentz, Miquel-Clopés et al. 2022).

There are also increasing number of studies reporting the ability of BEVs to modulate host immune responses (Shen, Giardino Torchia et al. 2012, Al-Nedawi, Mian et al. 2015, Fábrega, Rodríguez-Nogales et al. 2017, Ahmadi Badi, Khatami et al. 2019), CNS function and gene expression (Kahn, Barany et al. 1983, Zakharzhevskaya, Vanyushkina et al. 2017, Choi, Kim et al. 2019, Han, Choi et al. 2019, Lee 2020), intestinal epithelium integrity (Kim, Yoon et al. 2013) and ENS stimulation (Al-Nedawi, Mian et al. 2015) (Fig. 1.4). Furthermore, the detection of BEVs in the blood circulation (Jang, Kim et al. 2015, Park, Choi et al. 2017, Lee, Choi et al. 2020, Tulkens, Vergauwen et al. 2020, Jones, Stentz et al. 2021) indicate their ability to cross the host intestinal epithelial and reach tissues beyond the GIT (Wispelwey, Hansen et al. 1989, Furuta, Tsuda et al. 2009, Shen, Giardino Torchia et al. 2012, Han, Choi et al. 2019, Ha, Choi et al. 2020, Jones, Booth et al. 2020). In the gut, BEVs utilise several mechanisms and pathways to cross the intestinal epithelium including transcellular and paracellular transport, macropinocytosis, clathrin-mediated endocytosis and caveolin-

mediated endocytosis (O'Donoghue and Krachler 2016, Stentz, Carvalho et al. 2018, Jones, Booth et al. 2020). It is also possible that they are acquired in a size-dependent manner (Amano, Takeuchi et al. 2010, Turner, Bitto et al. 2018) in addition to some studies suggesting the structure of LPS present on the surface affects OMV internalisation (O'Donoghue, Sirisaengtaksin et al. 2017).

Depending on whether BEVs are derived from pathogenic or commensal bacteria, and the cargo they carry influences their effects on host cells. Pathogens can utilise BEVs for the long distance delivery of virulence factors (Bomberger, Maceachran et al. 2009) and toxins (Kesty, Mason et al. 2004, Bielaszewska, Rüter et al. 2013, Elmi, Nasher et al. 2016). Other examples of potentially harmful effects to host physiology include the transfer of plasmids containing antibiotic resistance genes to other gram-negative bacteria by *Neisseria gonorrhoeae* (*N. gonorrhoeae*) OMVs (Dorward, Garon et al. 1989) and chronic gastritis and autoimmune diseases caused by the presence of LPS on the surface of *Helicobacter pylori* (*H. pylori*) OMVs (Hynes, Keenan et al. 2005). Recent evidence also demonstrates that OMVs from pathogenic bacteria can directly or indirectly promote CNS functional and cognitive alterations (Han, Choi et al. 2019, Lee, Kim et al. 2020, Wei, Peng et al. 2020). In comparison to these harmful effects, OMVs derived from commensal and probiotic bacteria have been shown to confer differential effects on host cell viability dependent on strain (Canas, Fabrega et al. 2018, Ling, Dayong et al. 2019). Probiotic OMVs derived from *Escherichia coli* (*E. coli*) Nissle 1917 strain have been shown to enhance immunomodulation and antimicrobial activity of macrophages *in vitro* (Hu, Lin et al. 2020). Moreover, BEVs from probiotic gram-positive *Lactobascillus rhamnosus* (*L. rhamnosus*) *JB-1* have been shown to reproduce the effects of the parent bacterium by indirectly altering the amplitude of nerve-dependent colon migrating motor complexes in an *ex vivo* model of peristalsis (Al-Nedawi, Mian et al. 2015).

In addition to these diverse effects, growth conditions can alter the overall yield as well as the metabolic and proteomic profile of vesicles (Keenan, Davis et al. 2008, Bitto, Zavan et al. 2021). As a result of heterogeneity and high diversity in the formation, protein expression and cargo selection, challenges are faced when making comparisons between studies. Clearer identification and standardisation of protocols can help towards better understanding of these vesicles in host physiology.

1.5.3. Tryptophan metabolites and neurotransmitters

Tryptophan is an essential amino acid and precursor of many microbial and host metabolites (Alkhalaf and Ryan 2015). Certain gut microbes, including *Peptostreptococcus russellii* (Wlodarska, Luo et al. 2017) and species from *Lactobacillus* genera (Zelante, Iannitti et al. 2013, Lamas, Richard et al. 2016) can metabolise intestinal tryptophan, indole and its derivatives, many of which including indole-3-aldehyde (IAld), indole-3-acetic acid (IAA), indole 3 propionic acids (IPA), are ligands for aryl hydrocarbon receptor (AhR) (Hubbard, Murray et al. 2015, Alexeev, Lanis et al. 2018). AhR signalling is critical for maintaining intestinal barrier homeostasis and immune signalling and is associated with cellular responses to environmental stimuli (Lamas, Natividad et al. 2018).

Some gut microbes can also produce a diverse array of neurotransmitters and neural substances such as GABA, 5-HT and catecholamines, relaying signals via intestinal enterochromaffin (EC) cells and enteric neurones. Some species from the *Escherichia*, *Streptococcus* and *Enterococcus* genus can synthesise 5-HT (Tsavkelova, Klimova et al. 2006), whereas *Lactobacillus brevis* (*L. brevis)* and *Bifidobacterium dentium* (*B. dentium*) can produce GABA, a inhibitory neurotransmitter in the CNS (Barrett, Ross et al. 2012). The catecholamines, including NE and DA are both used in the CNS and peripheral nervous system and have roles in regulating host functions such as gut motility, cognition and mood (Eisenhofer, Kopin et al. 2004). The importance of bacterial-derived catecholamines is demonstrated in germfree (GF) models that display significantly lower caecal and luminal catecholamines compared to specific pathogen free (SPF) mice (Sudo, Chida et al. 2004, Asano, Hiramoto et al. 2012). Bacterial β-glucuronidase is important for the generation of biologically active free form of NE and DA (Asano, Hiramoto et al. 2012). Colonisation of GF mice with a mixture of *Clostridia* species or microbiota from SPF mice, results in significantly increased luminal levels of NE, DA and β-glucuronidase activity (Sudo, Chida et al. 2004). Furthermore, the identification of DA receptors on intestinal epithelial cells indicates an important physiological role of luminal catecholamine (Lam, App et al. 2003).

1.6. The gut-microbiota-brain axis

Early studies in the field of gut-brain axis focused on digestive functions and satiety (Taché, Vale et al. 1980, Konturek, Konturek et al. 2004, Berthoud 2008). With research in this field of discipline growing exponentially over the last few decades, a strong connection between the gut and brain and its implication in immune, endocrine, behavioural and cognitive functions have been reported (Mayer, Knight et al. 2014, Schmidt 2015, Smith 2015, Jenkins, Nguyen et al. 2016). The ability of the gut microbiota to affect host physiology outside the GIT, especially the brain, has led to increasing interest in the gut-microbiota-brain axis (Mayer, Knight et al. 2014, Burokas, Moloney et al. 2015, Mayer, Tillisch et al. 2015, Dinan and Cryan 2017, Cryan, O'Riordan et al. 2019).

The nature of the bidirectional communication networks in the microbiota-gut-brain axis, enables signals from the brain to influence the motor, sensory and secretary functions of the GIT, but also allows signals from the gut to alter brain function and behaviour (Dinan and Cryan 2017). Alterations in these signalling pathways have been implicated in gastrointestinal disorders (Rhee, Pothoulakis et al. 2009, Mayer 2011), metabolism related disorders (Backhed, Ding et al. 2004, Cani, Everard et al. 2013), dysfunction of immune system (Petersen and Round 2014) and more recently in neurological disorders including autism spectrum disorder (ASD) and depression, as well as neurodegenerative diseases including Parkinson's disease (PD) and Alzheimer's disease (AD) (Cryan and Dinan 2012, Park, Collins et al. 2013, Mayer, Knight et al. 2014). Although there is accumulating evidence implicating the gut-brain axis in health and disease, there is controversy regarding the magnitude of influence on individual's health, mechanisms and mediators involved. Not only do changes in gut microbiota during adulthood and later stages of life affect brain function and behaviour, but the microbiota also influences the development and maturation of the CNS (Diaz Heijtz, Wang et al. 2011). This evidence comes from a range of approaches ultilising GF models, probiotic/prebiotic treatments, infection models, antibiotic treatments, faecal microbial transplant (FMT) as well as some emerging clinical trials in human population. GF animals display alterations in neuroendocrine, neurochemical, and gastrointestinal systems, and show abnormal behavioural development (Desbonnet, Garrett et al. 2010, Diaz Heijtz, Wang et al. 2011, Hoban,

Moloney et al. 2016), They provide a useful tool to determine the extent of microbial involvement in regulation of these communication pathways. Probiotics have long been used, with benefits to host gastrointestinal and immunological function (Kondo, Xiao et al. 2010, Savilahti 2011) and prevention of obesity (Kondo, Xiao et al. 2010), but also more recently CNS function and behaviour (Sampson and Mazmanian 2015). The mechanisms and pathways involved with these functions often integrate several, complex pathways that can act directly and/or indirectly on the components of the gut-brain axis.

1.7. Signalling within the gut-microbiota-brain axis

The gut-microbiota-brain axis combines the neural, endocrine and immunological signalling pathways that allow signalling between the enteric microbiota and host organ systems (Mayer, Tillisch et al. 2015). It consists of "bottom-up" signalling where the signals from gut microbiota reach the brain, and "top-down" signalling where the brain signals to the gut and its' microbiota. Bottom-up signalling mainly occurs via the neuroendocrine and neuroimmune systems and involves the intestinal mucosal barrier and the BBB (Martin, Osadchiy et al. 2018). The gut is the largest endocrine and immune organ in the human body (Rehfeld 1998, Raybould 2010) and therefore it is unsurprising that the gut microbiota can influence CNS function and behaviour. This can occur through endocrine and neural signalling pathways mediated by neuroendocrine cells, neuropeptides, neuroactive molecules, hypothalamic-pituitary-adrenal (HPA) axis and vagal signalling (Holzer and Farzi 2014, Sudo 2014, Farzi, Fröhlich et al. 2018), as well as regulation of innate and adaptive immune systems (Jarchum and Pamer 2011, Erny, Hrabe de Angelis et al. 2015, Palm, de Zoete et al. 2015, Fung, Olson et al. 2017, Ratajczak, Rył et al. 2019). Top-down signalling occurs via the neuroanatomical pathways (ANS) and regulation of the intestinal barrier and neurotransmitters (Saunders, Santos et al. 2002, Mayer, Knight et al. 2014).

1.7.1. Barriers

There are two natural barriers within the gut-microbiota-brain axis, the intestinal barrier, and the BBB. Gut microbiota can directly or indirectly modulate barrier permeability, thereby affecting homeostatic processes. It is therefore important that the integrity of the barriers is tightly regulated and maintained.

1.7.1.1. The intestinal barrier

The intestinal barrier is a biophysical, biochemical, and immunological barrier. A single layer of epithelial cells covered by the mucus layer, provides a physical barrier against luminal microbes and the epithelium. SCFAs production from microbial fermentation can affect mucus production. Butyrate for example, induces MUC2 gene expression via selective histone acetylation/methylation (Hatayama, Iwashita et al. 2007, Burger-van Paassen, Vincent et al. 2009). The production of SCFAs in the gut also help to supress growth of pathogenic bacteria by lowering pH and influencing bacterial composition (Cherrington, Hinton et al. 1991, Duncan, Louis et al. 2009). The mucus layer, therefore, is an integral component in maintaining intestinal homeostasis and breakdown of the mucus layer is implicated in several gastrointestinal-related disorders (Johansson, Gustafsson et al. 2014).

Around 90-95% of luminal SCFAs are absorbed by the gut mucosa (McNeil, Cummings et al. 1978), either via passive diffusion or through specific transporters. MCT1 is considered the primary nutrient transporter for butyrate, expressed predominantly on the apical (luminal) membrane of colonocytes but can also be found on the basolateral membrane (Gill, Saksena et al. 2005, Iwanaga, Takebe et al. 2006). Whereas SMCT1 is exclusively expressed on apical membrane (Iwanaga, Takebe et al. 2006, Cresci, Thangaraju et al. 2010). Interestingly, butyrate has been shown to induce the expression of SLC16A1 mRNA that encodes MCT1 (Cuff, Lambert et al. 2002, Borthakur, Priyamvada et al. 2012), and similar findings have also been observed in healthy animal (pig) models following dietary supplementation with resistant starch for 2-weeks. Unaltered expression of SMCT1 and other metabolite-sensing receptors in this study indicate that the effects of butyrate are specific to the nutrient sensing receptor, MCT1 (Haenen, Zhang et al. 2013). In addition to effects on gene expression, resistant starch supplementation results in increased caecal and colonic SCFA levels, corresponding to increased abundance of butyrate-producing *F. prausnitzii* and reduced abundances of pathogenic *E. coli* and species from the *Pseudomonas* genus (Haenen, Zhang et al. 2013).

Butyrate can activate FFAR2 (GPR43) expressed on intestinal enterocytes, inducing the production of the AMPs such as β-defensin and RegIIIγ (Zhao, Chen et al. 2018). Butyrate also inhibits proliferation of intestinal stem cells via selective acetylation of the transcription factor Foxo3 (Kaiko, Ryu et al. 2016), implying that the normal structure of colonic epithelium and mucus layer are needed to ensure limited exposure of crypt-based stem cells to microbial SCFAs. Indeed, a natural SCFA gradient exists along the crypt-villus axis, with differentiated mature enterocytes further up the villus being exposed to higher concentration of microbial SCFAs (Kaiko, Ryu et al. 2016). Furthermore, the metabolism of butyrate by colonocytes reduces the amount of butyrate exposure for crypt-based stem cells (McNeil 1984).

The importance of the normal gut microbiota in regulating intestinal homeostasis is demonstrated by reduced expression of functional proteins (including tight junction and adherens junction proteins) in the absence of gut microbiota in mice (Shimada, Kinoshita et al. 2013). Via SCFA production the gut microbiota can modulate intestinal epithelial permeability through the regulation of junctional complexes, gene expression and epithelial cell proliferation. In intestinal epithelial cell monolayers, butyrate enhances the expression of tight junction protein claudin-1 (Wang, Wang et al. 2012) and has been shown to improve the damaging effects of LPS (Yan and Ajuwon 2017). These effects on tight-junction integrity are mediated via the activation of AMP-activated protein kinase (AMPK) (Peng, Li et al. 2009) and are largely concentration dependent, with higher concentrations having paradoxical effects (Peng, Li et al. 2009, Wang, Wang et al. 2012, Yan and Ajuwon 2017). Similar findings are also observed in animal models following dietary fibre interventions. Mice receiving dietary fibres have increased faecal SCFAs and increased expression of tight junction proteins, including ZO-1, occludin, claudin-7 and junctional adhesion molecule A (JAKA) (Hung and Suzuki 2018). Moreover, in an obese murine model, a prebiotic-rich diet was shown to improve intestinal epithelial integrity through increased expression of ZO-1 and occludin (Cani, Possemiers et al. 2009).

It is apparent from these studies that microbial-derived butyrate has a wide range of roles in maintaining host intestinal barrier integrity in health and disease. Translation to clinical studies has also provided insight into the possibility of using prebiotics to modulate microbial fermentation and production of metabolites to improve intestinal

permeability. For example, supplementation with resistant starch and pectin for 1-week resulted in significantly reduced intestinal permeability and faecal output in children with persistent diarrhoea (Rabbani, Teka et al. 2004). The beneficial effects of prebiotics however are not consistent across clinical studies. No improvements in intestinal permeability was observed in type-2 diabetic patients receiving galacto-oligosaccharide (GOS) for 12-weeks (Pedersen, Gallagher et al. 2016). Similarly, no added beneficial effects on intestinal barrier function were reported in healthy volunteers following 2-week supplementation with fructo-oligosaccharide (FOS) (Wilms, Gerritsen et al. 2016). Clearly further human interventional trials are needed to assess the effects of different prebiotic and probiotic formulations across different populations and diseases. Consistency and conflicting clinical findings can be improved by validation of tools and multifaceted approaches used to conduct analysis of intestinal permeability to provide a better indication of intestinal barrier function.

In addition to microbial fermentation products, BEVs from probiotic bacterial strains have been shown to positively modulate the intestinal barrier. For example, the improvement in Caco-2 cell monolayer barrier integrity induced by the probiotic *E. coli* Nissle 1917 can be reproduced by its OMVs, resulting in upregulation of tight junction proteins, ZO-1 and claudin-14 (Alvarez, Badia et al. 2016). The mechanisms underlying *E. coli* OMV regulation of tight junction proteins aren't known, but it could involve OMPs. For example, microintegral membrane proteins (MIMPs) of *Lactobascillus platarum* (*L. plantarum*) have been shown to increase the expression of tight junctional proteins (Yin, Yan et al. 2018). On the other hand, OMVs from enterohemorrhagic *E. coli* has been shown to enter Caco-2 cells via dynamin-dependent endocytosis, trafficked to intracellular lyso-endosomal compartments, where upon acidification the associated hemolysin toxin is separated from the OMV. The disassociated hemolysin then targets mitochondria causing permeabilization of mitochondrial membranes and subsequent activation of caspase-3 and caspase-9 leading to apoptosis (Bielaszewska, Rüter et al. 2013).

These studies provide strong evidence of microbial fermentation products and membrane vesicles in modulating host intestinal barrier integrity (Fig. 1.5).

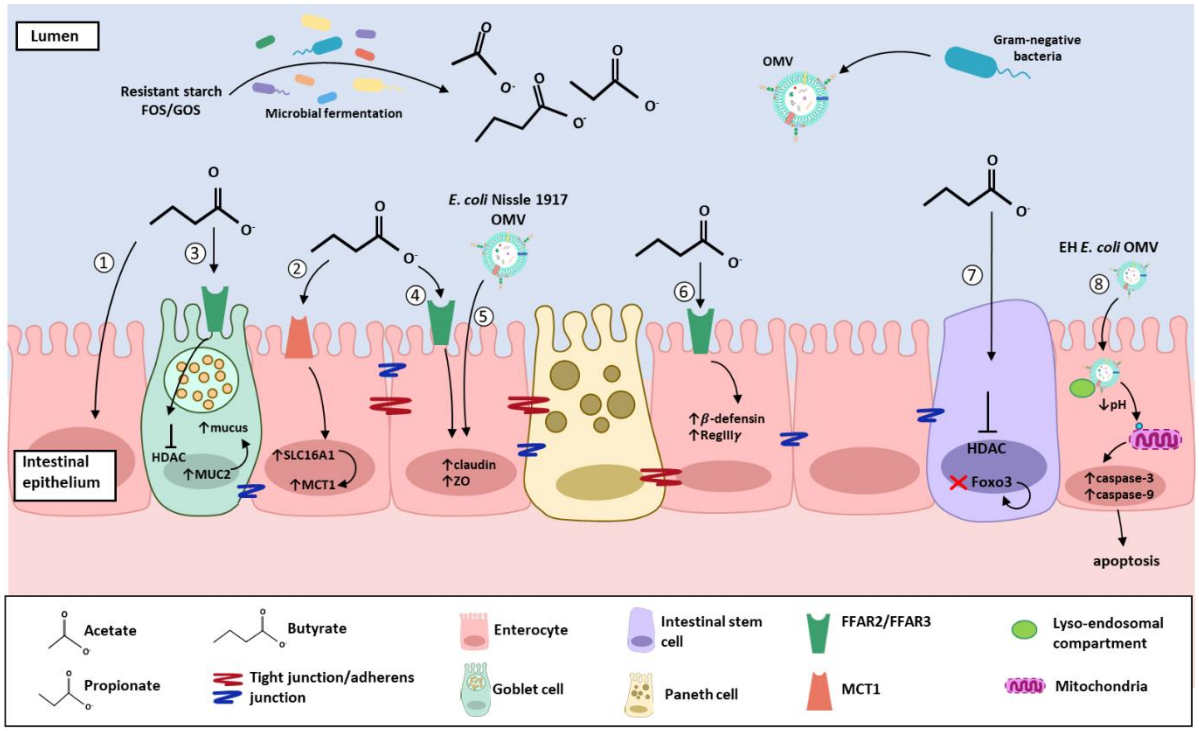

Figure 1.4 Microbial signalling and modulation of the intestinal epithelial barrier. Microbial fermentation productions (SCFAs; acetate, propionate, and butyrate) are key mediators involved in cellular interactions within the intestinal epithelium. SCFAs can enter cells via (1) passive diffusion or via (2) MCT1 expressed on the apical membrane, upregulating expression of MCT1. (3) Butyrate can also induce the expression of MUC2 in goblet cells (4) and tight junction proteins such as ZO-1 and claudins. (5) OMVs derived from probiotic *E. coli* Nissle 1917 strain increase the expression of tight junction proteins. (6) Via FFAR2, butyrate can induce the production of anti-microbial proteins. (7) Butyrate can inhibit proliferation of intestinal stem cells via selective histone acetylation of the transcription factor Foxo3. (8) OMVs derived from the enterohemorrhagic *E. coli* strain can be taken up by epithelial cells via dynamin-dependent endocytosis, where they are trafficked to lyso-endosomal compartments. Following separation of hemolysin toxin and targeting to the mitochondria causes permeabilization of mitochondrial membranes and subsequent activation of caspase-3 and caspase-9 leading to apoptosis of the cell.

1.7.1.2. The blood-brain barrier

The BBB is a highly dynamic and regulated cellular system that is part of the neurovascular unit (NVU) (Hawkins and Davis 2005, Parker, Fonseca et al. 2020). It's function is to provide a barrier between the brain and the blood circulation, protecting the CNS against potential blood borne toxins, providing a homeostatic environment for the proper functioning of neurones and aiding to meet the metabolic requirement of the brain (Andreone, Lacoste et al. 2015). The BBB consists of brain microvascular endothelial cells (BMECs), pericytes, astrocytes, neurones, microglia and extracellular matrix (ECM) (Hawkins and Davis 2005) (Fig. 1.6). The backbone component of the BBB is the endothelial microvessel formed by a single layer of BMECs. (Abbott, Rönnbäck et al. 2006). Pericytes are found wrapped around the CNS microvessel (Rustenhoven, Jansson et al. 2017) where they modulate the diameter of the capillary, maintain endothelial stability and blood flow (Winkler, Bell et al. 2011). Astrocytes are in contact with pericytes and BMECs and hence provide additional physical support to the BBB (Abbott, Rönnbäck et al. 2006), but they also have roles in neurotransmitter clearance and recycling and modulating immune responses (Gee and Keller 2005). Astrocytes have also been shown to enhance endothelial barrier function by upregulating tight junction protein formation in *in vitro* cell culture experiments (Hayashi, Nomura et al. 1997). Surrounding endothelial cells is the ECM, composed of collagen, laminin, nestin, heparin sulphate proteoglycan and other glycoproteins, supporting interactions been adjacent BMECs, pericytes and astrocytes (Thomsen, Routhe et al. 2017). Transport across the BBB is highly regulated. BMECs are different to endothelial cells found in other peripheral tissues as they have continuous tight junction complexes composed of four types of integral membrane proteins (occludin, claudins, junctional adhesion molecules and cell selective adhesion molecules) that are connected by cytoplasmic ZO proteins to the cytoskeleton (Dejana, Tournier-Lasserve et al. 2009). This results in low rates of paracellular movement of macromolecules from the blood circulation to the CNS (Abbott, Rönnbäck et al. 2006). The overlap of BMECs results in a high-resistance barrier, effectively preventing the transport of macromolecules through the tight junctions (Vorbrodt and Dobrogowska 2003).

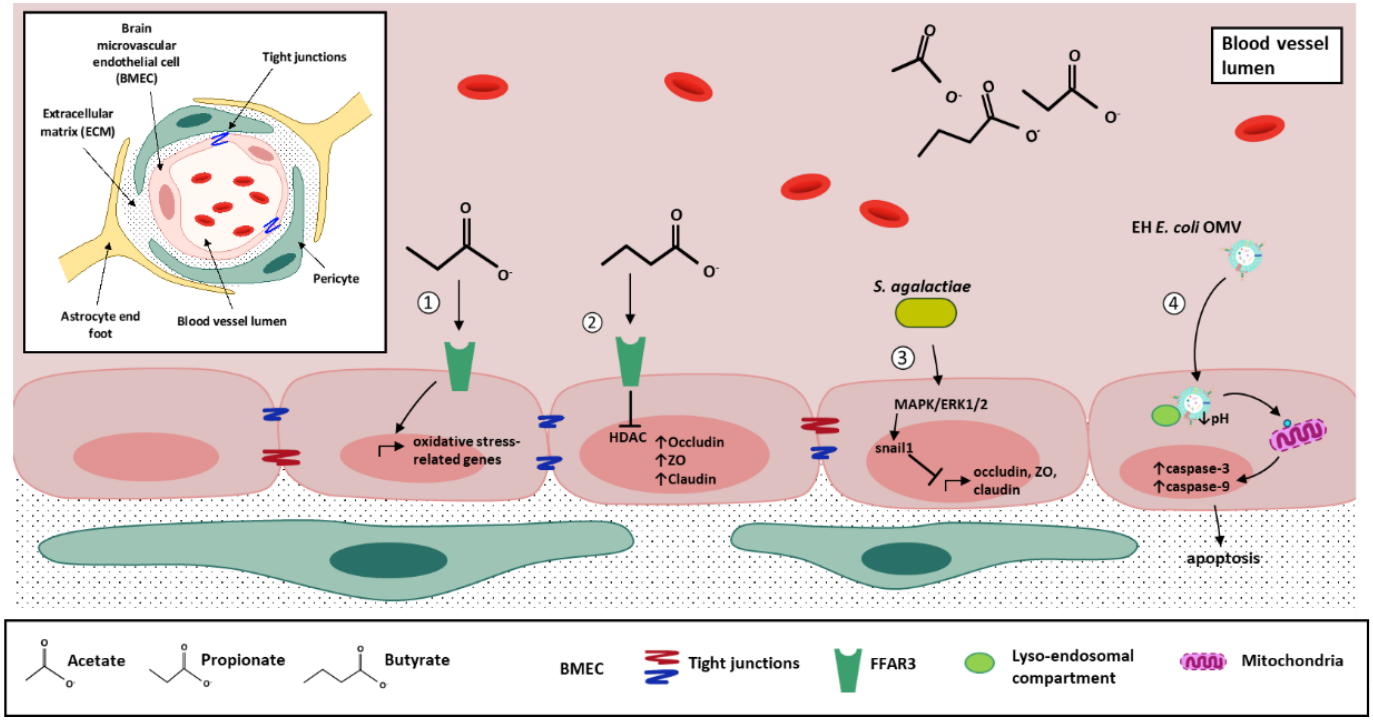

Figure 1.5 Microbial modulation of the blood brain barrier. The BBB is considered an integral component of the NVU and consists of BMECs, pericytes, astrocytes, tight junctional complexes, and ECM and together with surround neurons and microglia, support the function and integrity of the BBB. (1) propionate can activate FFAR3 expressed on endothelial cells to alter the gene expression. (2) Through its HDAC inhibitory properties, butyrate can upregulate the expression of tight junction proteins in BMECs. (3) The meningeal pathogen *S. agalactiae* can inhibit the expression of tight junction proteins via MAPK/ERK1/2 mediated induction of the transcriptional repressor Snail1, promoting BBB disruption. (4) OMVs derived from the enterohemorrhagic (EH) *E. coli* strain can be taken up by endothelial cells via dynamin-dependent endocytosis, where they are trafficked to lyso-endosomal compartments. Following separation of hemolysin toxin and targeting to the mitochondria causes permeabilization of mitochondrial membranes and subsequent activation of caspase-3 and caspase-9 leading to apoptosis of the cell.

BBB dysfunction and permeability can lead to imbalance in ions, transmitters and metabolic products that can alter neuronal activity. There are numerous neurological diseases where BBB breakdown is a pathological hallmark feature including epilepsy (Janigro 2012), AD (Sagare, Bell et al. 2012), PD (Bartels, Willemsen et al. 2008, Lu and Hu 2012) and multiple sclerosis (MS) (Engelhardt and Ransohoff 2012). But it is unclear whether BBB breakdown is part of the initial events that leads to neuronal loss or whether it is a downstream consequence.

Embryologically, the BBB begins to develop around week 2 with permeability beginning to reduce around day 15 in mouse embryos. GF mouse embryos show increased permeability of BBB, demonstrated by Evans blue staining not only in the blood vessel but in the brain parenchyma, which persists after birth and throughout adulthood. Accompanying alterations to BBB permeability, reduced tight junction protein expression, specifically claudin-5 and occludin are also reported in GF mice (Braniste, Al-Asmakh et al. 2014). Interestingly, conventionalisation of GF mice with flora from SPF mice restores BBB integrity by reducing its permeability. Impaired maturation and function of microglia cells in GF mice could also contribute to BBB impairment (Erny, Hrabe de Angelis et al. 2015). Together these results demonstrate the importance of the gut microbiota in the development and function of the BBB (Braniste, Al-Asmakh et al. 2014).

Given that over 200 different microbial metabolites have been identified in the peripheral circulation of healthy animals and humans (Zheng, Xie et al. 2011, Russell, Hoyles et al. 2013, Boets, Gomand et al. 2017), it is plausible that gut microbial fermentation products could target the BBB (Fig. 6). Indeed, the effects of mono-colonisation of GF mice with *Clostridium tyrobutyricum* (*C. tyrobutyricum*) were mimicked by oral gavage with sodium butyrate for 3-days in GF mice and was associated with specific upregulation of tight junction protein occludin in the frontal cortex and hippocampus (no effect on the expression of claudin-5 was observed). The effects of *C. tyrobutyricum* and butyrate were also associated with increased histone acetylation (Braniste, Al-Asmakh et al. 2014). Therefore, the effects on BBB permeability by *C. tyrobutyricum* could be explained by its production of butyrate as its primary metabolite (Louis and Flint 2009) and its associated inhibition of histone deacetylase (Levenson, O'Riordan et al. 2004). Although the histone deacetylase

inhibitory effects of butyrate have been associated with promoting of neurogenesis in rats (Kim, Leeds et al. 2009), the consequences of increased BBB permeability in GF mice on neuronal function during development and adulthood have not been fully explored and therefore, results from GF models should be interpreted cautiously.

In addition to butyrate, propionate too has been shown to modulate BBB permeability. Exposure of hCMEC/D3 cell monolayers to physiological relevant concentration of propionate (1 μM) for 24 hr resulted in significant alterations in gene expression profile namely those associated with oxidative stress responses, possibly involving signalling via the FFAR3 that is expressed on endothelial cells (Hoyles, Snelling et al. 2018). Although immortalised endothelial cell lines such as those used in this study are widely used to model the BBB, they are associated with several limitations. In particular, they display inherently higher permeability than that to their *in vivo* counterpart (Eigenmann, Xue et al. 2013).

In murine models of traumatic brain injury (TBI), intragastric administration with *Clostridium butyricum* (*C. butyricum*) also improved BBB impairment through the upregulation of the tight junction proteins (occludin and ZO-1) that correlated with improved neurological dysfunction, intestinal barrier integrity, increased colonic GLP-1 levels and elevated expression of the GLP-1 receptor (GLP-1R) in the cerebrum. These results suggests that GLP-1 signalling in the gut-brain axis could in part contribute to the mechanisms that underpins the neuroprotective effects of *C. butyricum* in TBI mice (Li, Sun et al. 2018). In line with previous studies, butyrate replicated the neuroprotective effects of *C. butyricum* treatment in mice following TBI (Li, Sun et al. 2016). The role of SCFAs in BBB permeability potentially holds clinical relevance as defects in BBB integrity have been linked to impaired cognitive function and implicated in the progression of several neurological disorders (Bowman, Kaye et al. 2007, Taheri, Gasparovic et al. 2011, Montagne, Barnes et al. 2015). Further studies are needed to elucidate the precise mechanistic details of how SCFAs contribute to the regulation of tight junctions in BBB development and adulthood. It is also unclear whether SCFAs directly influence endothelial cell tight junction expression or whether it via indirect mechanisms involving other cellular components of the NVU.

As SCFAs alone cannot fully recapitulate the neuroprotective effects of conventionalisation in GF animals on BBB permeability (Braniste, Al-Asmakh et al. 2014, Fröhlich, Farzi et al. 2016), it is likely that there are other possible mediators or mechanisms involved in microbial regulation of BBB. Indeed, enterohemorrhagic (EH) *E. coli* derived OMVs have been shown to be internalised by human brain microvascular endothelia cells (HBMECs), triggering acidification of lyso-endosomal compartments and apoptosis (Bielaszewska, Rüter et al. 2013), suggesting a possible route for the delivery of virulence factors by gut pathogens to the brain. The meningeal pathogen *Streptococcus agalactiae* (*S. agalactiae*) causes disruption of the barrier integrity in HBMECs evidenced by reduced ZO-1, claudin-5 and occludin at both transcript and protein levels. *S. agalactiae* mediates its disruptive effects on BBB through the induction of the transcriptional repressor Snail1 that inhibits the expression of tight junction protein genes, and was shown to be dependent on activation of the ERK1/2 and MAPK signalling pathways and components of the bacterial cell wall (Kim, Hancock et al. 2015). The pilus tip adhesion (PilA) protein and the glycoprotein Srr-1 expressed on *S. agalactiae* are thought to mediate these effects, as they have been demonstrated to bind to collagen and fibrinogen, promoting BBB disruption (Banerjee, Kim et al. 2011, Seo, Mu et al. 2012).

1.7.2. Neural pathways

There are between 30,000-80,000 vagal afferent neurons that innervate the mucosal and muscular layers of the GIT. Intrinsic primary afferent neurons (IPANs) of the ENS are the predominant sensory fibres innervating the intestinal mucosa (Blackshaw, Brookes et al. 2007, Wang and Powley 2007). Increased intrinsic excitability of colonic IPANs is observed in rats following 9-day feeding with *Lactobascillus reuteri* (*L. reuteri*) (Kunze, Mao et al. 2009). Similarly *Bifidobacterium fragilis* (*B. fragilis*) polysaccharide A (PSA) was shown to reproduce the effects of the parent bacterium by inducing excitability of IPANs in an *ex vivo* mucosa myenteric plexus preparation (Mao, Kasper et al. 2013), shedding light onto possible mechanisms behind microbial involvement in IPAN signalling. Further studies are needed to determine whether PSA acts via gut endocrine cells and the subsequent secretion of neuromodulators (such as neurotransmitters and cytokines) that act on mucosal IPAN terminals in a paracrine manner, or if PSA can translocate across the epithelium, possibly via the production

of OMVs to activate receptors on the afferent nerve terminals. The latter could potentially be at play here, as *B. fragilis* has been shown to produce OMVs containing PSA that reproduces the immunological effects of the parent bacterium (Shen, Giardino Torchia et al. 2012). The importance of the gut microbiota in regulating the ENS is further highlighted the investigation of the electrophysiological properties of neurones in the *ex vivo* myenteric plexus from SPF and GF mice. Results from this experiment demonstrated that GF mice have a reduced IPAN excitability, evidenced by a lower resting membrane potential and reduced number of action potentials (McVey Neufeld, Mao et al. 2013). GF mice also display alterations in neural innervation in the GIT, colonic motility (Aktar, Parkar et al. 2020).

Some of the first studies demonstrating the role of the vagus nerve in modulating behaviour come from sickness behaviour induced by bacterial endotoxins. In these experiments, LPS was used to induce a consistent, dose-dependent sickness behaviour in rodents that was inhibited by vagotomy (Bluthé, Walter et al. 1994, Bret-Dibat, Bluthé et al. 1995, Layé, Bluthé et al. 1995). There is also strong evidence from animal studies suggesting that gut microbiota can activate the vagus nerve. For example, *Salmonella typhimurium* induced the expression of a neuronal activation marker, c-FOS, in the hypothalamic paraventricular nuclear (PVN) and supraoptic nucleus (SON) which was inhibited by subdiaphragmatic vagotomy (Wang, Wang et al. 2002). Infection with *Campylobacter jejuni* (*C. jejuni*) in mice also resulted in increased c-FOS expression in the nucleus of the solitary tract (NTS) as well as brain regions associated with primary viscerosensory pathways and central autonomic networks within 12 hr (Goehler, Gaykema et al. 2005). These alterations were accompanied by behavioural changes but no changes in peripheral inflammatory markers (Goehler, Park et al. 2008), demonstrating that even in the absence of an associated inflammatory response, peripheral sensory neurones contribute to early signalling to the brain following infection. In addition to pathogens, non-pathogenic bacteria can also modulate brain signalling from the gut via vagal signalling. Oral administration with the probiotic, *L. rhamnosus* JB-1 has been shown to alter the normal behaviour of adult mice, correlating to alterations in central GABA receptor expression. These effects were abolished in subdiaphragmatic vagotomised mice (Bravo, Forsythe et al. 2011). Similarly, the anti-anxiety-like behaviours associated with treatment with the probiotic *Bifidobacterium longum* (*B. longum*) NCC3001 in dextran sodium sulphate (DSS)

induced mouse model of colitis, were shown to be vagally mediated and independent of its anti-inflammatory properties (Bercik, Park et al. 2011). Furthermore, 48 hr incubation of neuroblastoma cells with the fermentation products of *B. longum* NCC3001 revealed no effect on basal brain derived neurotrophic factor (BDNF) mRNA expression but were shown to decrease the excitability of perfused ileal enteric neurones (Bercik, Park et al. 2011), indicating the ENS may serve in this bacterium's method to signal to the host CNS. A detailed analysis of microbial communities before and after treatment in these studies is lacking, and therefore it remains to be determined whether the behavioural and functional alterations observed in these studies are associated directly with the administered bacterium or is contributed by alterations in overall microbial composition. The ability of gut microbiota to regulate neurotransmitter-related gene expression in the brain is further demonstrated by Heijtz et al., (Diaz Heijtz, Wang et al. 2011), with Neufeld et al., also demonstrating reduced mRNA expression of the N-methyl-D-aspartate (NMDA) receptor subunit and 5-HT receptor 1A (5-HT1A) in the central amygdala and hippocampus, respectively, in GF mice (Neufeld, Kang et al. 2011). These studies collectively demonstrate that microbial induced behavioural changes are accompanied by neurochemical alterations in the brain.

1.7.3. Immune signalling

Evidence highlighting the important role of the immune system in gut-brain signalling is expanding. The intestinal immune system is critical in maintaining gut bacterial homeostasis, providing immunity against pathogenic microbes and tolerance to gut luminal contents, food, and microbiota derived antigens (Salvo-Romero, Stokes et al. 2020). Additionally, the gut microbiota regulates the development and function of innate and adaptive immunity, and ultimately, neuroinflammatory responses in the CNS that can alter brain function and behaviour (Round and Mazmanian 2009, Neuman, Debelius et al. 2015, Palm, de Zoete et al. 2015, Powell, Walker et al. 2017, Shi, Li et al. 2017, Gao, Xu et al. 2018). Imbalance in either side of this sensitive system predispose to both local and systemic disease. Indeed, chronic activation of immune and inflammatory responses is a pathological feature of many human diseases (Cryan, O'Riordan et al. 2019).

Comprising of a vast cell population in the intestinal mucosa and mucosa-associated lymphoid tissues, the innate immune system provides essential first line defence functions, effectively discriminating between commensal antigens and potential pathogenic bacteria via pathogen recognition receptors (PRRs). In mammals, PRRs are expressed on many cells including macrophages, dendritic cells, epithelial cells, and neutrophils. Toll-like receptors (TLRs) form a major class of PRRs, that recognised bacterial LPS and other microbiota-association molecular patterns (MAMPs) (Akira 2001). Other PRRs, such as the NOD-like receptors (NLR), NOD1 and NOD2, recognise bacterial peptidoglycans. Activation of these receptors initiates the downstream inflammatory signalling cascades (Medzhitov and Janeway 2000, Tlaskalova-Hogenova, Tuckova et al. 2005). Gut microbiota can regulate the expression and signalling cascade of PRRs (Thaiss, Levy et al. 2014). For example, *B. fragilis* requires the activation of TLR-2 on CD4+T cells to modulate regulatory T (Treg) cell and T helper 17 responses (Round, Lee et al. 2011). It has been suggested that the microbial community regulates NOD2 expression in the intestine, as evidenced by NOD2-deficient mice displaying increased abundance of gut commensals (Petnicki-Ocwieja, Hrncir et al. 2009). As highly expressed on Paneth cells, NOD2 signalling is associated with AMP secretion and regulation of host-microbial homeostasis (Petnicki-Ocwieja, Hrncir et al. 2009) and NOD2-deficient mice have reduced levels of α-defensins (Kobayashi, Chamaillard et al. 2005, Vaishnava, Behrendt et al. 2008). However, the downstream signalling cascades resulting secretion of AMPs remain to be fully elucidated.

SCFAs can also activate immune cells. For example, in the colon, butyrate has been shown to enhance histone 3 acetylation to induce differentiation of colonic Treg cells (Shi, Wang et al. 2011, Arpaia, Campbell et al. 2013, Furusawa, Obata et al. 2013). Butyrate also induces anti-inflammatory effects against LPS-induced activation of macrophages, via inhibition of HDAC leading to reduction in the expression and secretion of inflammatory mediators (Chang, Hao et al. 2014).

In the CNS, glial cells, including astrocytes and microglia are the first line of defence against pathogens and their toxic products. The major glial cell-type in the brain, astrocytes, have essential roles in the development, functioning and survival of neurones, but they have also been suggested to function as immune effector cells

(Bowman, Rasley et al. 2003). For example, murine astrocytes are activated upon exposure to the meningeal pathogen *Neisseria meningitidis* inducing the secretion of IL-10 (Rasley, Tranguch et al. 2006), possibly through the activation of TLR-2 (Esen, Tanga et al. 2004). Microglia are regarded as CNS resident macrophages. When activated they accumulate to the site of damage and upregulate the production and secretion of proinflammatory cytokines and chemokines (Rasley, Anguita et al. 2002). Several studies have also demonstrated the increased mRNA and protein expression of TLRs by microglia following exposure to microbial pathogens (Kielian, Mayes et al. 2002, Rasley, Bost et al. 2002). These results indicate that CNS glial cells can modulate responses upon exposure to infections or damage through the production of inflammatory mediators and expression of PPRs. Further research is needed to determine the extent to which the complex microbial community in the gut reaches a self-sustaining equilibrium.

1.7.4. Neuroendocrine system

Neuroendocrine pathways in the gut-brain axis often involve the vagus nerve (Bravo, Forsythe et al. 2011, Tolhurst, Reimann et al. 2012, Singh, Roth et al. 2016) and is mediated via several microbial derived molecules including products of microbial fermentation and neuroactive molecules (Wikoff, Anfora et al. 2009, Tolhurst, Heffron et al. 2012, Yano, Yu et al. 2015). Intestinal enteroendocrine cells (EECs) are the primary sensors of these microbial products, propagating the signal via the production and secretion of various peptide hormones. In addition to this, microbial products can also cross the intestinal epithelium entering the peripheral circulation where they can be transported to various sites around the body (Samuel, Shaito et al. 2008, Yano, Yu et al. 2015). It remains unclear whether these microbial products act directly on brain regions or transmit responses via vagal signalling (Goehler, Gaykema et al. 2005, Bravo, Forsythe et al. 2011). The HPA axis is thought to be the major contributor to the function of the neuroendocrine system (Sudo 2014).

1.7.4.1. Enteroendocrine cells

EEC are specialised chemosensory cells scattered throughout the epithelium of the GIT, making up around 1% of the total epithelial cells in the mucosa. Despite their scarcity, they are considered the largest endocrine organ in the human body (Gribble

and Reimann 2016, Latorre, Sternini et al. 2016). EECs establish transepithelial signal transduction pathways in response to luminal nutrients and microbial metabolites through the production and secretion of various peptide hormones stored in cytoplasmic granules. The expression of metabolite receptors, including FFAR2 and FFAR3, make them primed for their key role as chemical sensors (Reimann, Tolhurst et al. 2012, Tolhurst, Reimann et al. 2012, Akiba, Inoue et al. 2015, Psichas, Reimann et al. 2015, Gribble and Reimann 2016, Martin, Lumsden et al. 2017, Lu, Gribble et al. 2018). The hormones expressed in EECs include cholecystokinin (Cck), GLP-1, GLP-2, glucose-dependent insulinotropic peptide (GIP), PYY and 5-HT. These have a wide range of physiological functions within and outside the GIT, such as appetite regulation, gastrointestinal motility, and neurotransmission (Gribble and Reimann 2016).

Classically, EECs are divided into subtypes depending on the peptide hormones they secrete (Solcia, Sessa et al. 1990). For example, L cells contain large secretory vesicles that co-express GLP-1 and PYY (Ali-Rachedi, Varndell et al. 1984, Böttcher, Alumets et al. 1986), which have roles in appetite regulation and insulin secretion (Mace, Tehan et al. 2015). Whereas K cells are identified by GIP containing vesicles (Buffa, Polak et al. 1975). Recent evidence has brought to light the heterogeneity in peptide hormone expression within individual subsets of EECs. For example, CCK cells have been shown to co-express the functionally related peptides GLP-1, GIP, PYY, neurotensin and secretin (Egerod, Engelstoft et al. 2012). EEC peptide co-expression can also vary depending on intestinal region they are located (Habib, Richards et al. 2012) and the crypt-villus bone morphogenetic protein (BMP) gradient (Beumer, Artegiani et al. 2018). These studies demonstrate that the classical classification system for EECs based on their peptide content is unreliable and inaccurate, but deespite this still remains the most widely used nomenclature for EECs.

Amongst the peptide hormones expressed in EECs, 5-HT has a central role as a neurotransmitter in the CNS (Kim and Camilleri 2000). Over 90% of the body's 5-HT is synthesised in the gut (Gershon and Tack 2007), primarily by enterochromaffin cells (EC), but also by mast cells and enteric neurones (Gershon and Tack 2007), mediated by tryptophan hydrolase (Tph) 1 (non-neuronal) or Tph2 (neuronal), converting tryptophan to 5-HT (Walther, Peter et al. 2003). 5-HT activates 14 different

5-HT receptor subtypes expressed on enterocytes (Hoffman, Tyler et al. 2012), enteric neurones (Mawe and Hoffman 2013) and immune cells (Baganz and Blakely 2013). Upon secretion, 5-HT has a diverse range of functions from regulation of gastrointestinal motility and secretion (Gershon and Tack 2007) to bone development (Chabbi-Achengli, Coudert et al. 2012) and immune modulation (Baganz and Blakely 2013). They can also be taken up by platelets in the peripheral circulation allowing distribution to sites outside of the GIT (Amireault, Sibon et al. 2013). Despite these features, the role of EC cells in signalling via the vagus nerve to modulate brain functions has not been well studied.

Investigating the fine ultrastructure of murine EECs have revealed that most EECs have axon-like basal processes called neuropods that contain neurofilaments similar to those in axons (Bohorquez, Samsa et al. 2014, Bohórquez, Shahid et al. 2015), highlighting their possible role in neurotransmission. Indeed, GLP-1 and 5-HT can activate receptors expressed on IPAN and extrinsic afferent neurones axon terminals (Raybould 2010). Further research has demonstrated that EEC neuropods can make direct connections with enteric glial cells and glial derived neurotrophic factors enhanced the development and structure of EEC neuropods in *in vitro* organoid based models (Bohorquez, Chandra et al. 2011, Bohórquez, Samsa et al. 2014). It was later identified that EECs expressed several genes encoding synaptic proteins suggesting that EECs could participate directly in neurotransmission (Bohórquez, Shahid et al. 2015). Whether EECs form direct connections with local enteric neurons remains to be determined. As epithelial turnover in the intestine is approximately 5-days, it means that there is only a small time window for them to form connections with neurones (Bertrand 2009). Other studies have reported that the lifespan of EECs is longer than surrounding enterocytes as they migrate slower along the crypt-villus axis (Tsubouchi and Leblond 1979), with some EECs reported to be tracked after 60 days (Bohórquez, Shahid et al. 2015). It seems unlikely therefore that these slow migrating EECs make direct connects to enteric neurones, as the slow migration rate can inhibit synapsing. It more likely that EECs secrete neuropeptides, activating receptors on nerve terminals in a paracrine manner (Wade and Westfall 1985, Pan and Gershon 2000, Bertrand 2004).

Specific gut microbiota profiles have been demonstrated to influence EEC network and secretion of peptide hormones. Experiments carried out in Chapter 2 show that the presence of a normal gut microbiota is critical in maintaining the distribution of specific subsets of EECs (including GLP-1, GIP and 5-HT expressing cells) along the GIT, and that a single commensal bacterial species (Bt) can normalise the altered distribution and hormone secretion of these cells observed in GF mice (Modasia, Parker et al. 2020). The ability of gut microbiota to influence EEC networks and hormone secretion is further supported by several other studies (Wichmann, Allahyar et al. 2013, Aktar, Parkar et al. 2020). Similarly, dietary supplementation in mice with fermentable carbohydrates increases the density of PYY cells and PYY secretion in a FFAR2 dependent manner, highlighting the role of SCFAs in mediating these effects (Brooks, Viardot et al. 2017). Indeed, the findings in Chapter 2 show that the effects of Bt on EEC networks and populations in GF mice are replicated by administering the major fermentation products of Bt (acetate, propionate and succinate) (Modasia, Parker et al. 2020). The ability of SCFAs to directly modulate hormone secretion has also been demonstrated in several other studies. For example, acetate, propionate and butyrate have been shown to induce GLP-1 secretion from *in vitro* primary murine L cell cultures through the activation of FFAR2 and FFAR3 (Tolhurst, Heffron et al. 2012). Similarly, in human enteroendocrine cell lines, propionate and butyrate activated FFAR3 to induce PYY gene expression and secretion (Larraufie, Martin-Gallausiaux et al. 2018). Intriguingly, GLP-1 and PYY co-expressed in the vast majority of L cells, have been shown to be situated in separate granular vesicles, suggesting that L cells can selectively release different hormones. Whether the hormone secreted depends on the signal or receptor pathways activated requires further research (Cho, Robinson et al. 2014). In addition, subcellular analysis of EEC granules revealed that the relative number of vesicles differ considerably amongst EECs (Fothergill, Callaghan et al. 2017). SCFA-induced secretion of peptide hormones has also been demonstrated *in vivo*. Intravenous administration of SCFAs has been shown to induced secretion of GLP-1 and PYY in rodents (Dumoulin, Moro et al. 1998). In a pilot study, rectal administration of sodium acetate resulted in significantly increased plasma GLP-1 and PYY within 60 min (Freeland, Wilson et al. 2010). Given that the majority of GLP-1 is degraded following secretion by dipeptidyl peptidase 4 (DPPIV) (Hansen, Deacon et al. 1999) resulting in less than 10% of gut-derived GLP-1 entering the systemic circulation (Holst 2007), it is more likely that GLP-1 acts locally in a

paracrine manner (Kakei, Yada et al. 2002). The identification of GLP-1 receptor (GLP-1R) expression in murine colonic neurons support this. Moreover, GLP-1 has been shown to stimulate IPANs in culture, suggesting that GLP-1R expressing neurones may serve as sensory neurones responding indirectly via the secretion of GLP-1 from neighbouring L cells to respond to luminal microbial metabolites (Amato, Cinci et al. 2010).

The expression of FFARs has been reported to differ between EEC subtypes, with regional variations also observed. For example, in the proximal small intestine, FFAR3 expression is enriched in Cck, GIP and secretin expressing cells, whereas throughout the colon, FFAR3 is strongly expressed in the majority of GLP-1 and PYY expressing cells (Nohr, Pedersen et al. 2013). Interestingly, analysis of fluorescent activated cell sorted (FACs) purified murine EC cells revealed that small intestinal EC cells lack nutrient receptors, whereas EC cells from the colon expressed several GPCR sensors of microbial metabolites, including FFAR2 (Lund, Egerod et al. 2018). GLP-1R was identified to be highly expressed in EC cell populations, further supporting the paracrine role for GLP-1. Indeed, 5-HT secretion from EC cells is stimulated following exposure to a GLP-1R agonist. This indicates that in the small intestine at least, luminal nutrients and microbial metabolites do not stimulate EC cells directly and instead through paracrine pathways involving GLP-1 secretion (Lund, Egerod et al. 2018). This is in line with previous studies demonstrating that SCFAs do not effectively stimulate 5-HT secretion from isolated EC cells (Martin, Lumsden et al. 2017, Martin, Lumsden et al. 2017).

Although, EC cells are the main producers of 5-HT in the body, some bacterial species from the *Escherichia*, *Streptococcus* and *Enterococcus* genus can also synthesise 5-HT (Tsavkelova, Klimova et al. 2006), raising the question of whether these microbes, through *de novo* synthesis contribute to host 5-HT levels. For example, plasma 5-HT levels are significantly lower in GF mice compared to conventionally raised (CONV-R) mice (Wikoff, Anfora et al. 2009, Sjogren, Engdahl et al. 2012, Yano, Yu et al. 2015), with GF rats also displaying morphologically larger EC cells compared to SPF rats (Uribe, Alam et al. 1994), suggesting that gut microbiota can possibly affect the development and function of EC cells. Other components in the serotonergic system are also implicated in GF mice. For example, plasma concentrations of tryptophan are

increased by 40-60% in GF mice compared to CONV-R mice (Wikoff, Anfora et al. 2009). Yano et al., also demonstrated that GF mice have reduced colonic expression of Tph1, elevated expression of the 5-HT transporter SLC6A4 and increased concentration of tryptophan in serum and faecal samples, suggesting that the reduction in serum 5-HT levels are a result of disruption to 5-HT synthesis (Yano, Yu et al. 2015). Colonisation of GF with microbiota isolated from SPF mice restores the disruption of 5-HT levels, Tph1 and SLC64A expression seen in GF mice (Yano, Yu et al. 2015). They also demonstrated that mice colonised with spore-forming bacteria isolated from SPF mice also restores the disruptions in GF mice (Yano, Yu et al. 2015). Further research using an *in vitro* model of EC cells (RIN14B cell) demonstrated that several microbial metabolites of spore-forming bacteria, including butyrate and propionate enhanced 5-HT levels and Tph1 expression (Yano, Yu et al. 2015). EC cells also express GABA receptors and can directly sense luminal GABA produced by some bacteria species, such as the gram-positive *L. brevis* and *B. dentium* (Nakajima, Tooyama et al. 1996, Barrett, Ross et al. 2012). GABA receptor activation has also been shown to induce 5-HT secretion from EC cells (Schwörer, Racké et al. 1989). EECs are also able to detect other signals from the gut microbiota through TLRs that recognised bacterial products such as LPS. In human and murine EECs, TLR-2 and TLR-4 are expressed on the apical surface. Moreover, in the STC-1 enteroendocrine cell line, LPS has been demonstrated to induce TLR-4 dependent secretion of CCK (Bogunovic, Davé et al. 2007).

Taken together, these studies support the role of EECs in the sensing of luminal microbial products, modulating communication between the gut and the brain (Fig. 1.7). Further research is needed to determine the full extent of heterogeneity within and across EEC populations, particularly in terms of hormone co-expression, granular storage, and receptor expressions, and to determine if these are also regulated by gut microbiota and their products.

1.7.4.2. The HPA axis

The HPA axis regulates processes in response to physiological and physical stress (Vale, Spiess et al. 1981, Rivier and Vale 1983). The key regulators of the stress response are localised in the PVN of the hypothalamus, pituitary gland and adrenal

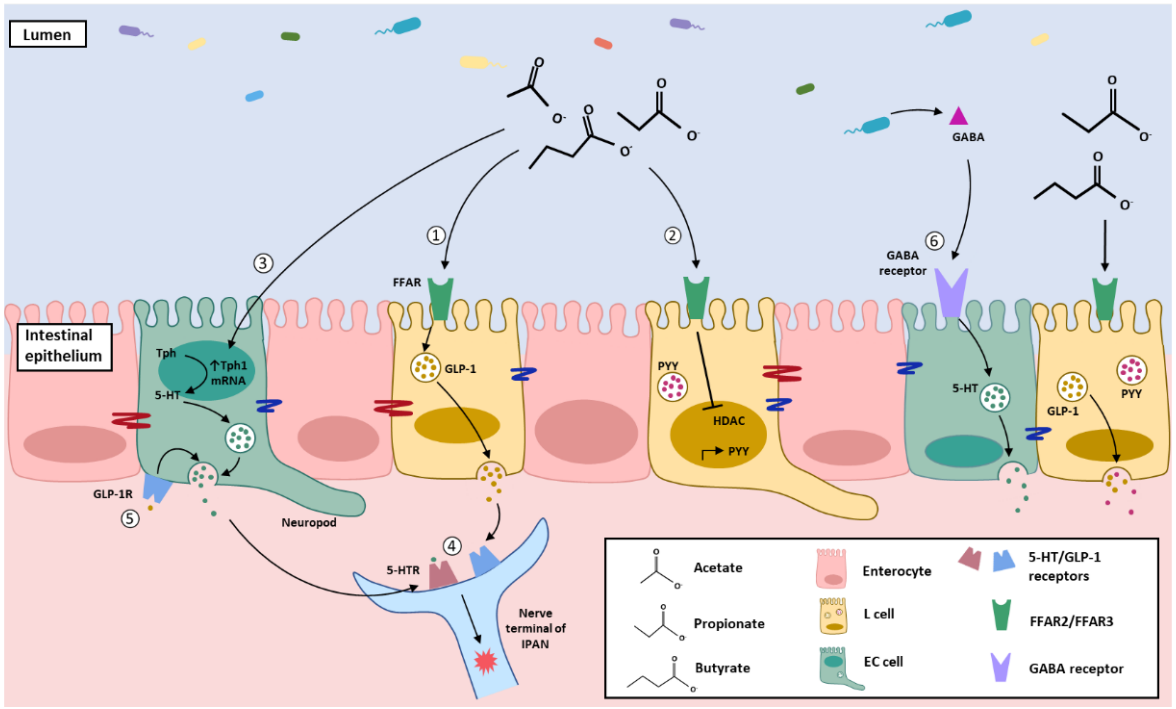

Figure 1.6 Microbial signalling via intestinal enteroendocrine cells. (1 and 2) Through activation of FFARs expressed on the apical membrane of EECs, microbial fermentation products (namely SCFAs) can induce the expression, production, and secretion of GLP-1 and PYY from L cells. GLP-1 and PYY have been shown to be stored in separate secretory granules in L cells, suggesting that L cells can selectively secrete different hormones. (3) SCFAs have been shown to upregulate the expression of Tph1 in small intestinal EC cells, thereby increasing serotonin production and secretion. (4) GLP-1 and 5-HT receptors expressed on afferent nerve terminals of IPANs indicate that GLP-1 and 5-HT can act in a paracrine manner to induce excitability in IPANs. (5) Small intestinal EC cells lack many of the nutrient receptors of their apical surface. The presence of GLP-1 receptor expressed on the basolateral membrane of EC cells indicate that SCFAs can indirectly induce secretion of 5-HT through the secretion of GLP-1 from adjacent L cells. (6) EC cells also express GABA receptors to directly sense luminal GABA produce by some bacterial species, including *L. brevis* and *B. dentium*, to induce 5-HT secretion.

gland (Smith and Vale 2006). Hypophysiotropic neurones localised in the PVN of the hypothalamus, produce and secrete corticotrophin-releasing factor (CRF) in response to stress (Vale, Spiess et al. 1981, Rivier and Vale 1983). The binding of CRF to CRF-receptors in the pituitary gland stimulates the secretion of adrenocorticotrophic hormone (ACTH) into the systemic circulation, primarily targeting the adrenal cortex and inducing glucocorticoid synthesis and secretion. Glucocorticoids, cortisol in humans and corticosterone in rodents, are steroid hormones that regulate metabolic, cardiovascular, immune and behavioural responses (Sapolsky, Romero et al. 2000) and are the main downstream effectors of the HPA axis (Munck, Guyre et al. 1984). They play a key role in regulating the magnitude and duration of HPA axis stimulation (Keller-Wood and Dallman 1984).

Comparisons of HPA responsiveness in GF and SPF mice show distinct differences in HPA sensitivity modulated by the absence of the gut microbiota in GF mice. Moreover, male GF mice display a more sensitive HPA response and reduced protein expression of BDNF in the cortex and hippocampus. Whereas female GF mice exhibit increased hippocampal BDNF mRNA (Neufeld, Kang et al. 2011, Neufeld, Kang et al. 2011, Clarke, Cryan et al. 2012), suggesting that some of these differences in GF mice are sex dependent. Comparisons between GF and SPF mice have revealed increased exploratory behaviour in GF mice in an open-field activity box and reduced anxiety-like behaviour in light-dark box test (Diaz Heijtz, Wang et al. 2011). These results are replicated when BALB/c mice are orally administered a course of antibiotics for 7-days (Bercik, Denou et al. 2011), also resulting in alterations in BDNF levels. Alterations in intestinal microbial composition are also observed, particularly increased abundance of *Lactobacillus* and reduction of γ-Proteobacteria. These alterations are not associated in changes in intestinal inflammatory cytokines, suggesting that the microbial-associated changes in behaviour and expression are not dependent on intestinal inflammation (Bercik, Denou et al. 2011).

1.8. Alzheimer's disease (AD)

AD is the most prevalent form of dementia worldwide (Karlawish, Jack et al. 2017). The main pathophysiological characteristics of AD include extracellular accumulation of amyloid-β (Aβ) plaques and intracellular accumulation of hyperphosphorylated tau

protein, that begins in the trans-entorhinal cortex, progressing to the hippocampus and cortical regions (Braak, Braak et al. 1993, Sperling, Aisen et al. 2011). Despite the significant public health issue that AD poses, only a handful of treatments are available and only act to enhance the quality of life by controlling symptoms rather than controlling the progression of disease (Briggs, Kennelly et al. 2016). At present, genetic, and environmental factors are considered to play a role in AD pathogenesis, however, the underlying mechanisms of Aβ deposition leading to neurodegeneration and cognitive decline remain to be fully elucidated.

1.8.1. Microbial alterations in AD

Recent studies have identified a link between the gut microbiota and the onset and progression of AD, summarised in Table 1.1. One of the first studies to characterise bacterial taxonomic composition in faecal samples from AD patients, revealed that compared to healthy age-matched controls, the gut microbiome in AD participants had reduced microbial diversity and decreased relative abundance of the phyla Firmicutes and Bacteroidetes and increased *Bifidobacterium* genus. These correlated with cerebrospinal fluid (CSF) biomarkers of AD pathology (Vogt, Kerby et al. 2017). Microbial compositional changes in AD are also observed, although different, in another clinical study. Reported here were reductions in the phyla Bacteroidetes and increases in Actinobacteria, but also alterations at a family level, specifically increases in abundance of *Ruminococcaceae*, and *Enterococcaceae*, and reductions in *Lachnospiraceae*, *Bacteroidaceae* and *Veillonellaceae* (Zhuang, Shen et al. 2018). Comparisons of faecal microbiomes between individuals showing cognitive decline associated with brain amyloidosis (+Amy), cognitive decline associated with no brain amyloidosis (-Amy) and healthy controls participants also revealed significantly reduced abundances in *Eubacterium rectale* and increases in *Escherichia/Shigella* in +Amy patients compared to -Amy and healthy controls, but also a reduction in *B. fragilis* compared to healthy controls (Cattaneo, Cattane et al. 2017). Microbial composition has also been associated with altered cognition, with increased proportions of Firmicutes and Verrucomicrobia associated with beneficial effects on cognitive function, whilst higher proportions of Bacteroidetes and Proteobacteria suggested to be less beneficial (Manderino, Carroll et al. 2017).

The apolipoprotein E (APOE) genotype is considered a strong genetic risk factor for AD (Neu, Pa et al. 2017, Pontifex, Vauzour et al. 2018). Indeed, Vogt et al., reported a high prevalence of the APOE4 genotype in their AD participants (72 %) compared to healthy controls (20 %) (Vogt, Kerby et al. 2017). The ApoE protein is involved in multiple physiological processes including lipoprotein metabolism (Raffai, Dong et al. 2001) and neuronal modulation (Hui, Harmony et al. 1980, Jofre-Monseny, Minihane et al. 2008). Until recently the impact of APOE genotype on intestinal barrier function and microbiome composition were unknown. Tran et al., investigated the faecal microbiome composition of human APOE genotypes with healthy matched individuals. Although no significant differences were observed in the overall microbe composition, specific associations between individual APOE genotypes were observed. For example, the human APOE3/E3 carries were associated with higher relative abundances of *Prevotellaceae*. Whereas APOE2/E3 genotype was associated with higher relative abundances of *Ruminococcaceae* compared to human APOE4 carriers (Tran, Corsini et al. 2019). In line with previous reports of a negative correlation of these bacterial taxa with AD (Vogt, Kerby et al. 2017). Further analysis conducted in APOE-genotyped mice revealed lower overall concentrations of SCFAs in APOE4 mice, similar to that observed in human APOE4/E4 carriers (Tran, Corsini et al. 2019). This warrants further investigation as SCFAs have been demonstrated to prevent Aβ aggregation *in vitro* (Ho, Ono et al. 2018).

Another major risk factor for AD is age. Analysis of the faecal microbiome from elderly participants revealed a significant shift towards a *Bacteroides*-predominated population (Claesson, Cusack et al. 2011) and in centenarians, reductions in the anti-inflammatory *F. prausnitzii* are also reported (Biagi, Nylund et al. 2010). Proinflammatory cytokines are associated with cognitive impairment (Soares, Potter et al. 2012, Leung, Proitsi et al. 2013, Tan, Yu et al. 2013, Chen, Na et al. 2015, Clark and Vissel 2015) and increased levels of IL-6, IL-1β, NLR family pyrin domain containing 3 (NLRP3) and chemokine ligand 2 (CXCL2) have been reported in patients showing brain amyloidosis-associated cognitive decline (Cattaneo, Cattane et al. 2017).

1.8.2. Role of gut microbiota in AD pathology

There are several mouse models of AD that provide a useful tool to study microbial alterations and targeted interventions for AD, summarised in Table 1.2. The APP/PSI transgenic mouse model that overexpresses Aβ is commonly used to model amyloid pathology associated with AD. CONV-R APP/PSI mice begin to develop Aβ-aggregates in cerebrum as early as 6-weeks of age and continue to accumulate throughout development and adulthood (Radde, Bolmont et al. 2006). Accompanying this are compositional changes in the gut microbiota including significant reductions in abundance of Firmicutes, Verrucomicrobia and Proteobacteria, and increased abundance of Bacteroidetes (Harach, Marungruang et al. 2017) in line with those observed in human clinical studies of AD (Vogt, Kerby et al. 2017, Zhuang, Shen et al. 2018). It is important to note that whilst there are many similarities in physiology and anatomy of the GIT of rodents and humans, there are significant differences with respect to resident microbial communities (Nguyen, Vieira-Silva et al. 2015). Therefore, comparing taxa and abundances between animal and human studies may not be advantageous. To further understand the association between the gut microbiome and Aβ pathology, GF APP/PSI mice have been studied to reveal significant reductions in Aβ accumulations in the cerebrum. Alterations in neuroinflammation are also observed in these mice, with a reduction in microglia activity as demonstrated by reduced Iba-1^{+} immunostaining and levels of proinflammatory cytokines (Harach, Marungruang et al. 2017). Furthermore, GF APP/PSI mice have significantly higher levels of Aβ-degrading enzymes, neprilysin degrading enzyme (NDE) and insulin degrading enzyme (IDE), thus providing some insight into mechanisms by which Aβ deposition is reduced in GF APP/PSI mice (Harach, Marungruang et al. 2017). Early postnatal antibiotic treatment in APP/PSI mice also demonstrate alterations in gut microbial composition (reduced α-diversity accompanying increased abundance of genus *Akkermansia* and family *Lachnospiraceae*), inflammatory mediators that correlate to a reduction in Aβ pathology (Minter, Hinterleitner et al. 2017). These alterations are also accompanied by a reduction in plaque-localised glial cell reactivity in the brain, suggesting that alterations in astrocytes and microglia may affect the cellular activity surround Aβ plaques following antibiotic treatment (Minter, Hinterleitner et al. 2017). A reduced microbial diversity has been implicated in major alterations in microglia morphology

and homeostasis (Erny, Hrabe de Angelis et al. 2015) and alterations in microglia-related genes, including CD86, are associated with AD (Hollingworth, Harold et al. 2011, Naj, Jun et al. 2011), indicating glial cell involvement in AD pathology. To further confirm microbial involvement in amyloid pathology, colonisation of GF APP/PSI mice with microbiota from CONV-R APP/PSI mice resulted in increased Aβ pathology. Accompanying these changes in Aβ pathology were microbial alterations, specifically reduced abundance in *Rikenellaceae*, *Ruminococcus* and *Bacteroides* and an overall slower development of microbial diversity (Harach, Marungruang et al. 2017). These studies indicate that specific microbes might be involved in the progression of Aβ deposition.

Probiotic administration in a murine model for IgA Nephropathy (ddY mice) has been shown to induce protective effects against Aβ induced pathology. Here, *B. breve* administration in Aβ-ddY mice was shown to prevent Aβ induced cognitive dysfunction, which correlated to increased plasma acetate levels and short-term alterations in microbial composition, namely proportions of Bifidobacteriaceae, *Odoribacteraceae* and *Lachnospiraceae*, suggesting that other mechanisms maybe involved in Aβ pathology (Kobayashi, Sugahara et al. 2017). Whether *B. breve* administration supressed Aβ accumulation in these mice was not explored in this study. Moreover, the AD murine model used in this study doesn't reflect the pathology of AD like other transgenic murine models do therefore the potential therapeutic effects of *B. breve* should be explored in other AD models (such as APP/PSI and 5xFAD models) (Kobayashi, Sugahara et al. 2017). Similar effects are observed following *C. butyricum* treatment in APP/PSI mice. *C. butyricum* significantly ameliorated cognitive deficits, reduced deposition of Aβ42 in the brain, supressed microglia activation and reduced levels of inflammatory IL-1β and TNF-α in the brain. These molecular and biochemical alterations from *C. butyricum* administration were accompanied by changes in the gut microbiota, including reduced abundances of *Helicobacteraceae* and increased abundances of *Alloprevotella*, coinciding with increased faecal butyrate levels (Sun, Xu et al. 2020).

Human clinical trials studying the effects of probiotic supplementation in AD patients however has varying outcomes. In a randomised double-blind controlled trail, 12-week probiotic supplementation, containing a mixture of *Lactobacillus and Bifidobacterium*

strains/species, improved cognitive function in AD patients, although no significant effect on the biomarkers on oxidative stress and inflammation (Akbari, Asemi et al. 2016). However, this conflicts with another similar study whereby probiotics containing mixtures of *Lactobacillus* and *Bifidobacterium* strains given for 12-weeks to AD patients. No improvement in cognitive function was observed in the probiotic supplemented group and serum inflammatory cytokines, including TNF-α, IL-6 and IL-10, and serum nitric oxide levels were also unaltered. The patients recruited in this study however, displayed severe AD pathology. It is possible that severe AD patients are insensitive to probiotic supplementation and it could be the case that longer treatment periods and/or higher dosage is needed for significant effects on cognitive function in AD patients displaying severe disease symptoms (Agahi, Hamidi et al. 2018). It is also worth noting that accompanying microbial analysis was not undertaken in these clinical trials. These varying effects reiterate the requirement for further clinical trials to assess whether probiotics can have a clinically significant effect on cognitive outcomes and biomarkers in AD.

1.8.3. Microbial mediators in AD pathology

The mechanisms that gut microbiota utilise to regulate AD pathology are unclear, but bacterial metabolites are believed to be potential mediators. A summary of studies investigating the role of microbial fermentation products in AD pathology are displayed in Table 1.3. Supplementation with prebiotics enhances the formation of SCFAs. For example, mannose oligosaccharide (MOS) supplementation in a transgenic 5xFAD murine model of AD was shown to increase serum and faecal butyrate levels through alterations in the gut microbiota, principally increased abundances of butyrate-producing bacteria including *Clostridium pasteuridrum*, *Lachnospira*, *Phascolarctobacterium* and Veillonellaceae (Liu, Xi et al. 2021). These changes were accompanied by improved cognitive function, spatial memory, and anxiety-like behaviours and histopathological changes including reduction of Aβ accumulation in the brain, Iba-1^{+} microglia, mRNA expression of TNF-α and IL-6, serum corticosterone (CORT) levels and increased serum NE levels and gut barrier integrity. These results indicate that MOS supplementation alleviate neuroinflammation and restore hormonal imbalance in the HPA axis in AD mice (Liu, Xi et al. 2021). SCFAs have also been shown to restore microglia abnormalities in GF

mice (Erny, Hrabe de Angelis et al. 2015) supporting the role of microbial metabolites as mediators in gut-brain signalling in neurodegenerative diseases. Indeed, butyrate has been identified as a potent neuromodulator in the CNS, with recent evidence indicating it's anti-inflammatory and neuroprotective effects in *in vivo* and *in vitro* models of AD (Chuang, Leng et al. 2009). The anti-inflammatory effects of *C. butyricum* can also be replicated directly by butyrate treatment. In Aβ-induced BV-2 cells, butyrate treatment suppressed the activation of microglia (Sun, Xu et al. 2020). The neuroprotective effects of butyrate are further supported by studies demonstrating its ability to readily cross the BBB (Cremer, Lai et al. 1977, Collins, Pearson et al. 1995, Butler and Bates 2006) and has been shown to reverse spatial memory deficits in another transgenic murine model of AD (Tg2576 mice) through mechanisms involving increased acetylation of histone 4 (AcH4), reduced phosphorylation of hippocampal tau via the glycogen synthase kinase 3β (GSK3β) pathway and enhanced expression of synaptic plasticity markers GluR1 and post-synaptic density protein 95 (PSD95) (Ricobaraza, Cuadrado-Tejedor et al. 2009). These results indicate butyrate regulates histone modification to recover the loss of memory deficits in AD mouse models and support the feasibility of modulating gut SCFAs for potential treatments for AD.

In addition to microbial fermentation products, dysfunction of the GABAergic system may contribute to the cognitive impairment observed in AD patients (Lanctôt, Herrmann et al. 2004). Post-mortem analysis of AD patients has revealed that GABA levels are reduced in the frontal, temporal, and parietal cortex (Lanctôt, Herrmann et al. 2004, Solas, Puerta et al. 2015). Whether reduced GABA levels correspond to a reduction in GABA-producing species (*L. brevis* and *B. dentium*) are not known. The increased abundances of *Bifidobacterium* in AD patients reported (Vogt, Kerby et al. 2017) requires further investigations to determine specific species are increased, and whether there are other mechanisms contributing to reduced GABA levels in AD patients, such as impaired production of GABA by metabolism of glutamate.

Loss of tight junctions leading to a compromised BBB has been linked to AD (Banks, Robinson et al. 2003, Deane, Wu et al. 2004, Bell, Sagare et al. 2007, Jaeger, Dohgu et al. 2009). Transport across the BBB plays a key role in regulating the composition of brain interstitial fluid (ISF) and under normal physiological conditions, Aβ

concentration in the ISF is regulated by BBB transporters receptor for advanced glycation end products (RAGE) and low-density lipoprotein receptor related protein-1 (LRP-1) (Shibata, Yamada et al. 2000, Deane, Du Yan et al. 2003, Deane, Wu et al. 2004). RAGE is the primary influx transporter, allowing uptake of Aβ from the blood to the brain, whereas LRP-1 is the main Aβ efflux transporter, clearing Aβ from the brain (Deane, Wu et al. 2004). Indeed, hippocampal RAGE expression is increased (Miller, Tavares et al. 2008) and LRP expression is reduced in AD patients (Donahue, Flaherty et al. 2006). Therefore, the impaired BBB transport of Aβ peptides, which could contribute to accumulation of Aβ in the brain, has been proposed to play a role in AD pathogenesis. The underlying cause of altered Aβ transport across the BBB is not clear but neuroinflammation has been suggested to play a role.

LPS has been put forward as a causative agent for inducing neuroinflammation in AD pathology (Zhan, Stamova et al. 2016, Cattaneo, Cattane et al. 2017). Table 1.4 outlines key studies demonstrating the role of LPS in AD pathology. Systemic inflammation induced by repeated intraperitoneal administration of LPS in mice was shown to increase brain uptake and reduce brain clearance of I-Aβ, that was shown to be dependent on transport via LRP. In addition to this, various serum cytokines, including IL-10, IL-6, regulated on activation normal T cell expressed secreted protein (RANTES) and macrophage chemotactic peptide-1 (MCP-1), were also increased. Further analysis using BMEC cell monolayers revealed that LPS does not affect I-Aβ efflux *in vitro*, indicating that LPS doesn't act directly upon brain endothelial cells to affect I-Aβ efflux *in vivo*, suggesting that other mechanisms, including regulation of peripheral cytokines, that LPS could induce to indirectly alter transport across the BBB (Jaeger, Dohgu et al. 2009).

LPS also induces concentration and time dependent Aβ fibrillogenesis and toxicity *in vitro* culture with Aβ fragments. These results were replicated upon incubation of Aβ fragments with *E. coli*, verifying the effects of LPS were reproducible by bacteria (Asti and Gioglio 2014). The role of bacterial endotoxins in AD pathology is further highlighted by presence of endotoxins within senile plaque lesions of AD brains (Schwartz 2013, Asti and Gioglio 2014). Further evidence supporting interactions between bacterial LPS and Aβ peptides is demonstrated in mice intraperitoneally injected with LPS resulting in increased levels of hippocampal Aβ, central and

peripheral proinflammatory cytokines levels and cognitive dysfunction (Kahn, Kranjac et al. 2012).

Although elevated levels of LPS have been detected in AD brains, a very small amount of peripheral LPS passes through the BBB (Singh and Jiang 2004, Banks and Robinson 2010), suggesting LPS could be delivered via alternate means such as BEVs (Zhan, Stamova et al. 2016). Recent attempts to characterise BEVs in AD patients revealed significant alterations in the metabolic profile of BEVs in AD patients compared to age and sex matched healthy controls, of which levels of aspartate, L-glutamate and imidazole-4-acetate in particular were significantly upregulated in AD-BEVs (Wei, Wei et al. 2019). BEVs isolated from faecal samples from AD patients and administered in mice demonstrated the involvement of AD-BEVs in cognitive function, enhancement of hippocampal tau phosphorylation via activation of GSK-3β and increased astrocyte and microglia activation. It was further uncovered that AD-BEVs induce these effects via disruption of the BBB as evidenced by increased Evans blue concentration and reduction in claudin-5 expression in the brain (Wei, Peng et al. 2020). Recently, BEVs isolated from *Paenalcaligens hominis* (*P. hominis*), an abundant species found in elderly patients, delivered in C57BL/6 mice resulted in impairment of cognitive function and increased microglia activation as demonstrated by increased numbers of $NFkB^{+}$/Iba-1^{+}, $TLR4^{+}$/Iba-1^{+} and LPS^{+}/Iba-1^{+} cells, suggesting *P. hominis* derived BEVs acts via activation of NFkB and TLR-4 pathways. Levels of hippocampal BDNF were also shown to be reduced by *P. hominis* BEVs. Vagotomy inhibited *P. hominis* BEVs induced cognitive decline and reduced levels of bacterial 16S rDNA in the hippocampus, implicating the role of the vagus nerve in BEV-induced cognitive decline (Lee, Kim et al. 2020). A summary of these two studies is displayed in Table 1.5.

1.8.4. EEC signalling in AD pathology

The enteroendocrine pathway has also been proposed as a contributor in gut-CNS signalling implicated in AD pathology, summarised in Table 1.6. Indeed, mRNA expressions of colonic FFAR2 and FFAR3 were upregulated in MOS treated 5xFAD-mice, consistently with increased GLP-1 mRNA expression (Liu, Xi et al. 2021).

Signalling via GLP-1R in the brain is associated with enhanced learning and neuroprotection (During, Cao et al. 2003).

Moreover, T2D has been implicated as a significant risk factor for AD. Brain levels of insulin and insulin receptor (IR) are lower in AD patients (Moloney, Griffin et al. 2010) and murine models of AD (Lester-Coll, Rivera et al. 2006). Supporting this, Aβ oligomers induce loss of IRs in neuronal processes of primary rat hippocampal cultures, demonstrating that CNS neurones display major alterations in insulin signalling upon exposure to Aβ oligomers (Zhao, De Felice et al. 2008). It is likely that Aβ accumulation in AD brain also induces similar effects on IRs, however, the nature of Aβ binding and the downstream mechanisms of how it triggers loss of IRs remain unclear. Analysis of brains from AD patients revealed increased serine phosphorylation of the insulin receptor substrate 1 (IRS-1/pSer) (Bomfim, Forny-Germano et al. 2012), a central feature of peripheral insulin resistance (Hirosumi, Tuncman et al. 2002). Intracerebroventricular administration of Aβ oligomers in non-human primates, also induces neuronal IRS-1/pSer in the hippocampus and temporal cortex, (Bomfim, Forny-Germano et al. 2012). ISR-1/pSer is associated with the activation of c-Jun-N-terminal kinases (JNK) (Hirosumi, Tuncman et al. 2002), and increased hippocampal pJNK have been detected in AD human brains and non-human primates administered Aβ (Bomfim, Forny-Germano et al. 2012). Further analysis in APP/PSI murine model of AD also revealed increased hippocampal IRS-1/pSer and pJNK, which is significantly reduced upon Exendin-4 (a GLP-1R agonist) administration, accompanied by improved cognitive function and Aβ plaque load in the brain (Bomfim, Forny-Germano et al. 2012). Further studies utilising knockout models of IRS-1 or JNK can provide mechanistic insight into insulin resistance in AD pathology.

Similarly, intraperitoneal injection with a GLP-1R agonist, liraglutide (commonly used as a T2D treatment), was shown to significantly reduce the histological hallmarks of AD and brain APP levels in APP/PSI mice, implying that Aβ synthesis is likely reduced. It is also possible that Aβ clearance is enhanced although expression of LRP-1 in the brain wasn't measured. Liraglutide treatment also reduced neuroinflammation, enhance neurogenesis and synaptic plasticity and increased GLP-1 levels in the brain of APP/PSI mice (McClean, Parthsarathy et al. 2011). Similar findings are also

observed following Val(8)GLP-1 (a stable analogue for GLP-1) administration in APP/PSI mice. Strong induction of long-term potentiation (LTP) in CA1 region of the hippocampus is also observed in this study (Gengler, McClean et al. 2012). Furthermore, Val(8)GLP-1 was shown to prevent the detrimental effects of Aβ peptides fragments have on hippocampal LTP in rats (Gault and Hölscher 2008). These studies demonstrate the direct effects of GLP-1R signalling in neurotransmission in the brain. In a double-blind clinical trial with AD patients, 6-month treatment with liraglutide, was shown to significantly reduce the decline of brain glucose metabolism in the cingulate cortex and cerebellum although no significant changes in cognitive function were reported between treatment and placebo control groups (Gejl, Gjedde et al. 2016) (Table 1.7). The lack of significant results in this study could be due to the advanced stage of AD in the patient's cohorts together with uneven randomisation of disease duration in patients (Gejl, Gjedde et al. 2016), reiterating the need for large long-term controlled clinical trials to evaluate the efficacy of GLP-1R agonists in AD cohorts.

1.8.5. Constraints in current AD research and future directions

Control matching AD participants is critical in microbiome studies. Although most of the studies age and sex match participants recruited, medication intake is often disregarded. For example, Vogt et al., reported differences in the participants recruited with respect to the use of selective serotonin reuptake inhibitor (SSRIs) and AD mediations (Vogt, Kerby et al. 2017). Despite not reporting any difference in microbial richness, diversity or relative abundance between AD participants taking medication or not, the impact of medication on the results cannot be completely ruled out, especially as some AD medication is known to cause some side effects (Hansen, Gartlehner et al. 2008). Most AD clinical studies use faecal sampling alone to determine intestinal microbial composition, and many studies do not look at other factors such as diet, environment and other medical diagnosis that influence the human microbiome. The method used to analyse microbiome populations is also important. Broader 16S rRNA sampling approaches generally deemed better than quantitative PCR-based approaches.

Overall, animal models provide a valuable tool in AD research, but it is important to interpret the results with caution. It is important that AD models simultaneously display neuropathological and behavioural alterations as observed in AD patients (Kokjohn and Roher 2009). The development of APP/PSI transgenic line provides a promising mouse model for AD, as mice develop both Aβ plaques and behavioural deficits (Jankowsky, Fadale et al. 2004). The transgenic 5xFAD line provides a more rapidly progressing model of AD, with mice displaying amyloid pathology and cognitive deficits by 4 months of age. Moreover, 5xFAD mice display neuronal loss that is not observed in APP/PSI mice (Oakley, Cole et al. 2006). Both APP/PSI and 5xFAD models are commonly used as they reliably recapitulate amyloid pathology. However, a major drawback is that they don't display tau pathology often associated with AD pathology in humans (Naseri, Wang et al. 2019). The 3xTag line combines the APP/PSI and tau transgenes with mice progressively displaying amyloid and tau pathology. Although the tau mutation in 3xTag line is not associated in human AD, this line better mimics AD neuropathology (Lewis, Dickson et al. 2001, Oddo, Caccamo et al. 2003) and therefore provides a progressive tool for use in pre-clinical trials. Intracerebroventricular injection with Aβ can also be used to model AD-like pathology and cognitive impairment and can help focus on the downstream pathology arising from amyloid abnormalities (Kim, Lee et al. 2016). Compared to transgenic mouse models of AD, intracerebroventricular injection of Aβ allows researchers to determine and control the concentration of Aβ and enables simultaneous testing of a large group of animals. A caveat to this method is the acute surge in Aβ that doesn't faithfully recapitulate the chronic progression of AD in humans (Kim, Lee et al. 2016). Further studies could also develop approaches to co-administrate amyloid peptides and tau proteins, to better mimic the pathology of AD.

The results discussed here demonstrate complex and varying interactions between the gut microbiota and progression and severity of AD pathology. Epidemiological studies have provided the first steps to uncover to what extent the gut microbiota affects AD pathology and studies have begun to assess disease progression and outcome through modulation of the microbiota via diet or probiotic interventions. Larger interventional trials are warranted, particularly with participants in the early stages of AD progression. Further animal experiments and longitudinal human studies using multidisciplinary approaches are needed to determine the cause-effect relationship

between the gut microbiota and the neuropathogenesis of AD. Understanding this will help develop novel microbial interventional approaches for the treatment and prevention of AD.

1.9. Parkinson's disease (PD)

PD is a progressive neurodegenerative disease characterised by tremor, rigidity of movement and progresses to cognitive and motor decline, leading to the onset of dementia (Cheng, Ulane et al. 2010, Ding, Ding et al. 2015). The molecular and biochemical hall marks of PD include degeneration of dopaminergic neurones and accumulation of α-synuclein containing cytoplasmic inclusions called Lewy bodies, (Angot and Brundin 2009, Bisaglia, Mammi et al. 2009). Non-motor symptoms such as constipation, defecation dysfunction, weight loss and gastroparesis (Sung, Park et al. 2014, Kim, Kim et al. 2015) are prevalent in PD patients. Current treatments for PD include dopamine replacement, which although initially effective in reducing motor symptoms, it serves to delay and not prevent the progression of PD. Evidence of α-synuclein inclusions in the ENS prior to PD onset suggests that these progress to the CNS via the vagus nerve, suggesting that the pathogenesis of PD begins in the gut (Braak, Rüb et al. 2003, Shannon, Keshavarzian et al. 2012).

1.9.1. Microbial alterations in PD

It is now well recognised that gastrointestinal dysfunction occurs in many PD patients prior to the onset of CNS motor-symptoms (Abbott, Petrovitch et al. 2001, Jost 2010, Liddle 2018). Microbial alterations in the gut microbiome of PD patients is evident and is summarised in Table 1.8 (Hasegawa, Goto et al. 2015, Keshavarzian, Green et al. 2015, Scheperjans, Aho et al. 2015, Unger, Spiegel et al. 2016, Bedarf, Hildebrand et al. 2017, Hill-Burns, Debelius et al. 2017, Vascellari, Palmas et al. 2020). One of the first clinical studies looking at the microbiome in PD patients reported that the reduced abundance of *Prevotellaceae* and increased abundance in *Enterobacteriaceae* is positively associated with severity of PD symptoms including postural instability and gait. This suggests that specific alterations within bacterial communities may be associated with PD pathology and disease severity (Scheperjans, Aho et al. 2015). Other studies have also reported similar alterations in the gut microbiome of PD patients (Unger, Spiegel et al. 2016, Bedarf, Hildebrand et al. 2017). The Enterotype

Prevotella is associated with increased abundance of neuroprotective SCFAs and the biosynthesis of vitamins folate and thiamine (Arumugam, Raes et al. 2011, Cryan and Dinan 2012), which could also help explain deficiencies in folic acid and thiamine frequently observed in PD patients (Leung, Proitsi et al. 2013). Interestingly, hydrogen sulphide secreted by *Prevotella* spp. have neuroprotective effects in rodent models of PD (Hu, Lu et al. 2010, Kida, Yamada et al. 2011).

Taxonomic level analysis of colonic bacterial composition has revealed significant differences in faecal and mucosal microbial community in PD patients compared to control subjects and provides evidence of proinflammatory dysbiosis in PD. Keshavarzian et al., reported that butyrate-producing bacteria from the genera *Blautia*, *Coprococcus* and *Roseburia* were significantly less abundant in PD patients compared to healthy controls. Whereas in the colonic mucosa, reduction in *Faecalibacterium* and increase in Proteobacteria where reported, indicating a potential pro-inflammatory shift in PD patients that could trigger α-synuclein mis-folding and aggregation (Keshavarzian, Green et al. 2015). A larger case-control study by Hill-Burns et al., undertook a systematic analysis of 39 variables as potential factors over 327 subjects. The also examined the microbiome at global, taxonomic, and functional levels, thus helping to eliminate inconsistent reports of microbial alterations. They reported increased abundance of *Akkermansia*, *Ruminococcaceae*, *Lactobacillus* and *Bifidobacterium* and reduced abundance of species from the family *Lachnospiraceae* in faecal samples from PD patients (Hill-Burns, Debelius et al. 2017). Interestingly, Hasegawa et al., reported higher abundances in bacteria from the genus *Lactobacillus*, including *L. gasseri*, *L. brevis*, *L. casei*, *L. fermentum* and *L. reuteri* (Hasegawa, Goto et al. 2015). They also reported reduced members from the genus *Clostridium* (namely *C. coccoides* and *C. leptum*) (Hasegawa, Goto et al. 2015), which conflicts with a recent study reporting increased abundance of *Clostridium* in PD patients (Vascellari, Palmas et al. 2020). Whist certain findings across microbiome studies in PD have been replicated, the inconsistency in some results could be due to variations in study design and methodological approaches used to determine microbial composition, in addition to natural variation in microbial compositions across populations, lifestyles and diet, all of which have the potential to confound the results (Boertien, Pereira et al. 2019). Recently, Romano and colleagues (Romano, Savva et al. 2021) conducted a meta-analysis reanalysing several 16S microbiome datasets from PD studies to evaluate

whether there were common alterations between different cohorts. In agreement with previous studies (Hasegawa, Goto et al. 2015, Keshavarzian, Green et al. 2015, Hill-Burns, Debelius et al. 2017, Vascellari, Palmas et al. 2020), key differences identified included enrichment of the genera *Lactobacillus*, *Akkermansia* and *Bifidobacterium* whilst members belonging to the family *Lachnospiraceae* and genus *Faecalibacterium* were less abundant (Romano, Savva et al. 2021). Intriguingly, *Akkermansia* spp. were reported to be more abundant in patients with constipation (Gobert, Sagrestani et al. 2016, Vandeputte, Falony et al. 2016) and constipation is a key non-motor symptom that is often associated with PD (Sung, Park et al. 2014, Kim and Sung 2015). As a major mucin-degrading bacteria genera, increased abundances of *Akkermansia* may lead to depletion of the intestinal mucus layer, promoting barrier functional disruption (Desai, Seekatz et al. 2016, Cao, Liu et al. 2017). It is important to note however that different strains from species can have different effects on modulation of host responses (Zhai, Xue et al. 2019). The reduction in butyrate-producing species, such as those belonging to the genera *Faecalibacterium* may also correlate to a reduction in colonic-health promoting butyrate observed in PD patients (Hamer, Jonkers et al. 2008, Unger, Spiegel et al. 2016, Schwiertz, Spiegel et al. 2018).

Gut microbial dysbiosis has also been reported in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) murine models of PD (Sun, Zhu et al. 2018). MPTP is a neurotoxin often used to model neurodegeneration of dopaminergic neurones in PD (Smeyne and Jackson-Lewis 2005). Here key alterations including decreases in abundance of Firmicutes and the order Clostridiales, accompanied by increases in Proteobacteria, order Turicibacterales and Enterobacterales (Sun and Shen 2018), in line with those observed in PD patients (Keshavarzian, Green et al. 2015, Unger, Spiegel et al. 2016). Whether microbial dysfunction is a cause or downstream effect of dopaminergic neurodegeneration leading to progressive cognitive decline is still unclear. α-synuclein lesions have been identified in the ENS at very start of disease progression and CNS motor symptoms, indicating that α-synuclein pathology could begin in the ENS then propagating to the CNS via the vagus nerve (Braak, de Vos et al. 2006). This is supported by a *in vivo* study whereby α-synuclein injected into the intestinal wall of rats resulted in transportation of α-synuclein via the vagus nerve to the dorsal motor nucleus of the vagus located in the brainstem (Holmqvist, Chutna et al. 2014). The mechanism of α-synuclein transportation remain unclear, however

dysfunctional BBB has been suggested to play a role in PD pathogenesis, particularly as there are a growing number of studies reporting α-synuclein presence in the CSF and blood plasma of PD patients (Borghi, Marchese et al. 2000, El-Agnaf, Salem et al. 2003, Lee, Lee et al. 2006, Tokuda, Salem et al. 2006, Mollenhauer, Locascio et al. 2011). Conversely, there are also reports that CNS damage itself could result in gastrointestinal dysfunction. For example, direct injection of 6-hydroxydopamine (6-OHDA) in the substantia nigra (SN) in rats was shown to result in impaired gastric motility and emptying. Furthermore, these effects were inhibited in vagotomised rodents supporting the role of the vagus nerve in CNS communication to the gut (Zheng, Song et al. 2014).

1.9.2. Role of gut microbiota in PD pathogenesis

It is unclear what the exact causes of microbial dysbiosis in PD patients are, whether they are caused by dysfunction in CNS resulting in GI dysfunction or extrinsic factors such as diet. Preclinical studies have provided pathophysiological evidence of microbial associations with PD development (Table 1.9). Transgenic α-synuclein over-expressing (ASO) mice can be used to model PD as they display progressive defects in motor function and GI dysfunction (Rockenstein, Mallory et al. 2002, Chesselet and Carmichael 2012). At the molecular level ASO-mice display α-synuclein aggregation, increased microglia activation and increased levels of proinflammatory cytokines (Sampson, Debelius et al. 2016). Generation of ASO-mice under GF conditions inhibits α-synuclein mediated motor and GI dysfunction, reduces α-synuclein pathology in the caudoputamen (CP) and SN and reduced microglia activation. These results are also replicated in ASO-mice treated with antibiotics, indicating that active gut-brain signalling by gut microbiota contributes to α-synuclein mediated disease pathology (Sampson, Debelius et al. 2016).

FMT in ASO mice using microbiota from human PD patients (ASO-PD) exacerbates α-synuclein induced motor deficits with mice also displaying gut microbial dysbiosis including increased abundance of species from the genera *Proteus, Bilophila* and *Roseburia* and decreased abundance of members from the family *Lachnospiraceae*, *Rikenellaceae, Peptostreptococcaceae* and genus *Butyricicoccus*, accompanied also by lower faecal SCFA concentrations (Sampson, Debelius et al. 2016). No

exacerbation of motor deficits was observed in ASO mice transplanted with the gut microbiome from healthy human donors, indicating that a specific repertoire of the gut microbiota in PD patients drives disease outcome in genetically susceptible hosts (Sampson, Debelius et al. 2016).

FMT from healthy mice to MPTP-PD mice has also been shown to exert neuroprotective effects, ameliorating motor impairment through reduced dopaminergic neuronal loss, glial cell activation, striatal TLR-4, NFκB and TNF-α expression and increasing striatal neurotransmitter levels. These effects were suggested to be mediated, in part, by the restoration of gut microbiota dysbiosis and SCFAs concentrations (Sun and Shen 2018), indicating that specific repertoire of microbiota regulates neuroinflammation resulting in neurodegeneration in a MPTP-PD model. Intragastric administration of *C. butyricum* in MPTP-mice also reversed microbial dysbiosis and improved motor deficits by preventing loss of dopaminergic neurones and microglia activation (Sun, Li et al. 2021) *C. butyricum* was also shown to increase synapsin-1 levels, indicating reduced synaptic dysfunction (Sun, Li et al. 2021) which is implicated with the onset of PD (Bouchard, Malykhin et al. 2008). These studies suggest that modulation of the gut microbiota to inhibit excessive microglial activation, neuronal loss synaptic dysfunction, could help alleviate PD impairment.

Recently the novel probiotic formulation containing mixtures of lactic acid bacteria and Bifidobacteria, SLAB51, were tested to determine their neuroprotective effects in 6-OHDA induced model of PD (Castelli, d'Angelo et al. 2020). Here, in neuroblast-like cell line (5H-SY5Y cells) treated with 6-OHDA, SLAB51 was shown to increase the expression of neuroprotective BDNF, and peroxisome proliferator activated receptor γ (PPARγ) (Castelli, d'Angelo et al. 2020). Furthermore, 2-week SLAB51 pre-treatment in an *in vivo* 6-OHDA-mice resulted in recovery of injury to dopaminergic neurones and neuroinflammation, demonstrated by increased tyrosine hydrolase (TH) immunoreactivity and reduced Iba-1^{+} and $GFAP^{+}$ cells, respectively. Moreover, the expression of NFκB and PPARγ in the SN and striatum was observed in SLAB51 pre-treated PD-mice. In addition to neuroprotective and anti-neuroinflammatory effects, SLAB51 induced antioxidant effects demonstrated by restoration of nuclear transcription factor-erythroid 2 related factor (Nrf2), which is a

transcription factor involved in cellular redox status, and its target gene heme oxygenase-1 (HO-1). Reduced apoptosis in the SN further confirmed the neuroprotective effects of SLAB51 (Castelli, d'Angelo et al. 2020). These neurochemical alterations were accompanied by inhibition of behavioural impairments induced by 6-OHDA inoculation, analysed using the cylinder test and elevated body swing test (EBST) to evaluate locomotor asymmetry in rodent models. Collectively these findings indicate that this probiotic formulation elicits inhibitory effects on *in vitro* and *in vivo* 6-OHDA-PD models by modulating neuroprotective, anti-inflammatory and antioxidative pathways, providing some insight into the molecular pathways used to modulate PD behavioural and neuropathology.

In clinical trials however, there is discrepancy in outcomes from probiotic treatment in PD patients (Table 1.10). Positive outcomes in the Movement Disorders Society Unified PD rating score (MDS-UPDRS) were reported in a 12-week clinical trial with PD patients taking probiotic formulation containing *L. acidophilus*, *B. bifidum*, *L. reuteri* and *L. fermentum*, compared to placebo controls. In addition, improved insulin metabolism was also reported (Tamtaji, Taghizadeh et al. 2019). The anti-inflammatory effects of this probiotic formulation are further demonstrated in another clinical trial with PD patients. Here, 12-week probiotic supplementation resulted in reduced expression of IL-1, IL-8 and TNF-α mRNA and increased expression of transforming growth factor β (TGF-β) and PPARγ mRNA in peripheral blood mononuclear cells (PBMC) (Borzabadi, Oryan et al. 2018). A draw back to this study is that it didn't examine cognitive function in PD patients, so it is unclear whether these anti-inflammatory effects had beneficial effects on cognition. Anti-inflammatory and antioxidant effects are also observed following co-culture with PBMCs isolated from PD patients with a probiotic mixture containing species from the *Lactobacillus* and *Bifidobacterium* genus (Magistrelli, Amoruso et al. 2019).

What is needed now is analysis of gut microbial composition in prebiotic and placebo treated PD patients to identify specific alterations in gut microbiota that could be responsible for inducing anti-inflammatory and neuroprotective effects, and to provide further insight into how the gut microbiota influences PD pathology.

1.9.3. Role of microbial mediators in PD pathogenesis

Systemic inflammation and elevated activation of microglia are becoming increasingly recognised as important factors in the progression of PD (McGeer, Itagaki et al. 1988, Hirsch, Vyas et al. 2012).

Recently, the first case-control study of plasma SCFAs in PD patients revealed increases in peripheral venous concentrations of acetate, but not butyrate and propionate (Shin, Lim et al. 2020) whereas previous studies reported decreases in faecal SCFAs in patients (Unger, Spiegel et al. 2016). It is important to note that faecal SCFAs differ considerably with venous SCFA concentrations, and in addition to markers for intestinal inflammation and intestinal permeability not being measured, could contribute to these contradictory findings. In addition, participants recruited were on anti-parkinsonian mediation (Shin, Lim et al. 2020), all of which contribute to difficulties in accurately determining the possible role of SCFAs in PD pathogenesis. *Lactobacillus* and *Bifidobacterium* are acetate producers (Sun and Shen 2018) and there are reports of increased acetate-producing bacteria in PD (Unger, Spiegel et al. 2016, Hill-Burns, Debelius et al. 2017), which could explain the increased plasma acetate reported by Shin et al., (Shin, Lim et al. 2020). Conversely, there are other studies that reported a reduction in acetate producing bacteria (Sun and Shen 2018) raising questions regarding the biological functions of SCFAs in PD pathology. These studies highlight the need for more comprehensive methods to analyse SCFAs concentrations in PD patients.

Despite this, there are several studies that have explored the role of SCFAs in development and progression of PD, using various *in vitro* and *in vivo* models. Sodium butyrate in one study was demonstrated to improve α-synuclein induced DNA damage via inhibition of histone deacetylation and upregulation of DNA-repair genes in the LHUMEs cell line (Paiva, Pinho et al. 2017). In contrast, *in vivo* sodium butyrate treatment exacerbated MPTP-induced motor dysfunction, striatal dopamine, and 5-HT levels, and neuroinflammation in mice (Qiao, Sun et al. 2020). Similarly, in BV-2 cells, a murine microglia cell line, sodium butyrate treatment exacerbated LPS-induced inflammations demonstrated by significantly increase nitric oxide production, and expression of proinflammatory cytokines including IL-1β and IL-18 (Qiao, Sun et al.

2020). In another *in vitro* model using rotenone-induced neurodegeneration in primary midbrain dopaminergic neuronal cell culture from embryonic rats, showed that propionic acid did not have any neuroprotective effects (Ostendorf, Metzdorf et al. 2020). Moreover, treatment with SCFAs alone in GF-ASO mice is sufficient to promote α-synuclein mediated neuroinflammation and motor deficits, suggesting that gut microbiota could potentially mediate PD pathology via the production of SCFAs (Sampson, Debelius et al. 2016), particularly as microbial dysbiosis and alterations in SCFAs are observed in many PD patients (Unger, Spiegel et al. 2016, Hill-Burns, Debelius et al. 2017, Sun and Shen 2018, Wallen, Appah et al. 2020). Despite these discordant findings, the consensus is a potentiating proinflammatory effect of microbial LPS and SCFAs in PD models. Further studies should focus on delimitating the signalling pathways involved.

1.9.4. Role of enteroendocrine hormones in PD pathogenesis

There is growing interest in the potential neuroprotective role of the incretin hormone GLP-1 in both preclinical and clinical studies (Bertilsson, Patrone et al. 2008, Li, Perry et al. 2009, Athauda, Maclagan et al. 2017, Yun, Kam et al. 2018, Glotfelty, Olson et al. 2020, Li, Tu et al. 2020, Zhang, Zhang et al. 2020, Sun, Li et al. 2021, Wang, Kuo et al. 2021). An overview of key studies demonstrating this are summarised in Table 1.11 and 1.12. Evidence that GLP-1 may be of clinical importance in PD comes from a recent study showing dysregulated postprandial secretion of GLP-1 in PD patients (Manfready, Engen et al. 2021) which could also provide an explanation for metabolic disorders, including diabetes and obesity, being significant risk factors for PD that are characterised by reduced systemic GLP-1 levels (Bagger, Christensen et al. 2011, Biosa, Outeiro et al. 2018, Brauer, Wei et al. 2020, Jeong, Han et al. 2020). PD patients also show downregulated expression of GLP-1R in the SN compared to healthy controls (Yun, Kam et al. 2018). Therefore, altered gut microbial composition and reduced SCFA concentrations in PD patients (Keshavarzian, Green et al. 2015, Unger, Spiegel et al. 2016, Sun and Shen 2018) could contribute to disrupted GLP-1 levels also observed in these patients.

The probiotic *C. butyricum* has been shown to increase GLP-1 secretion, upregulate GLP-1R expression in the brain, improving motor function and reducing the loss of

dopaminergic neurones in a MPTP-induced murine model of PD (Sun, Li et al. 2021). Regional variations in GLP-1R mRNA expression in the brain have been recently reported, with GLP-1R highly expressed in striatum, hippocampus, ventral midbrain, and brainstem (Yun, Kam et al. 2018). In addition, GLP-1R-immunoreactivity is localised to Iba-1^{+} and $TREM119^{+}$ microglia cells, with neuronal cells expressing lower levels of GLP-1R (Yun, Kam et al. 2018) suggesting that microglia may be the natural target of GLP-1 and its analogues. There is evidence also of microglia being a source of GLP-1 secretion induced by LPS-stimulated inflammation (Kappe, Tracy et al. 2012). GLP-1R activation of the AKT pathway is a major contributor to the regulation of physiological ageing, modulating several cellular processes that are found to be disrupted in PD including apoptosis, oxidative stress, inflammation, mitochondrial biogenies, protein synthesis and autophagy (Baggio and Drucker 2007).

Exendin-4 is a GLP-1R agonist with a longer plasma half-life (~2 hrs) compared to mammalian GLP-1, which is rapidly degraded by DPPIV (Kieffer, McIntosh et al. 1995). Moreover, Exendin-4 has been demonstrated to readily cross the BBB and induce neuroprotective effects in *in vitro* and *in vivo* models of PD (Bertilsson, Patrone et al. 2008, Harkavyi, Abuirmeileh et al. 2008, Li, Perry et al. 2009). In a MPTP-murine model of PD, Exendin-4 pre-treatment was shown to inhibit dopaminergic neuronal cell loss and increase levels of DA and its metabolites in the striatum. These neuroprotective effects were also accompanied by improved motor function (Li, Perry et al. 2009). *In vitro* cell culture experiments suggest that Exendin-4 is a regulator of neuronal stem cell fate and highlights its potential as a neurogenesis modulator *in vivo* (Bertilsson, Patrone et al. 2008). Further studies have also demonstrated the ability of Exendin-4 to reverse effects of 6-OHDA induced neuronal damage and behaviour deficits (Harkavyi, Abuirmeileh et al. 2008).

NLYO1 is a pegylated form of Exendin-4 with a half-life of ~38 hrs in mice (Yun, Kam et al. 2018) and its transport across the BBB is limited (Zhang, Zhang et al. 2020, Lv, Xue et al. 2021). However, NLY01 was shown to effectively cross the BBB in hA53T mice (a transgenic mouse model of PD that overexpresses α-synuclein (Graham and Sidhu 2010)), but this may be due to a breakdown of BBB integrity observed in these mice (Yun, Kam et al. 2018). The neuroprotective effects of NLY01 treatment in PD models observed (Luk, Kehm et al. 2012, Mao, Ou et al. 2016, Yun,

Kam et al. 2018) therefore must be interpreted with caution, as accompanying measurements of barrier permeability was not carried out. This is supported by evidence showing Exendin-4 as a more effective single-receptor agonist than NLY01 in MPTP mouse model of PD due to its ability to readily cross the BBB (Salameh, Rhea et al. 2020, Lv, Xue et al. 2021).

The "sister" incretin hormone of GLP-1, GIP, also shows promising results in animal models of PD. Like GLP-1, is it degraded rapidly by DPPIV (Kieffer, McIntosh et al. 1995) and therefore several enzyme-resistant GIP analogues have been developed. GIP receptors (GIP-R) are found in multiple brain regions, including the hippocampus, cerebellum, SN and brainstem and its activation in the CNS promotes proliferation of neural progenitor cells (Nyberg, Anderson et al. 2005), axonal regeneration (Buhren, Gasis et al. 2009) and modulates synaptic activity (Gault and Hölscher 2008). In a recent study, the potential therapeutic effects of a novel long lasting GIP analogue, D-Ala2-GIP-GLU-PAL was explored in MPTP-induced PD mouse model. Similar to the effects of GLP-1 analogues (Bertilsson, Patrone et al. 2008, Harkavyi, Abuirmeileh et al. 2008, Li, Perry et al. 2009, Glotfelty, Olson et al. 2020, Wang, Kuo et al. 2021), D-Ala2-GIP-GLU-PAL promoted neurogenesis and synaptogenesis, reduced chronic neuroinflammation, and improved locomotor and exploratory activity in MPTP-mice (Li, Liu et al. 2016, Li, Liu et al. 2017). The use of GLP-1/GIP dual receptor agonists provides a more effective treatment for diabetes (Finan, Ma et al. 2013) and has also been therapeutically applied to animal models of PD demonstrating increased effectiveness compared to single GLP-1/GIP analogues in reducing neuroinflammation, improving mitochondrial activity and normalising autophagy (Feng, Zhang et al. 2018, Zhang, Zhang et al. 2020, Lv, Xue et al. 2021). Compared to Exendin-4, DAS-CH was demonstrated to cross the intact BBB more readily as evidenced by using fluorescently labelled peptides injected peripherally in wild-type SPF mice and analysing fluorescent in various brain sections. Higher immunofluorescence was detected in DA5-CH injected mice compared to Exendin-4 injected mice (Lv, Xue et al. 2021). A more comprehensive analysis of incretin analogues for the treatment of PD models are needed and need to consider the transport across the BBB, especially as BBB integrity is compromised in PD patients (Gray and Woulfe 2015).

In a recent randomised placebo-controlled phase II clinical trial, Exenatide (a synthetic form of Exendin-4) was found to have beneficial effects on motor function following 48-week treatment (Athauda, Maclagan et al. 2017). Analysis was conducted following a 12-week wash-out period and highlighted potential pathways implicated including the protein kinase B (Akt) signalling pathways (a downstream target of IRS-1). Other measures including quality of life, mood and non-motor symptoms showed no significant clinical difference but did show a favourable trend towards Exenatide treatment (Athauda, Maclagan et al. 2017, Athauda, Gulyani et al. 2019). In a smaller randomised controlled clinical trial, 12-month follow-up revealed significant improvements were also reported in the MDS-UPDRS Part 3 score in PD patients that received 12-month Exenatide treatment compared to randomised controls (Aviles-Olmos, Dickson et al. 2014). Whilst these clinical trials don't provide evidence of neuroprotective effects of GLP-1 analogues, they do provide encouraging evidence for the use of GLP-1 analogues to improve motor function in PD. Larger clinical trials are needed to provide comparisons, in addition to analysis of biomarkers in the serum and CSF and alterations in gut microbiome that could help provide insight into molecular mechanisms that underpin GLP-1R signalling in the CNS. Further studies are needed to identify causes of dysregulated GLP-1 signalling in PD and determining the effects of medication.

1.9.5. Constraints in current PD research and future directions

It is evident that significant microbial alterations exist in the gut microbiome of PD patients. What is needed now is comprehensive verification of consistent alterations across cohorts, particularly in studies where the heterogenous methodological approaches and sampling criteria create confounding results (Romano, Savva et al. 2021).

The neuroprotective actions of incretin receptor signalling are translated across several PD models. The ability for drugs to cross the BBB is an important factor to consider when developing novel drugs. Some of the discrepancies in results may be reflective of the ability of the receptor analogues to effectively cross the BBB. This needs to be considered when comparing studies that use treatments with different BBB penetrative abilities. Moreover, differences between human and rodent BBB exist and is

understudied. Combined with different methods for studying the BBB integrity in rodents (radioactivity and brain tissue analysis) and humans (imaging and CSF sampling), could contribute to conflicting and variable results seen between preclinical and clinical trials (Salameh, Rhea et al. 2020).

The use of *in vitro* methods to study the underlying molecular pathways to control PD pathology have some drawbacks. Cell lines provide useful insights into processes occurring in single dopaminergic neurones, but as an isolated model, they do not reproduce the clinical conditions of PD. Development of PD rodent models provide a valuable, low-cost tool in evaluative behavioural and pharmacological effects of innovative therapeutic approaches. Toxic PD models such as those induced by 6-OHDA and MPTP, do not replicate many of the neuropathological features associated with PD in humans, and there are many differences in processes leading to dopaminergic cell loss, thus limiting its value as an effective model for PD pathology (Simola, Morelli et al. 2007). Moreover, these approaches are dominated by studies of dopaminergic neurodegeneration and its consequences, and do not address the more subtle non-motor symptoms that often precede the characteristic motor deficits. ASO mice, that over-express human wild-type α-synuclein driven by the murine Thy-1 promotor (also referred to as Thy-1-αSyn mice) (Rockenstein, Mallory et al. 2002) more accurately recapitulate the progressive age-related motor deficits and colonic motor alterations observed in PD humans. They overexpress α-synuclein to levels comparable to human individuals with familial PD, and therefore are unlikely to cause non-specific toxic effects. Although they don't produce accumulative loss of dopaminergic neurones, they provide a useful tool to study the pathological accumulation of α-synuclein (Chesselet and Carmichael 2012).

1.10. Depression

Mental health is a growing concern worldwide, with common mental health disorders such as depression increasing significantly in the last few decades, markedly amongst the youth (Carrellas, Biederman et al. 2017, Friedrich 2017, Jorm, Patten et al. 2017). Depression is a major neuropsychiatric disease affecting around 350 million people worldwide and is associated with reduced life span and quality of life (Bosnyák, Kamson et al. 2015, Wachholz, Eßlinger et al. 2016). Characterised by

psychophysiological changes including low mood and anhedonia (Belmaker and Agam 2008), depression is now viewed as a systemic disease, with many patients also suffering from other non-phycological disorders such as GI-related disorders (Forsythe, Sudo et al. 2010, Dash, Clarke et al. 2015, Evrensel and Ceylan 2015, Kundu, Blacher et al. 2017). Most anti-depressants currently available work to restore levels of monoamine neurotransmitters, but many have a delayed onset of action (Taylor, Freemantle et al. 2006) and can have limited beneficial psychotropic and psychological effects in healthy human participants (Serretti, Calati et al. 2010). Intriguingly, some antidepressants are known to have antimicrobial effects (Munoz-Bellido, Munoz-Criado et al. 2000, Lieb 2004). Conversely, certain antibiotics have suggested to possess antidepressant-like properties (Miyaoka, Wake et al. 2012, Mello, Monte et al. 2013). The molecular mechanisms underlying the physiological characteristics of depression are not clearly understood, but it thought to involve dysfunction within the CNS, the HPA axis, and immune system. Growing evidence now suggests a role for the microbiota-gut-brain axis in the pathophysiology of depression (Neufeld, Kang et al. 2011, Foster and McVey Neufeld 2013, Simpson, Diaz-Arteche et al. 2021).

1.10.1. Microbial alterations in depression

Numerous studies have reported gut microbial alterations in patients with depressive disorders compared to healthy controls, summarised in Table 1.13. At a phylum level, Jiang et al., reported increases in relative abundance of Bacteroidetes, Proteobacteria and Actinobacteria, and reduced relative abundance of Firmicutes in patients with major depressive disorder (MDD). Severity of depressive symptoms were reported to be correlated to *Faecalibacterium* and reduced levels of BDNF (Jiang, Ling et al. 2015). In addition, significant taxonomic differences between MDD and healthy control participants reported include reductions in the families *Prevotellaceae* and increases in *Thermoanaerobacteriaceae*, and at the genus level reductions *Prevotella* and *Dialister* (Kelly, Allen et al. 2017). Similarly, a larger population cohort study reported significant reductions in *Dialister* and *Coprococcus* amongst depressed patients (Valles-Colomer, Falony et al. 2019). Reduced microbial diversity and richness reported in MDD patients in one study (Kelly, Allen et al. 2017), conflicts with the outcomes observed in another study (Naseribafrouei, Hestad et al. 2014).

Methodological inconsistencies and lack of consideration for confounding factors (such as diet and antidepressant medication) may explain some of the discrepancies observed between studies.

A multilevel omics approach provides an in-depth method for exploring the gut microbiota and metabolomic contributors in the pathogenesis of depression. Using this approach, Yang et al., reported that MDD participants were predominantly characterised by increased abundance of *Bacteroides* and decreased *Blautia* and *Eubacterium* and alterations in 50 faecal metabolites that were mapped to γ-aminobutyrate, phenylalanine and tryptophan metabolism (Yang, Zheng et al. 2020). Moreover, in a recent study characterising microbial DNA in faecal samples from depressed and non-depressed participants revealed that gut microbial DNA contributes to the depression phenotype (Stevens, Roesch et al. 2021). A recent systematic review undertaken reported that anxiety and depressive disorders associated with higher relative abundances of proinflammatory microbial species and lower abundance of SCFA-producing bacteria (Simpson, Diaz-Arteche et al. 2021) in line with previous studies (Jiang, Ling et al. 2015, Kelly, Allen et al. 2017, Valles-Colomer, Falony et al. 2019).

1.10.2. Microbial modulation of depression

Evidence of microbial modulation of the immune, neuroendocrine, and neurochemical systems involved in the pathophysiology of depression has been explored in several animal studies, summarised in Table 1.14 (Sudo, Chida et al. 2004, Girard 2008, Desbonnet, Garrett et al. 2010, Diaz Heijtz, Wang et al. 2011). GF mice display increased motor activity and reduced anxiety-like behaviour compared to SPF mice, which is normalised upon introducing SPF microbiota in early stages of development (Diaz Heijtz, Wang et al. 2011). These effects were not observed in mice colonised in later stages of development indicating that there is a time-sensitive period during development for gut microbiota to affect brain function and behaviour later in life (Diaz Heijtz, Wang et al. 2011). Hyperactivity of the HPA axis is characterised by increased levels of cortisol, ACTH and CRH, and has been shown to play a core role in progression of depression (Bao, Hestiantoro et al. 2005, Swaab, Bao et al. 2005, Wang, Kamphuis et al. 2008). GF mice exhibit elevated levels of CORT and ACTH

compared to SPF mice in response to restraint stress, indicating an exaggerated HPA stress response. This can be normalised upon reconstitution with *B. infantis*, highlighting the ability of a specific bacterium to regulate the HPA stress response (Sudo, Chida et al. 2004). In line with Diaz-Heijtz et al., reconstitution of GF mice with SPF faecal microbiota partially normalised the HPA response but only when administered at an early stage in development (Sudo, Chida et al. 2004).

Striatal levels of synaptic related proteins, synaptophysin and PSD-95, are elevated in GF mice (Diaz Heijtz, Wang et al. 2011), and can be used as an indirect marker for synaptogenesis (Ulfig, Setzer et al. 2000). Reduced levels of synaptic related proteins could contribute to deficits in neuroplasticity and neuronal connections that are often associated with depressive symptoms (Fuchs, Czéh et al. 2004, Pittenger and Duman 2008, Malykhin and Coupland 2015). These results provide insight into the mechanisms involved microbial modulation of brain function but they do not explain the region-specific variations observed in the CNS (Diaz Heijtz, Wang et al. 2011).

Neurotransmitters are crucial for brain function and behaviour, and deficiency in the monoamine neurotransmitters, 5-HT, DA and NE, are linked with symptoms of depression (Cryan and Leonard 2000, Hirschfeld 2000, Berger, Gray et al. 2009, Hamon and Blier 2013). 5-HT in particular is considered the main biological substrate in the serotonergic system (Lucki 1998). Evidence of microbial modulation of the serotonergic system is clearly demonstrated by GF models. Compared to SPF mice, GF mice display increased plasma concentrations of tryptophan, and increased brain concentration of 5-hydroxyindoleacetic acid (5-HIAA) (Diaz Heijtz, Wang et al. 2011, Clarke, Grenham et al. 2013) as well as increased hippocampal mRNA expression of 5-hydroxytryptamine 1A (5-HT1A) receptor and 5-HT2A receptor. Moreover, GF mice display a blunted immune response and exaggerated stress response (Clarke, Grenham et al. 2013). Colonisation of GF mice normalises plasma tryptophan levels and reduces anxiety-like behaviours. Intriguingly, alteration in hippocampal BDNF is sex-specific, with only male GF mice displaying significantly reduced hippocampal expression, increased hippocampal 5-HT and 5-HIAA levels and plasma tryptophan concentrations (Clarke, Grenham et al. 2013).

Wikoff et al., used mass-spectrometry-based metabolomics to compare plasma metabolites in GF and SPF mice. Indole derivatives were found to be significantly altered in GF mice, in particular a 1.7-fold increase in tryptophan, 2.4-fold increase in N-acetyl tryptophan and a 2.8-fold decrease in 5-HT (Wikoff, Anfora et al. 2009). A subset of enteric bacteria including *E. coli*, express tryptophanase that converts tryptophan to indole (Botsford and Demoss 1972), therefore it seems likely that the increase in plasma tryptophan and N-acetyl tryptophan in GF mice is a direct result from lack of microbiota to metabolise tryptophan. The decrease in plasma 5-HT could also be a direct effect of host-microbiota interaction as EC cells are largest source of 5-HT production and express microbial metabolite receptors (Gershon 2013).

FMT in rodents with faecal microbiota from MDD patients has been demonstrated to increase depression- and anxiety-like behaviours compared to FMT with faecal microbiota from healthy participants (Zheng, Zeng et al. 2016, Kelly, Allen et al. 2017). Accompanying these behavioural alterations in MDD-FMT rodents are increased intestinal motility, plasma kynurenine, but no significant alterations were observed in plasma 5-HT, CORT, or inflammatory cytokines (Kelly, Allen et al. 2017). Although microbial alterations were observed in these rodents, the overlap of specific taxa between MDD patients and FMT-MDD rodents is not observed possibly due to inherent translational differences between humans and rodent models used in these studies (Zheng, Zeng et al. 2016, Kelly, Allen et al. 2017). MDD-FMT rodents also display alterations in microbial genes and host metabolites involved in carbohydrate and amino acid metabolism (Zheng, Zeng et al. 2016) and display increased faecal acetate (Kelly, Allen et al. 2017) and succinate levels (Zheng, Zeng et al. 2016). These "humanised" animal models provide a valuable tool in demonstrating that depression- and anxiety-like behaviours are transmissible via the gut microbiome and result in alterations in host metabolism and signalling pathways.

Several preclinical studies have been undertaken to explore the potential of psychobiotic treatment to reduce anxiety-like and depression-like behaviours. Rats subjected to maternal separation (MS) display long-lasting behavioural alterations associated with behavioural despair accompanied by biochemical alterations including increased NA levels in the brain, increased 5-HIAA levels in amygdala cortex and increased IL-6 secretion in response to immune stimulation (Desbonnet, Garrett et al.

2010). In addition increased CRF mRNA expression is increased in the amygdala cortex (Desbonnet, Garrett et al. 2010), in line with reports of increased CRF immunoreactivity in depressed suicidal patients (Austin, Janosky et al. 2003). *B. infantis* treatment in MS-rats attenuated MS-induced behavioural alterations, and levels of 5-HIAA in amygdala cortex (Desbonnet, Garrett et al. 2010). Further analysis of the anti-depressant properties of *B. infantis* revealed significant inhibition of LPS or concanavalin A (ConA) induced pro-inflammatory cytokine secretions in peripheral whole blood immune samples (Desbonnet, Garrett et al. 2010). These results indicate the potential for *B. infantis* in improving depression-like behaviours, possibly through attenuating inflammatory pathways in MS-rats. The inflammatory response is an important factor in regulating brain function and behaviour and is implicated in depressive disorders (Maes 1995, Dantzer, O'Connor et al. 2008, Maes 2008). However, altered behaviours can also be induced by bacterial infection with no associated immune response. For example, mice infected with *Campylobacter jejuni* (*C. jejuni*) display increased time spent grooming indicating increased anxiety-like behaviours that is unaccompanied by changes in plasma inflammatory cytokines levels (Lyte, Varcoe et al. 1998). How *C. jejuni* increases this anxiety-like behaviour is unclear but could involve stimulation via vagal nerve or other microbial products, like BEVs.

The neuro-beneficial effects of probiotics are also observed upon administration to healthy animals. *In vivo* analysis revealed significant increases in plasma tryptophan and kynurenic acid and reductions in 5-HIAA content in the frontal cortex and 3, 4-dihydroxyphenylacetic acid (DOPAC) levels in amygdala cortex in *B. infantis* treated rats (Desbonnet, Garrett et al. 2010). The prefrontal cortex, hippocampus and amygdala are key regions involved in emotion, motivation, the stress response and cognitive function and altered functionality of these regions are reported in patients suffering from depression (Lawrence, Williams et al. 2004, Siegle, Thompson et al. 2007, Diener, Kuehner et al. 2012, Palmer, Crewther et al. 2014). In another study, chronic administration with the lactic acid bacteria *L. rhamnosus* JB-1 was shown to reduce stress-induced increases in corticosterone, and attenuate anxiety/depression-like behaviours, with region-specific alterations in GABA(B1b) and GABA(Aα2) mRNA expression in the brain. These effects were shown to be mediated via the vagus

nerve as the neurochemical and behavioural effects of JB-1 were not observed in vagotomised mice (Bravo, Forsythe et al. 2011).

When translated to human clinical trials, the psychobiotic effects of *L. rhamnosus* JB-1 are not observed (Table 1.15). Healthy human male participants given *L. rhamnosus* JB-1 daily for 4-weeks resulted in no overall effect of JB-1 on cytokine profile, cognitive measures, or anxiety/stress response (Kelly, Allen et al. 2017). Given that most anti-depressants have delayed onset of action (Taylor, Freemantle et al. 2006), it may be the case that longer period of psychobiotic treatment is needed to observe any biochemical and behavioural effects. This study highlights the difficulties in translating outcomes from preclinical studies to human clinical trials, possibly arising from inherent differences between rodent models and human.

Varying outcomes are observed in other clinical trials using multi-species probiotics, displayed in Table 1.15. Probiotics containing *B. bifidum, B lactis, L. acidophilus, L. brevis, L.casei, L. salivarius* and *Lactococcus lactis*, taken daily for 28 days by healthy participants was shown to reduce total cognitive reactivity to depression, specifically aggressive and ruminatory thoughts measured by the LEIDS-r, a self-report questionnaire aimed to assess what dysfunctional thoughts are activated when expressing mild dysphoria. (Steenbergen, Sellaro et al. 2015). Similarly, probiotic containing *L. helveticus* and *B. longum* administration in healthy participants alleviated psychological distress and reduced urinary free cortisol levels when compared to placebo controls (Messaoudi, Lalonde et al. 2011).

Prebiotic administration has also been shown to induce neuro-beneficial effects in humans. For example, GOS administration in healthy human participants results in reductions of the waking cortisol response compared to placebo controls (Schmidt, Cowen et al. 2015). This is in line with a preclinical study in which GOS administration in rats resulted in region-specific upregulation of BDNF, NMDA receptor (NR) 1A and NR2A mRNA in the brain as well as increased abundance of *Bifidobacterium* in faeces. These results suggest that GOS exerts neuro-beneficial effects through the gut *Bifidobacterium* species (Savignac, Kiely et al. 2014).

It is important to note that probiotic administration in a healthy population may serve to only promote gut homeostasis and function, and not directly affects overall microbial composition (Sanders 2016) and therefore it is necessary to assess the effects of probiotic administration in depressed patients. Akkasheh et al., were the first to study the effects of probiotics (containing *L. acidophillus, L. casei* and *B. bifidum*) on clinical symptoms and metabolic responses in MDD patients. They reported significant reduction in Beck Depression Inventory (BDI) total score and beneficial effects on markers of insulin metabolism in MDD patients taking probiotics compared to placebo groups. Additional dietary records conducted revealed no significant differences in dietary intake between the probiotic and placebo groups (Akkasheh, Kashani-Poor et al. 2016). Further analysis of individual strains for a longer period on clinical depression symptoms, as well as other biomarkers of inflammation and oxidative stress in high-risk/clinical groups are needed.

1.10.3. Role of microbial mediators and neuroendocrine signalling in depression

There is emerging evidence suggesting a role of SCFA and depression-related disorders (Table 1.16). A recent pilot study undertaken revealed significant associations between symptoms of depression accompanied by GI dysfunction and faecal SCFAs. In particular faecal acetate levels were positively correlated to symptoms of depression, whereas faecal butyrate and propionate were negatively correlated with depression symptoms (Müller, Rasmusson et al. 2021), in line with negative correlations between *Faecalibacterium*, prominent butyrate producers, and severity of depressive symptoms in MDD patients (Jiang, Ling et al. 2015). In another study, overall faecal SCFAs were reduced in depressed participants, and a negative correlation with severity of depression was observed between faecal acetate and propionate (Skonieczna-Żydecka, Grochans et al. 2018). However, over-representation of the depressed population (125 depressed participants verses 39 non-depressed participants) may contribute to the differences observed in this study (Müller, Rasmusson et al. 2021). Lack of dietary measures, which is an important factor in influencing SCFA levels, could also contribute to discrepancies amongst studies, although in a recent study, no significant correlation between faecal SCFAs and dietary fibre intake was reported (Skonieczna-Żydecka, Grochans et al. 2018). In

contrast, Kelly et al., reported no significant alterations in faecal SCFAs between MDD patients and health matched controls (Kelly, Allen et al. 2017). Most studies looking at SCFA concentrations association with psychiatric disorders, differ in design, analyse samples from different populations, have different sampling methods and either calculate relative or absolute SCFA levels, meaning that making comparisons between studies is often challenging and give rise to conflicting results. Comparing systemic and faecal SCFAs concentrations would also provide a better insight into the potential role of SCFA in depressed participants.

In vivo murine FMT models of depression also report positive associations between faecal acetate and succinate and depression/anxiety-like behaviours (Zheng, Zeng et al. 2016). Significant positive correlations between butyrate concentration and hippocampal 5-HTP and BDNF levels in prefrontal cortex is also reported (Tian, Wang et al. 2019). Here, caecal butyrate concentrations were found to be significantly reduced in depressed mice, and administration of the probiotic *B. longum* was able to normalise caecal butyrate levels, depression-like behaviours, stress-induced CORT and BDNF concentrations (Tian, Wang et al. 2019). Some *B. longum* strains used in this study also increased caecal acetate and propionate levels (Tian, Wang et al. 2019). Analysis of CSF in depressed macaques also showed increased levels of propanoic acid, acetic acid and butanoic acid (Deng, Pan et al. 2019), suggesting increased translocation of peripheral SCFAs across the BBB. The ability of SCFAs to induce 5-HT at both a transcript and protein levels is evidenced in RIN14B cell cultures. Increased 5-HT secretion and Tph1 mRNA expression following exposure to butyrate and propionate (Yano, Yu et al. 2015). Chronic restraint stress (CRS) is often used to model depression-like behaviours in animals. In CRS mice, faecal SCFAs are diminished, and hypothalamus neurotransmitter (NE, 5-HT and 5-HIAA) levels are reduced (Wu, Tian et al. 2020). These studies demonstrate the potential of SCFAs to modulation 5-HT secretion and role in neuroendocrine regulation. Under normal physiological conditions, gut derived 5-HT does not cross the BBB, suggesting that 5-HT may exert its effects in the CNS through indirect mechanism (El-Merahbi, Löffler et al. 2015).

Similar findings were also observed by Huang et al., where glycerol triacetate (GTA) supplementation in murine models of acute and chronic induced-depression, induced

anti-depressant-like effects through upregulation of downstream target genes involved in stress and depression, such as BDNF, NTF3 and nerve growth factor (NGF), improvement in impairment of synaptic structure in hippocampal CA1 regions. Plasma and brain levels of acetate are also increased in these mice, suggesting that the behavioural and molecular effects of GTA supplementation are mediated, in part, through increased acetate levels (Huang, Hu et al. 2021). The hippocampal CA1 region is important in modulating emotional behaviour and anxiety-responses (Spolidório, Echeverry et al. 2007, Zarrindast, Nasehi et al. 2012), therefore normalising dysfunction in this region via SCFAs may provide a potential therapeutic route.

Regulation of gene expression by histone deacetylation has been implicated in depression-like behaviours (Dalton, Kolshus et al. 2014). Indeed, elevated expression of HDAC2, 4, 5, 6, and 8 in peripheral blood have been reported in depressed patients (Hobara, Uchida et al. 2010, Abe, Uchida et al. 2011). Furthermore, inhibition of HDAC ameliorates depression-like behaviours in rodents (Covington III, Vialou et al. 2011), highlighting the potential of SCFAs as HDAC inhibitors in therapeutic treatment of depression. This has been demonstrated in *in vivo* murine models of chronic depression, where GTA supplementation increases brain levels of histone H3 and H4 acetylation and downregulation of HDAC mRNA expression (Huang, Hu et al. 2021). Combined these results indicate that gut microbiota plays a significant role in pathology of depression, in part, through the regulation of SCFAs which influence neurotransmitter levels, gene expression regulation via HDAC inhibition and anti-inflammatory effects. Table 1.12 provides a summary of these key studies.

An increased inflammatory profile reported in depressed patients, supports the current view of depression being a multifactorial disease (Maes 1995, Maes, Meltzer et al. 1995, Maes 2008). LPS administration recapitulates depression-like behaviours in rodent models (Yirmiya 1996, Kubera, Curzytek et al. 2013, Rodrigues, de Souza et al. 2018). Sodium butyrate pre-treatment in LPS-induced murine model of depression was shown to exert anti-depressive effects through inhibition of inflammatory and oxido-nitroactive stress (Qiu, Liu et al. 2020). IFN-γ induces activation of the enzyme indoleamine 2, 3-dioxygenase (IDO), expressed predominately on microglia cells and is involved in the kynurenic pathway that produces neurotoxic and neuroprotective

end-products (Maes, Mihaylova et al. 2007, Yadav, Burudi et al. 2007). One such neurotoxic end product, Quinolinic acid, is reported to be upregulated in depressed patients (Steiner, Walter et al. 2011), indicating a role of increased intestinal translocation of LPS to induce proinflammatory pathways involved in pathology of depression (Maes 2008, Stevens, Goel et al. 2018). This is further supported by *in vivo* evidence demonstrating LPS-induced chronic depression is accompanied by increased hippocampal microglia activation and proinflammatory cytokine levels, in addition to upregulated expression of hippocampal IDO-1 mRNA and increased levels of kynurenine and Quinolinic acid (Rodrigues, de Souza et al. 2018). Similar findings are also observed in rats receiving intravenous administration of LPS, but interestingly, these effects were shown to be sex-dependent, with only females displaying increased inflammatory profiles (Tonelli, Holmes et al. 2008).

There is also growing interest in associations between T2D and mental health disorders. Indeed, in a meta-analysis report, depression was found to be more prevalent in diabetic patients (17.6 %) compared to non-diabetic participants (9.8 %) (Ali, Stone et al. 2006). In addition to its central metabolic role, there is evidence to support the role of GLP-1 in anxiety and depression like disorders. For example, GLP-1 agonists have been demonstrated to exert anxiolytic and anti-depressant like effects and to reverse neuropathy in a mouse model of diabetes (Komsuoglu Celikyurt, Mutlu et al. 2014). The anxiolytic and anti-depressant like effects of GLP-1 agonist, can potentially be explained by the important neuroprotective role of GLP-1 in the CNS and may have direct trophic actions on the nervous system (McClean, Gault et al. 2010). As neuropeptides have been demonstrated to modulate catecholamine and 5-HT secretion from the hypothalamus, it is also plausible that GLP-1 could in acting indirectly on these systems (Perry, Lahiri et al. 2003). Indeed, GF mice have increased plasma and luminal GLP-1 concentrations, increased colonic proglucagon expression (Wichmann, Allahyar et al. 2013, Modasia, Parker et al. 2020) and increased GLP-1 expression in the hypothalamus compared to CONV-R mice (Schéle, Grahnemo et al. 2013). Moreover, probiotics such as *B. animalis* and *B. bifidum* and prebiotics have also been shown to increase intestinal and plasma GLP-1 levels in rodents (Hong, Kim et al. 2016, Aoki, Kamikado et al. 2017).

In a pilot study, the effect of liraglutide in individuals with MDD or bipolar disorder (BD) was investigated. Analysis revealed that 4-week treatment with the GLP-1R agonist was associated with cognitive improvements without altering metabolic parameters (Mansur, Ahmed et al. 2017). However, due to the small sample size, open-label design of the study and lack of placebo controls, the results from this study should be congenialized, but the findings are consistent with pre-clinical and clinical trials reporting the GLP-1R agonist-mediated improvements in cognition in AD and PD (see 1.8.4/1.9.4). Further clinical trials in patients with depressive disorders to determine whether GLP-1 agonists can offer beneficial effects on other symptoms of psychiatric disorders and whether longer-term treatments can reduce or prevent the reoccurrence of depressive symptoms. Understanding the modulatory role of GLP-1 in CNS is critical to determine to which extent which it can influence depression and anxiety.

There is potential for microbial manipulation to increase endogenous peptide hormone production and secretion (key studies are summarised in Table 1.17) , but further research is required to fully understand the mechanisms and mediators involved in these signalling pathways

1.10.4. Constraints in current neuropsychiatric research and further directions

The past few decades have seen progress in understanding the psychological processes underlying depression, made possible in part using rodent models that have enabled the study of neural and molecular pathways involved. Core symptoms of MDD including anhedonia and depressed mood and other associated conditions such as weight, appetite and anxiety can be easily assessed in animals, using a combination of behavioural observations and other behaviour/motor tests (Planchez, Surget et al. 2019) Most animal models of MDD are based on the application of stressors. Chronic mild stress (CMS) is possibly one of the most characterised and realistic models of depression that currently exist, that induces long-lasting behavioural, neurochemical, neuroimmune and neuroendocrinological alterations that closely resemble those observed in MDD patients (Willner 2005). MS is often used to model early-life stress and produces physiological and behavioural alterations that persist well into adulthood

(Newport, Stowe et al. 2002, Pryce, Rüedi-Bettschen et al. 2005). However this approach has been demonstrated to yield contradictory findings across studies (Daly 1973, Lehmann and Feldon 2000), possibly because of different methodological approaches used to create maternal-separation induced stress (Lehmann and Feldon 2000, Lehmann, Russig et al. 2002). Moreover, epidemiological studies clearly demonstrate sex-dependent differences in depression, with women deemed more vulnerable to stress-related psychopathologies than men. Intriguingly, sex-specific alterations are also observed in rodent models (Clarke, Grenham et al. 2013). However, most preclinical research is conducted with male animal models to try an eliminate possible confounding factors. As a result, it is still unclear why and how this sex difference exists. As depression has a strong genetic association, the use of transgenic lines that have implicated serotoninergic and noradrenergic systems and HPA axis regulation, provide a useful tool, but as highlighted by a recent meta-analysis between MDD and control participants, the impressive genetic complexity displayed in MDD, cannot fully be reproduced by a single gene mutation in transgenic models (Howard, Adams et al. 2019).

Ultimately, there is growing preclinical evidence of microbial manipulation in exerting beneficial effects on behaviour and cognitive function. What is lacking, is consistency in translating this to clinical trials. This could be a result of numerous factor, including types of analysis undertaken and size of sample groups. Most studies don't include dietary measures accompanying microbiome analysis and often rely on self-reported analysis that only provides indirect information of cognitive behaviour/function. What is needed are longitudinal studies in high-risk or clinical groups which ultimately are necessary to confirm the clinical effects of probiotics/prebiotics and to design a study to specifically test biological mechanisms that could be underly the beneficial cognitive effects of probiotics. Effort should also be put on delaminating possible confounding factors, standardising analytic pipelines and clinical diagnostic parameters.

1.11. Conclusion

The use of high-throughput 16S rRNA sequencing, metagenomics and metabolomics have accelerated the study of intestinal microbiota and their impacts amongst patients

suffering from neurodegenerative and neuropsychiatric disorders, providing evidence to support a role for the gut microbiota in gut-brain communication. Discrepancies in preclinical and clinical studies can arise from variability in sample sizes, misrepresentation of diseased cohorts and different methodological approaches used. This makes is challenging to provide direct comparisons and raises questions regarding the representativeness of the whole disease population. Larger and more comprehensive clinical investigations of the gut-microbiota-brain communication is needed, and consistency in participant recruitment, sampling and microbiome analysis approaches will help reduce some discrepancies between studies and help identify subtle differences and interactions between the gut microbiota and host. It is, however, evident that a well-balanced and diverse gut microbiota is necessary for the maintenance of host health. Gaps in current therapies for psychiatric and neurodegenerative disorders calls for a more detailed understanding of this relationship, facilitating the advancements in novel and personalised treatments that target these disorders from the gut microbiota prospective.

Some key questions surrounding microbial interactions with the host gut-brain axis remain unanswered. It is evident from studies exploring the role of microbial mediators, that SCFAs are multifunctional, influencing different host pathways and systems, inducing differential effects. EECs are amongst one of the first to detect luminal signals transmitting these beyond the GIT, via vagal afferent signalling or hormone secretion, and therefore are key modulators of gut-brain communication. Understanding how gut microbes interact with these chemosensory cells, and to what extent they influence their peptide secretion is important and will ultimately help towards developing strategies and therapies for various metabolic and neurological disorders associated with alterations to the neuroendocrine system. Studying the effects of gut microbiome in its entirety is challenging, and it can be useful to first eliminate potential influencing factors and focus on single species, particularly commensal or probiotic bacteria that have proven beneficial effects.

Bt is one such example. The experiments conducted in Chapter 2 aim to shed light onto the extent to which this gut symbiont regulates enteroendocrine networks and distribution. Using GF mice provides a valuable tool in assessing the role of the normal gut microbiota in maintaining the enteroendocrine system, but also allows mono-

colonisation to determine the importance and potential therapeutic value of Bt in singularly regulating this system *in vivo*.

Of course, it is unlikely that microbiota and their fermentation products work alone, especially when they can produce a vast array of neuroactive molecules and BEVs that can shelter and carry bacterial products over long-distances. Considering this, recent interest has shifted onto the role of BEVs as mediators involved in modulating host gut-brain signalling. To date there are relatively few studies demonstrating the ability of microbial derived vesicles, particularly those derived from commensals to be able to cross host epithelial and endothelial barriers to target the CNS and modulate cellular responses. Therefore, the results presented in Chapter 3 aim to determine the ability of Bt derived BEVs to cross gut epithelial and brain endothelial barriers and their subsequent uptake and cellular effects on brain neuronal and microglia cells, using *in vitro* cell culture-based models. These experiments will provide useful insight into the role of commensal BEVs in regulating CNS function, opening further avenues for research into this relatively unexplored field of research.

Table 1.1 Summary of clinical studies exploring microbial alterations associated with AD.

Study sample	Microbial alterations associated with AD	Reference
25 AD participants 25 HC participants	↓Firmicutes ↓Bacteroidetes ↑*Bifidobacterium*	(Vogt, Kerby et al. 2017)
43 AD participants 43 HC participants	↓Bacteroidetes ↑Actinobacteria ↓Verrucomicrobia ↑*Ruminococcaceae* ↑*Lactobascillaceae* ↑*Enterococcaceae* ↓*Lachnospiraceae* ↓*Veillonellaceae* ↓*Bacteroidaceae*	(Zhuang, Shen et al. 2018)
40 (+Amy) participants 40 (-Amy) participants 10 HC participants	+Amy vs. -Amy/HC: ↓*E. rectale* +Amy vs. HC: ↓*B. fragilis*	(Cattaneo, Cattane et al. 2017)
24 APOE4 carriers 32 APOE4 noncarriers	APOE4 carrier's vs noncarriers: ↓*Prevotellaceae* ↓*Ruminococcaceae* ↓SCFAs	(Tran, Corsini et al. 2019)

Abbreviations: ↓decreased abundance; ↑increased abundance; AD (Alzheimer's disease); HC (healthy control); +Amy (with brain amyloidosis); -Amy (without brain amyloidosis); SCFA (short chain fatty acid); APOE (apolipoprotein E).

Table 1.2 Summary of preclinical and clinical studies assessing the modulation of AD pathology by gut microbiota.

Preclinical studies				
Design	**Model**	**Treatment**	**Outcome**	**Reference**
In vivo	GF APP/PSI mice		↓Aβ levels in brain and blood ↓Aβ deposition in brain ↓Iba-1^{+} microglia in brain ↓IFN-γ, IL-2 and IL-5 in brain ↑NPE and IDE	(Harach, Marungruang et al. 2017)
		Colonisation with CONV-R APP/PSI mice microbiota for 8-weeks	↑Aβ levels in brain and blood ↓Microbial diversity Family: ↓*Rikenellaceae* Genus: ↓*Bacteroides* ↓*Ruminococcus*	
In vivo	CONV-R APP/PSI mice	Early post-natal antibiotic treatment for 1-week	↓Aβ deposition in brain ↑FoxP3^{+} Treg cells in brain and blood ↓plaque-localised glial reactivity ↓α-diversity ↑*Lachnospiraceae* ↑*Akkermansia*	(Minter, Hinterleitner et al. 2017)
In vivo	ddY mice	*B. breve* administration (oral gavage) for 2-days, followed by Aβ i.c.v injection	↓cognitive function ↑plasma acetate Phylum: ↑Actinobacteria Family: ↑*Bacteroidaceae* ↓*Lachnospiraceae* ↓*Odoribacteraceae*	(Kobayashi, Sugahara et al. 2017)
In vivo	APP/PSI mice	Daily intragastric administration of *C. butyricum* for 4-weeks	↓cognitive deficits ↓neurodegeneration ↓Aβ deposition in brain ↓IL-1β and TNF-α in brain ↓microglia activation Family: ↓*Helicobacteraceae* Genus: ↑*Alloprevotella* ↑Faecal butyrate	(Sun, Xu et al. 2020)
Clinical Trials				
Study design			**Outcome**	**Reference**
30 AD patients + probiotics (*L. acidophilus*, *L. casei*, *B. bifidum*, *L. fermentum*) -12-weeks 30 AD patients + placebo control -12-weeks			Probiotic vs. placebo: ↑cognitive function n.s. effect on biomarkers for oxidative stress or inflammation	(Akbari, Asemi et al. 2016)
30 AD patients + probiotics (mixture containing *L. fermentum*, *L. plantarum* and *B. lactis* or *L. acidophilus*, *B. bifidum* and *B. longum*) – 12 weeks 30 AD patients + placebo control – 12-weeks			Probiotic vs. placebo: n.s. effect on cognitive function n.s. effect on inflammatory biomarkers	(Agahi, Hamidi et al. 2018)

Abbreviations: ↑ (increased); ↓ (reduced); n.s. (no significant); AD (Alzheimer's disease) GF (germfree); SPF (specific pathogen free); CONV-R (conventionally raised); Aβ (amyloid-β); IFN-γ (interferon-γ); IL (interleukin); TNF-α (tumour necrosis factor-α); NPE (neprilysin degrading enzyme); IDE (insulin degrading enzyme); Treg (T regulatory); i.c.v (intracerebroventricular).

Table 1.3 Summary of preclinical studies exploring the role of microbial fermentation products in modulating AD pathology.

Preclinical studies				
Method	**Model**	**Treatment**	**Outcome**	**Reference**
In vivo	5xFAD mice	MOS in drinking water for 8 weeks	↓cognitive deficits ↓behavioural disorders ↓Aβ deposition in PFC, CA1 and amygdala ↓Iba-1^{+} microglia in PFC, CA1 and amygdala ↓TNF-α and IL-6 mRNA in brain ↓serum CORT and NE ↑colonic ZO-1 mRNA ↓serum LPS ↑butyrate producing bacteria: *C. pasteuridrum*, *Lachnospira*, *Phascolarctobacterium* and *Veillonellaceae* ↑Serum and faecal butyrate	(Liu, Xi et al. 2021)
In vivo	GF mice	SCFAs in drinking water for 4 weeks (sodium propionate, sodium butyrate and sodium acetate)	↓Ddit4 mRNA in microglia ↓Iba-1^{+} microglia in cerebral cortex ↓microglia malformation	(Erny, Hrabe de Angelis et al. 2015)
In vitro	BV-2 cells	Butyrate pre-treatment for 2 hrs followed by treatment with Aβ oligomers for 36 hrs	↓microglia activation ↓COX-2 expression ↓phosphorylation of NFκB p65	(Sun, Xu et al. 2020)
In vivo	Tg2576 mice	Daily 4-PBA i.p. administration for 5 weeks	↓spatial memory deficits n.s. effect of Aβ levels in brain ↓hippocampal phosphorylated tau ↑hippocampal phosphorylated GSK3β ↑AcH4 in frontal cortex ↑AcH4+/NeuN+ neurones in CA1 and CA3 regions ↑hippocampal GluR1 and PSD95	(Ricobaraza, Cuadrado-Tejedor et al. 2009)

Abbreviations: ↑ (increased); ↓ (reduced); GF (germ-free); SCFAs (short-chain fatty acids); MOS (mannan oligosaccharide); 4-PBA (sodium 4-phenylbutyrate); i.p. (intraperitoneal); Aβ (amyloid-β); PFC (prefrontal cortex); CA (cortical area); CORT (corticosterone); NE (norepinephrine); LPS (lipopolysaccharide); Ddit4 (DNA damage-inducible transcript 4); GSK3β (glycogen synthase kinase 3β); AcH4 (acetylation of histone 4); PSD95 (post-synaptic density protein 95); GluR1 (glutamate receptor 1); NFκB (nuclear factor kappa-B); TNF-α (tumour necrosis factor-α); IL (interleukin); CORT (corticosterone); NE (norepinephrine); ZO-1 (zonula occludin-1).

Table 1.4 Summary of preclinical studies exploring the role of microbial LPS in modulating AD pathology.

Preclinical studies				
Method	**Model**	**Treatment**	**Outcome**	**Reference**
In vivo	CD-1 mice	i.p. administration of LPS followed by i.v. administration of I-Aβ	↑BBB influx of I-Aβ ↓BBB efflux of I-Aβ ↓LRP protein and mRNA expression n.s. in RAGE protein or mRNA expression ↑serum IL-10, IL-6, MCP-1 and RANTES	(Jaeger, Dohgu et al. 2009)
In vitro	BMEC monolayers isolated from CD-1 mice	LPS pre-treatment for 4 hrs followed by I-Aβ addition to luminal chamber	n.s. effect on luminal to abluminal influx of I-Aβ ↓TEER	
In vitro	Aβ fragments	Incubation with LPS for 3-days	↑Aβ fibrillogenesis Helical fibrils	(Asti and Gioglio 2014)
		Incubation with intact cell suspension of *E. coli* for 3-days	Short irregular and scattered Aβ fibrils, closely associated with bacterial cell wall	
In vivo	C57BL/6 mice	i.p. administration of LPS for 1/7 days	<u>1-day LPS:</u> ↑hippocampal Aβ, IL-1β ↑sickness behaviour <u>7-day LPS:</u> Hippocampal Aβ returned to baseline n.s. effect on sickness behaviour	(Kahn, Kranjac et al. 2012)

Abbreviations: ↑ (increased); ↓ (reduced); n.s. (not significant); i.p. (intraperitoneal); i.v. (intravenous); LPS (lipopolysaccharide); BBB (blood brain barrier); Aβ (amyloid-β); LRP (low density lipoprotein receptor related protein-1); RAGE (receptor for advanced glycation end products); IL (interleukin); MCP-1 (macrophage chemotactic peptide-1); RANTES (regulated on activation normal T cell expressed secreted protein); TEER (transepithelial electrical resistance).

Table 1.5 Summary of preclinical studies exploring the role of microbial derived membrane vesicles in modulating AD pathology.

Preclinical studies				
Method	**Model**	**Treatment**	**Outcome**	**Reference**
In vivo	C57BL/6 mice	Tail vein injection of OMVs (50 μM) isolated from AD patients– daily for 8-weeks	↑ BBB permeability ↓claudin-5 ↓leaning and memory ↑GSK-3β and tau hyperphosphorylation in hippocampus ↑NFκB, IL-1β and TNF-α in hippocampus ↑microglia and astrocyte activation	(Wei, Peng et al. 2020)
In vivo	C57BL/6 mice	Oral gavage with *P. hominis* BEVs (10 μg/kg) daily for 5-days	↑NFκB/Iba1$^+$, LPS$^+$/Iba1$^+$ and IL-1R$^+$ in hippocampus ↓BDNF in hippocampus ↑IL-1β and LPS in blood ↑cognitive impairment ↑hippocampal bacterial 16S rDNA	(Lee, Kim et al. 2020)
	C57BL/6 mice with celiac vagotomy		↓cognitive impairment ↓NFκB/Iba1$^+$, LPS$^+$/Iba1$^+$ and IL-1R$^+$ in hippocampus ↓hippocampal bacterial 16S rDNA	

Abbreviations: ↑ (increased); ↓ (reduced); AD (Alzheimer's disease); OMVs (outer membrane vesicles); BEVs (bacterial extracellular vesicles); BBB (blood brain barrier); LPS (lipopolysaccharide); GSK3β (glycogen synthase kinase 3β); NFκB (nuclear factor kappa-B); TNF-α (tumour necrosis factor-α); IL (interleukin); BDNF (brain derived neurotrophic factor).

Table 1.2 Summary of preclinical studies exploring the role of gut peptides in modulating AD pathology.

Preclinical studies				
Method	**Model**	**Treatment**	**Outcome**	**Reference**
In vivo	5xFAD mice	MOS in drinking water for 8-weeks	↑colonic FFAR2/FFAR3 mRNA ↑colonic GLP mRNA ↑brain APP levels	(Liu, Xi et al. 2021)
In vivo	APP/PSI mice	Liraglutide (daily i.p. injection for 8-weeks)	↑memory ↑synaptophysin expression in hippocampus and striatum ↑brain GLP-1 ↓Aβ plaques in cortex ↓activated microglia in cortex	(McClean, Parthsarathy et al. 2011)
In vivo	APP/PSI mice	Val(8)GLP-1 (daily i.p. injection for 21-days)	↑LTP in CA1 of hippocampus ↓dense core plaque load in cortex n.s. in Aβ plaque load or microglia activation	(Gengler, McClean et al. 2012)
In vivo	Wister rats	i.c.v. administration of Aβ(25-35)	↓hippocampal LTP	(Gault and Hölscher 2008)
		i.c.v. administration of GLP-1 or Val(8)GLP-1	↑hippocampal LTP	
		30 min pre-treatment with Val(8)GLP-1 + i.c.v Aβ(25-35)	↑hippocampal LTP	
In vitro	Hippocampal and cortical neuronal cultures from Sprague-Dawley rats	Soluble Aβ oligomers (30 min)	↓IR activity upon insulin exposure ↓dendrite insulin receptors	
In vivo	Cynomolgus monkeys	i.c.v. injection Aβ oligomers	↑neuronal IRS-1/pSer in hippocampus and temporal cortex ↑hippocampal pJAK+/NeuN+	(Bomfim, Forny-Germano et al. 2012)
	APP/PSI mice		↑hippocampal IRS-1/pSer ↑hippocampal pJNK	
		Exendin-4 (daily i.p. injections for 3-weeks)	↓brain levels of IRS-1/pSer and pJNK ↑spatial memory ↓Aβ plaque load and soluble Aβ	

Abbreviations: ↑ (increased); ↓ (reduced); n.s. (not significant); AD (Alzheimer's disease); i.p. (intraperitoneal); i.c.v. (intracerebroventricular); MOS (mannose oligosaccharide); GLP-1 (glucagon-like peptide 1); FFAR (free-fatty acid receptor); APP (amyloid precursor protein); Aβ (amyloid- β); LTP (long-term potentiation); CA (cortical area); IR (insulin receptor); IRS-1 (insulin receptor substrate-1); JNK (c-Jun N-terminal kinase); JAKA (junctional adhesion molecule A).

Table 1.3 Summary of clinical trials exploring the role of gut peptides in modulating AD pathology.

Clinical Studies		
Study Design	**Outcome**	**Reference**
18 AD participants + liraglutide – 26 weeks 20 AD participants + placebo control – 26 weeks	Liraglutide vs. placebo: n.s. effect in total cognitive scores n.s. effect on fibrillary Aβ ↑% change in CMRglu between baseline and 26 weeks in cingulate cortex and cerebellum	(Gejl, Gjedde et al. 2016)
AD participant brains vs. non-cognitively impaired participants	AD vs. Non-cognitively impaired: ↑hippocampal ISR-1/pSER ↑hippocampal pJNK	(Bomfim, Forny-Germano et al. 2012)

Abbreviations: ↑ (increased); ↓ (reduced); n.s. (not significant); AD (Alzheimer's disease); Aβ (amyloid- β); IRS-1 (insulin receptor substrate-1); JNK (c-Jun N-terminal kinase).

Table 1.4 Summary of clinical studies exploring microbial alterations in PD.

Participants	Alterations in gut microbiota (PD vs. HC)	Reference
72 PD patients 72 HC patients	↓*Prevotellaceae* ↑*Enterobacteriaceae*	(Scheperjans, Aho et al. 2015)
34 PD patients 34 HC patients	↓Bacteroidetes ↓*Prevotellaceae* ↑*Enterobacteriaceae*	(Unger, Spiegel et al. 2016)
31 PD patients 28 HC patients	↑Firmicutes ↑*Verrucomicrobiaceae* (*A. muciniphila*) ↓*Prevotellaceae* (*P. copri*) ↓*Erysipelotrichaceae* (*E. bioforme*)	(Bedarf, Hildebrand et al. 2017)
38 PD patients 34 HC patients	↓*Faecalibacterium* ↑Proteobacteria (*Ralstonia*) ↓*Lachnospiraceae* (*Blautia, Coprococcus, Roseburia*)	(Keshavarzian, Green et al. 2015)
197 PD patients 130 HC patients	↓*Lachnospiraceae* (*Blautia, Roseburia*) ↓*F. prausnitzii* ↑Verrucomicrobia (*Akkermansia*) ↑*Bifidobacterium* ↑*Lactobacillus*	(Hill-Burns, Debelius et al. 2017)
52 PD patients 36 HC patients	↑*Lactobacillus* (*L. gasseri, L. brevis, L. casei, L. fermentum, L. reuter*i) ↓*Clostridium* (*C. coccoides, C. leptum*) ↓*B. fragilis*	(Hasegawa, Goto et al. 2015)
64 PD patients 51 HC patients	↓*Lachnospiraceae* (*Blautia, Coprococcus, Roseburia, Butyrivirio*) ↑*Clostridium* ↑*Veillonella* ↑*Streptococcus* ↑*Bifidobacterium* ↑*Verrucomicrobiaceae* (*Akkermansia*)	(Vascellari, Palmas et al. 2020)

Abbreviations: PD (Parkinson's disease); HC (healthy controls): ↓ (decreased abundance); ↑ (increased abundance).

Table 1.5 Summary of preclinical studies exploring the role of gut microbiota on PD pathology.

Preclinical studies				
Method	**Model**	**Treatment**	**Outcome**	**Reference**
In vivo	GF-ASO mice		↓motor and GI dysfunction ↓α-synuclein pathology in CP and SN ↓TNFα and IL-6 expression in $CD11b^+$ microglia ↓microglia morphological alteration	(Sampson, Debelius et al. 2016)
	SPF-ASO mice	Antibiotic treatment, 6-7 weeks	↓motor and GI dysfunction ↓microglia morphological alterations	
	GF-ASO mice	SCFAs (acetate, propionate and butyrate) in drinking water for 6-7 weeks	↑motor and GI dysfunction ↑microglia activation in SN and CP	
	SPF-ASO mice	FMT with microbiota from human PD patients	↑motor and GI dysfunction Families: ↑*Lachnospiraceae* ↑*Rikenellaceae* ↑*Peptostreptococcaceae* Genera: ↑*Roseburia* ↑*Bilophila* ↑*Proteus* ↓*Butyricicoccus* SCFAs: ↓acetate ↑propionate ↑butyrate	
In vivo	MPTP-C57BL/6 mice	FMT of microbiota from healthy mice	↓motor dysfunction ↓striatal DA, 5-HT, DOPAC, HVA and 5HIAA ↑dopaminergic neurones in SN ↓activated $GFAP^+$ astrocytes and Iba-1^+ microglia in SN ↓striatal and colonic TLR-4, NFκB and TNFα expression ↓*microbial dysbiosis*: ↑Firmicutes ↓Proteobacteria ↓faecal SCFAs	(Sun, Zhu et al. 2018)
In vivo	MPTP-C57BL/6 mice	Intragastric administration of Clostridium butyricum for 4 weeks	↓motor dysfunction ↓loss of dopaminergic neurones in SN ↑synapsin-1 and CB11b expression ↓microbial dysbiosis: ↑*Akkermansia* ↑*Helicobacter* ↑*Enterorhabdus*	(Sun, Li et al. 2021)
In vitro	6-OHDA treated 5H-SY5Y cells	SLAB51 probiotic treatment	↑cell viability ↑BDNF and PPARγ expression	
In vivo	MPTP-C57BL/6 mice	SLAB51 probiotic oral gavage for 5 weeks (starting 2 weeks prior to MPTP)	↓motor and behavioural dysfunction ↑dopaminergic neurones in SN and striatum ↑DAT in SN ↓Iba-1^+ and $GFAP^+$ cell fluorescent intensity ↑PPARγ expression in SN and striatum ↓NFκB expression in SN and striatum	(Castelli, d'Angelo et al. 2020)

Abbreviations: ↑increased; ↓reduced; GF (germ-free); SPF (specific-pathogen free); ASO (α-synuclein overexpressing); MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine); 6-OHDA (6-hydroxydopamine); SCFAs (short chain fatty acids); FMT (faecal microbial transplant); GI (gastrointestinal); CP (caudoputamen); SN (substantia nigra); TNFα (tumour necrosis factor α); TGF-β

(transforming growth factor-β); IL (interleukin); DA (dopamine); 5-HT (serotonin); DOPAC (3, 4-dihydroxyphenylacetic acid); HVA (homovanillic acid); 5HIAA (5-hydroxyindoleacetic acid); TLR (toll-like receptor); NFκB (nuclear factor kappa-B); BDNF (brain derived neurotrophic factor); PPARγ (peroxisome proliferator activated receptor γ); DAT (dopaminergic transporter).

Table 1.6 Summary of clinical trials investigating the role of microbial modulation by probiotics on PD pathology.

Clinical Studies		
Study design	**Outcome**	**Reference**
30 PD patients + probiotics (*L. acidophilus, B. bifidum, L. reuteri, L. fermentum*) – 12 weeks 30 PD patients + placebo control – 12 weeks	Probiotics vs. placebo: ↓MDS-UPDRS score ↓Insulin levels ↓insulin resistance ↑insulin sensitivity	(Tamtaji, Taghizadeh et al. 2019)
25 PD patients + probiotics (*L. acidophilus, B. bifidum, L. reuteri, L. fermentum*) – 12 weeks 25 PD patients + placebo control – 12 weeks Isolation of PBMCs	Probiotics vs. placebo: ↓IL-1, IL-8 and TNFα gene expression ↑TGF-β and PPARγ gene expression	(Borzabadi, Oryan et al. 2018)
Isolation of PBMCs from PD and HC patients and co-culture with individual probiotics from *Lactobacillus* and *Bifidobacterium* genera	PD vs. HC: ↓IL-6 secretion by *L. salvarius, L. acidophilus, L. plantarum, L. rhamnosus, B. breve* and *B. animalis* ↓TNFα secretion by *L. salvarius, L. acidophilus* and *B. animalis* ↑IL-10 secretion by *L. salvarius, L. plantarum, L. acidophilus, L. rhamnosus* and *B. animalis* ↓O_2- production by *L. salvarius, L. acidophilus, L . plantarum, L. rhamnosus* and *B. animalis*	(Magistrelli, Amoruso et al. 2019)

Abbreviations: ↑ (increased); ↓ (reduced); PD (Parkinson's disease); HC (healthy controls); MDS-UPDRS (Movement Disorders Society-Unified PD rating score PPARγ (peroxisome proliferator activated receptor γ); TNFα (tumour necrosis factor α); TGF-β (transforming growth factor-β); IL (interleukin); PBMC (peripheral blood mononuclear cell).

Table 1.7 Summary of preclinical studies exploring role gut peptides in modulating PD pathology.

Preclinical studies				
Method	**Model**	**Treatment**	**Outcome**	**Reference**
In vivo	MPTP-C57BL/6 mice	Intragastric *C. butyricum* administration for 4 weeks	↑colonic GLP-1 ↑colonic FFAR2/FFAR3 expression ↑cerebral GLP-1R expression	(Sun, Li et al. 2021)
In vitro	Neuronal stem cell culture (C57BL/6 mice)	Ex-4 treatment (4-6 days)	↑intracellular cAMP ↑neuronal differentiation	(Bertilsson, Patrone et al. 2008)
In vivo	6-OHDA-Wister rats	i.p. administration Ex-4 for 3 weeks	↑TH^+ neurones in SN ↑$VAMT2^+$ neurones in SN	
In vivo	6-OHDA-Wister rats	Administration of Ex-4 twice daily for 7-days	↓apomorphine-induced rotational behaviour ↑striatal DA and DOPAC ↑TH^+ neurones	(Harkavyi, Abuirmeileh et al. 2008)
In vitro	Primary rat embryonic CC and VM cultures + 6-OHDA	Incubation with Ex-4	↓caspase-3 activity ↑TH immunoreactivity	(Li, Perry et al. 2009)
In vivo	MPTP-C57BL/6 mice	Pre-treatment with Ex-4	↓motor dysfunction ↓dopaminergic damage in SN ↑DA, DOPAC and HVA in striatum	
In vivo	α-synuclein C57BL/6 mice	s.i. of NLYO1 twice weekly for 5 months	↓behavioural deficits ↑TH^+ and ↓Iba-1 cells in SNpc ↓α-synuclein immunoreactivity in striatum and VMB ↓DAT expression in VMB ↑DA, HVA and DOPAC ↓IL-1β and IL-6 expression in VMB ↓$GFAP^+$ cells in VMB	(Yun, Kam et al. 2018)
In vivo	hA53Tg mice	s.i. NLYO1 twice weekly for 4 months	↑lifespan ↓α-synuclein pathology ↓Iba-1^+ and $GFAP^+$ cell fluorescent intensity	

Abbreviations: ↑ (increased); ↓ (reduced); n.s. (not significant); s.i.(Subcutaneous injection); MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine); 6-OHDA (6-hydroxydopamine); VM (ventral mesencephalic); SN (substantia nigra); SNpc (SN pars compacta); VMB (ventral midbrain); Ex-4 (exendin-4); GLP-1 (glucagon-like peptide-1); FFAR (free-fatty acid receptor); GLP-1R (GLP-1 receptor); cAMP (cyclic AMP); TH (tyrosine hydrolase); DA (dopamine); DOPAC (3, 4-dihydroxyphenylacetic acid); HVA (homovanillic acid); DAT (dopaminergic transporter); IL (interleukin); MDS-UPDRS (Movement Disorders Society-Unified PD rating score).

Table 1.8 Summary of clinical trials exploring the role of gut peptides in PD pathology.

Clinical studies		
Study design	**Outcome**	**Reference**
31 PD patients + exenatide – 12 weeks 29 PD patients + placebo control – 12 weeks (Analysis at 48 weeks)	<u>Exenatide vs. placebo:</u> ↑MDS-UPDRS Part 3 score n.s. effect on mood, quality of life or non-motor symptoms	(Athauda, Maclagan et al. 2017)
20 PD patients + exenatide – 12 months 24 PD patients + randomised controls – 12 months (Analysis at 24 months)	<u>Exenatide vs. control:</u> ↑MDS-UPDRS Part 3 score	(Aviles-Olmos, Dickson et al. 2014)

Abbreviations: ↑ (increased); ↓ (reduced); PD (Parkinson's disease); HC (healthy controls); MDS-UPDRS (Movement Disorders Society-Unified PD rating score).

Table 1.9 Summary of clinical studies identifying microbial alteration associated with depression-related disorders.

Participants	Alterations in depressed patient's vs HC patients	Reference
29 MDD patients 30 HC patients	↑α-diversity ↑Bacteroidetes ↑Proteobacteria ↑Actinobacteria ↓Firmicutes ↓*Faecalibacterium* ↑*Alistripes*	(Jiang, Ling et al. 2015)
34 MDD-patients 33 HC patients	↓diversity and richness ↓*Prevotellaceae* ↑*Thermoanaerobacteriaceae* ↓*Prevotella* ↓*Dialister*	(Kelly, Allen et al. 2017)
Depressed cohort (n=121)	↓*Dialister* ↓*Coprococcus*	(Valles-Colomer, Falony et al. 2019)
37 depressed patients 18 HC patients	↑Bacteroidales ↓Lachnospiraceae	(Naseribafrouei, Hestad et al. 2014)
58 MDD patients 63 HC patients	↑Actinobacteria ↓Bacteroidetes n.s. Firmicutes ↑29 OTUs assigned to families: *Actinomycinaceae, Lactobascillaceae (Anaerostripes, Blautia, Dorea), Streptococcaceae, Coriobacteriaceae, Ruminococcaceae, Eubacteriaceae* ↓25 OTUs assigned to families: *Bacteroidaceae, Rikenellaceae, Lachnospiraceae (Coprococcus, Clostridium XIVa, Roseburia, Faecalibacterium), Veillonellaceae*	(Zheng, Zeng et al. 2016)
156 MDD patients 155 HC patients	↑*Bacteroides* ↓*Blautia* ↓*Eubacterium*	(Yang, Zheng et al. 2020)

Abbreviations: ↑ (increased abundance); ↓ (decreased abundance); n.s. (no significant); MDD (major depression disorder); HC (healthy control); OUT (operational taxonomic unit).

Table 1.10 Summary of preclinical studies exploring the role of the gut microbiota in pathology of depression-related disorders.

Preclinical studies				
Method	**Model**	**Treatment**	**Outcome**	**Reference**
In vivo	GF NMRI mice		↑motor activity ↓anxiety-like behaviour ↑NA, DA, 5-HT, DOPAC and HVA in striatum ↓BDNF mRNA in PFC, hippocampus, CC and amygdala ↓NGFI-1 mRNA expression in PFC, striatum, hippocampus, DG and amygdala ↑DA D1 receptor mRNA in hippocampus ↑synaptophysin and PSD-95 expression in striatum, FC and hippocampus	(Diaz Heijtz, Wang et al. 2011)
	GF NMRI mice	Conventionalisation with microbiota from SPF-NMRI mice	Normalisation of motor activity Normalisation of anxiety-like behaviours ↓synaptophysin and PSD-95 expression in striatum	
In vivo	GF BALB/c mice		↑stress-induced plasma ACTH and corticosterone ↓BDNF protein expression in cortex and hippocampus	(Sudo, Chida et al. 2004)
	GF BALB/c mice	Mono-colonisation with *B. infantis*	↓stress-induced plasma ACTH and CORT ↑plasma IL-6 (returned to baseline at 24 hrs)	
		Reconstitution with microbiota from SPF BALB/c mice at 6-weeks of age	↓stress-induced plasma ACTH and CORT	
Ex vivo	GF Swiss Webster mice (analysis from Male mice)		↓TNFα production following splenocyte stimulation with LPS	(Clarke, Grenham et al. 2013)
In vivo			↑acute stress induced plasma CORT	
			↓BDNF expression in hippocampus ↓kynurenine/tryptophan ratio ↑plasma tryptophan ↑hippocampal 5-HT and 5-HIAA	
In vivo	GF mice		↑plasma tryptophan, N-acetyl tryptophan ↓plasma 5-HT	(Wikoff, Anfora et al. 2009)
In vivo	GF mice	FMT with microbiota from MDD patients	↑depression and anxiety like behaviours ↓faecal lactate ↑faecal succinate ↓Phylum: Bacteroidetes (*Bacteroidaceae*, *Prevotellaceae*, *Porphynomonadaceae*) ↑Phylum: Actinobacteria (*Coriobacteriaceae)* ↑Phylum: Firmicutes (*Lachnospiraceae*, *Ruminococcaceae*)	(Zheng, Zeng et al. 2016)

In vivo	Sprague Dawley rats	Antibiotic treatment + FMT with microbiota from MDD patients	↑anxiety like behaviours ↑kynurenine/tryptophan ratio ↑intestinal motility ↑ faecal acetate ↓microbial richness and diversity Phylum: ↓Actinobacteria Family: ↓*Bifidobacteriaceae* ↓*Conobacteriaceae* ↓*Porphyromonadaceae* ↑*Propionibacteriaceae* Genus: ↓*Bifidobacterium* ↓*Roseburia* ↑*Peptococcus* ↑*Staphylococcus*	(Kelly, Allen et al. 2017)
In vivo	Sprague Dawley rats	MS for 2 weeks	↑stress induced immobility ↓NA in amygdaloid cortex ↓5-HIAA in Pons ↓5-HIAA:5-HT turnover in hippocampus ↑CRF mRNA in amygdaloid cortex	(Desbonnet, Garrett et al. 2010)
	Sprague Dawley rats	MS for 2 weeks + . infantis in drinking water for 45-days	↓stress induced immobility ↓NA and 5-HIAA in amygdaloid cortex ↑CRF mRNA in amygdaloid cortex	
In vivo	GF-1 mice	Subclinical infection (oral gavage) with C. jejuni	↑anxiety-like behaviours n.s. effect on IL-6 in peripheral blood	(Lyte, Varcoe et al. 1998)
In vivo	BALB/c mice	Oral gavage with *L. rhamnosus* (JB-1) daily for 28-days	↓stress-induced depression like behaviours ↑stress-induced anxiety like behaviours ↓stress induced plasma CORT ↑GABAB1b mRNA ↓GABAB1b mRNA DG, CA3, CA1 ↓GABAAα2 mRNA ↑ GABAAα2 mRNA in DG	(Bravo, Forsythe et al. 2011)
	Vagotomised BALB/c mice	Oral gavage with *L. rhamnosus* (JB-1) daily for 28-days	Inhibition of anxiolytic effects n.s. effects on GABAAα2 mRNA expression	
In vivo	Sprague Dawley rats	Oral gavage with GOS daily for 5 weeks	↑faecal Bifidobacterium ↑BDNF mRNA in CA3 and DG ↑NR1 and NR2A mRNA in DG ↑plasma PYY ↑Faecal amino acid concentrations ↑Amino acid (D-serine) in FC	(Savignac, Corona et al. 2013)

Abbreviations: ↑ (increased); ↓(reduced); n.s. (not significant); GF (germfree); SPF (specific pathogen free); MS (maternal separation); MDD (major depressive disorder); FMT (faecal microbial transplant); GOS (galaco oligosaccharide) NA (noradrenaline); DA (dopamine); 5-HT (serotonin); DOPAC (3, 4-dihydroxyphenylacetic acid); HVA (homovanillic acid); 5HIAA (5-hydroxyindoleacetic acid); BDNF (brain derived neurotrophic factor); NGFI-1 (neuronal growth factor inducible 1); PSD-95 (post-synaptic density protein 95); ACTH (adrenocorticotropic hormone); CRF (corticotropic releasing hormone); CORT (corticosterone); LPS (lipopolysaccharide); TNF-α (tumour necrosis factor-α); IL (interleukin); FC (frontal cortex); DG (dentate gyrus); CA (cortical area).

Table 1.11 Summary human clinical trials exploring the role of microbial modulation in pathology of depression-related disorders

Clinical studies		
Study design	**Outcome**	**Reference**
14 healthy participants + probiotic (*L. rhamnosus* JB-1) – 8 weeks 15 healthy participants + placebo – 8 weeks	n.s. anti-inflammatory effects n.s. effect on cognition n.s. effect on modulation of stress/HPA response	(Kelly, Allen et al. 2017)
20 healthy participants + probiotics (*B. bifidum, B. lactis, L. acidophilus, L. brevis, L. casei, L. salvarius, L. lactis*) – 28 days 20 healthy participants + placebo – 28 days	<u>Probiotics vs. placebo</u> ↓total cognitive reactivity to dysfunctional thoughts (LEIDS-r score)	(Steenbergen, Sellaro et al. 2015)
26 healthy participants + probiotics – 30 days 29 healthy participants + placebo – 30 days	<u>Probiotics vs. placebo:</u> ↓HADS score ↓HSCL-90 score ↓urinary free cortisol	(Messaoudi, Lalonde et al. 2011)
15 healthy participants + FOS – 3 weeks 15 healthy participants + GOS – 3 weeks 5 healthy participants + placebo – 3 weeks	<u>FOS/GOS vs. placebo:</u> ↑attentional vigilance to positive vs. negative stimuli <u>GOS vs. placebo:</u> ↓salivary cortisol awakening response	(Schmidt, Cowen et al. 2015)
20 MDD patients + probiotics (L. acidophilus, *L. casei*, B. *bifidum*)- 8 weeks 20 MDD patients + placebo – 8 weeks	<u>Probiotics vs. placebo:</u> ↓BDI total score ↓serum insulin and hs-CRP ↑plasma total glutathione	(Akkasheh, Kashani-Poor et al. 2016)

Abbreviations: ↑ (increased); ↓(reduced); n.s. (not significant); MDD (major depressive disorder); FOS (fructo-oligosaccharide); GOS (galacto-oligosaccharide); HPA (hypothalamic-adrenal-pituitary); CRF (corticotropic releasing hormone); BDI (Beck's Depression Inventory); HADS (Hospital Anxiety and Depression Scale); HSCL-90 (Hopkins symptom checklist-90).

Table 1.12 Summary of preclinical and clinical studies exploring the role of the gut microbiota and gut peptides in pathology of depression-related disorders.

Preclinical studies				
Method	**Model**	**Treatment**	**Outcome**	**Reference**
In vitro	RIN14B cells	Butyrate Propionate	↑5-HT secretion ↑Tph1 mRNA	(Yano, Yu et al. 2015)
In vitro	RIN14B cells	LAB strains	LAB E31 and M2CF22M7: ↑5-HTP secretion ↑Tph1 mRNA	(Tian, Wang et al. 2019)
In vivo	C57BL/6 mice subjected to CSDS (10 days)	GTA administered via oral gavage (3-days)	↓depression-like behaviours ↑hippocampal BDNF, NGF, c-FOS ↑Ac-H3 and Ac-H4 in hippocampus ↓HDAC2, HDAC5, HDAC7 and HDAC8 in hippocampus	(Huang, Hu et al. 2021)
In vivo	C57BL/6 mice + LPS induced depression	Sodium butyrate pretreatment (10-days)	↓depression-like behaviours ↓TNF-α, IL-6 and IL-1β in the hippocampus and PFC ↓oxido-nitrosative stress	(Qiu, Liu et al. 2020)
Clinical studies				
Study design			**Outcome**	**Reference**
19 participants with MDD or BD + liraglutide – 4 weeks			↑objective measures of cognition n.s. mood scores or metabolic parameters	(Mansur, Ahmed et al. 2017)

Abbreviations: ↑ (increased); ↓(reduced); n.s. (not significant); LAB (lactic acids bacteria); GTA (Glyceryl triacetate); CSFS (chronic social defeat stress); Ac-H (acetylation of histone); HDAC (histone deacetylase); PFC (prefrontal cortex); 5-HT (serotonin); 5-HTP (5-hydroxytryptophan); Tph (tryptophan hydrolase) BDNF (brain derived neurotrophic factor); NGF (nerve growth factor) TNF-α (tumour necrosis factor-α); IL (interleukin) MDD (major depressive disorder); BD (bipolar disorder)

2. Regulation of enteroendocrine cell networks by the major human gut symbiont *Bacteroides thetaiotaomicron*

2.1. Introduction

A mutualistic relationship exists between the intestinal microbiota and the host in which commensal microbes provide the host with essential protective and metabolic functions, including fermentation of complex plant-based carbohydrates to produce metabolites such as short chain fatty acids (SCFAs) that are an important energy source for host cells (McNeil 1984, Bergman 1990). In turn, the host provides the microbiota with nutrients essential for their colonization and survival (Savage 1977, Tremaroli and Backhed 2012). Chronic metabolic diseases including metabolic syndrome, obesity and diabetes have been associated with structural and/or functional changes in the intestinal microbiota and principally, the prokaryome (Carding, Verbeke et al. 2015). The causal nature of these associations remains to be determined, although alterations in energy extraction from food (Backhed, Ding et al. 2004, Turnbaugh, Ley et al. 2006), increased nutrient harvesting (Tremaroli and Backhed 2012) and appetite signalling (Plovier and Cani 2017, Covasa, Stephens et al. 2019) are potential mechanisms. Animal models and in particular germfree (GF) rodents have been instrumental in advancing the understanding of the complexity of the intestinal microbiota and providing mechanistic insights of microbial-host interactions at the epithelial interface (Tremaroli and Backhed 2012).

Enteroendocrine cells (EECs) are scattered throughout the entirety of the epithelium of the gastrointestinal tract (GIT) and are key sensors of microbial metabolites in the intestinal lumen. They sense changes within the luminal environment and relay signals via the production and secretion of peptide hormones. These act on local nerve endings of the enteric nervous system or other cells within the intestinal mucosa that converge on hypothalamic feeding circuits to regulate and coordinate metabolism and food intake (Beutler, Chen et al. 2017). Via the circulatory system and vagal nerves their influence can extend beyond the GIT, affecting the function of organs such as the brain, liver and adipose tissues (Gribble and Reimann 2016). EECs are divided into subgroups depending on their secreted hormones and location along the GIT. Prominent subsets include L, K and enterochromaffin cells (EC). L cells secrete mainly

glucagon-like peptide-1 (GLP-1) or peptide YY (PYY) and are found throughout the GIT but are more densely populated in the colon. K cells secrete glucose-dependent insulinotropic polypeptides (gastric inhibitory peptide, GIP) and are mainly found in the upper small intestine. ECs found throughout the GIT, make up the single largest population of endocrine cells in the intestinal epithelium and produce mainly serotonin or 5-hydroxytryptamine (5-HT) (Sjolund, Sanden et al. 1983).

GLP-1 and GIP are the primary incretin hormones that cause the release of insulin from pancreatic beta-cells following meal ingestion (Baggio and Drucker 2007). The administration of probiotic bacteria to obese and diabetic mice increases glucose tolerance, L cell number, intestinal proglucagon mRNA and plasma GLP-1 levels, suggesting that intestinal microbes can play a role in altering glucose homeostasis and EEC activity (Cani, Bibiloni et al. 2008). However, our understanding of how the intestinal microbiota initiates signalling in EEC is incomplete. Key insights have been obtained from studies using wildtype or transgenic strains of GF animals and by examining the impact of substrates and metabolites of microbiota metabolism. Evidence for the ability of the intestinal microbiota to influence L cells directly has come from GF mice expressing a proglucagon reporter gene in which conventionalisation with an unfractionated microbiota has been shown to modulate the L cell transcriptome in the ileum (Arora, Akrami et al. 2018). Amongst microbial metabolites, SCFAs have been the most intensively studied with those produced from the fermentation of dietary fibre increasing GLP-1 and peptide YY (PYY) levels in tissues and plasma (Keenan, Zhou et al. 2006, Zhou, Hegsted et al. 2006, Zhou, Martin et al. 2008). SCFAs signal through G-protein coupled receptors (GPCRs) that co-localize with EECs (Karaki, Mitsui et al. 2006), such as free fatty acid receptor (FFAR) 2 and 3 (also known as GPR43 and 41, respectively, expressed on L cells) (Tazoe, Otomo et al. 2008, Tazoe, Otomo et al. 2009, Tolhurst, Heffron et al. 2012, Nohr, Pedersen et al. 2013). FFAR2-deficient mice display lower GLP-1 plasma levels and reduced glucose tolerance highlighting the importance of these receptors in microbial signalling in EECs (Tolhurst, Heffron et al. 2012). In addition, oligofructose supplementation increases GIP plasma levels as well as altering microbial composition (Girard 2008, Tolhurst, Heffron et al. 2012). Furthermore, GIP is involved in fat metabolism (Yip and Wolfe 2000) and therefore is a potential target for microbiota modulation in obesity. Using two bacteria to conventionalise GF mice a role for the

FFAR3 receptor in regulating host energy balance has been identified in a process involving bacterial modulation of PYY expression (Samuel, Shaito et al. 2008). Aside from metabolic processes, the intestinal microbiota can affect neuronal and EEC signalling processes by altering 5-HT production. For example, the numbers of EECs are reduced in number in GF rats (Uribe, Alam et al. 1994), while the presence of indigenous spore-forming bacteria, mainly from the *Clostridium* spp., promotes 5-HT biosynthesis through increasing tryptophan hydrolase 1 (Tph1) expression, a rate-limiting enzyme involved in the biosynthesis of 5-HT tryptophan (Yano, Yu et al. 2015, Zelkas, Raghupathi et al. 2015). Conversely, 5-HT stimulates the growth in culture of bacterial species including *E. coli* and *Rhodospirillum* (Oleskin, Kirovskaia et al. 1998), suggesting a bi-directional relationship exists between EEC signalling and the gut microbiota.

Collectively, these studies suggest that the intestinal microbiota has profound effects on EECs including regulation of production and secretion of their peptide hormones, which may occur via products of microbial metabolism acting directly on EECs. Recent work undertaken demonstrated the critical role of ubiquitous and prominent commensal gut bacterium *Bacteroides thetaiotaomicron* (Bt) influencing colonic motility through regulating the neuronal and vagal afferent innervation of the mouse GIT (Aktar, Parkar et al. 2020). The experiments undertaken in this Chapter aim to obtain more evidence of these putative mechanisms using conventional and GF mice to investigate the role that Bt and its major metabolic output (acetate, propionate and succinate) (Hooper, Midtvedt et al. 2002, Wrzosek, Miquel et al. 2013, Curtis, Hu et al. 2014) have on EEC networks in the murine GIT.

2.2. Results

To determine the extent to which the gut microbiota regulates EEC populations a combination of SPF, GF, Bt conventionalised GF mice, *L. reuteri* (Lr) conventionalised GF mice, or GF mice administered the major fermentation products of Bt (acetate, propionate and succinate; APS) were used. A combination of methods including *in vivo* tissue analysis (including immunohistochemistry, faecal metabolite analysis and serum hormone levels), *ex vivo* hormone secretion assays and *in vitro* intestinal crypt derived

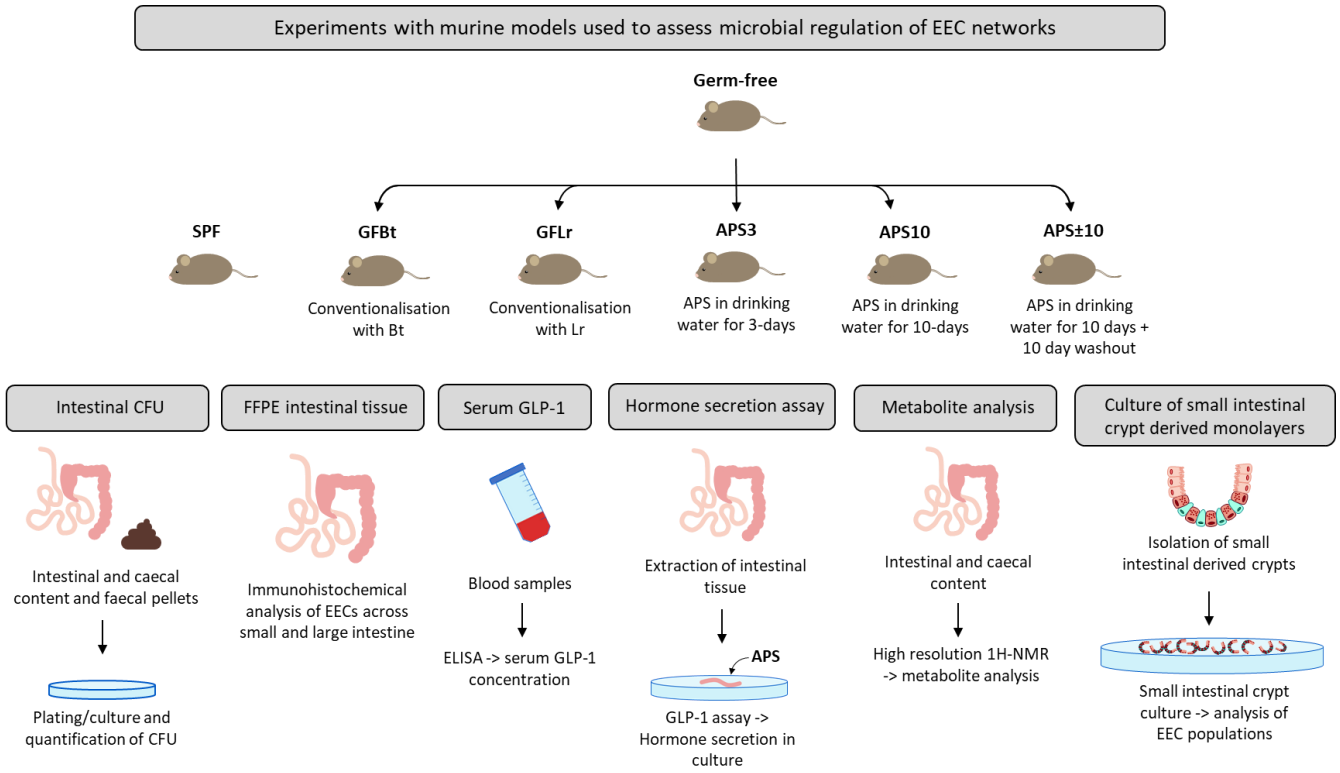

Figure 2.1 Experimental workflow for determining the extent of microbial modulation of EEC networks. In these experiments, SPF mice, GF mice, Bt or Lr conventionalised GF mice, or GF mice administered the major fermentation products of Bt (acetate, propionate and succinate; APS) were used to assess microbial impacts on EEC populations throughout the GIT. A combination of *in vivo* methods for tissue analysis (including immunohistochemistry, faecal metabolite analysis and serum hormone levels), *ex vivo* hormone secretion assays and *in vitro* intestinal crypt derived cultures were used to assess intestinal EEC networks in these mice.

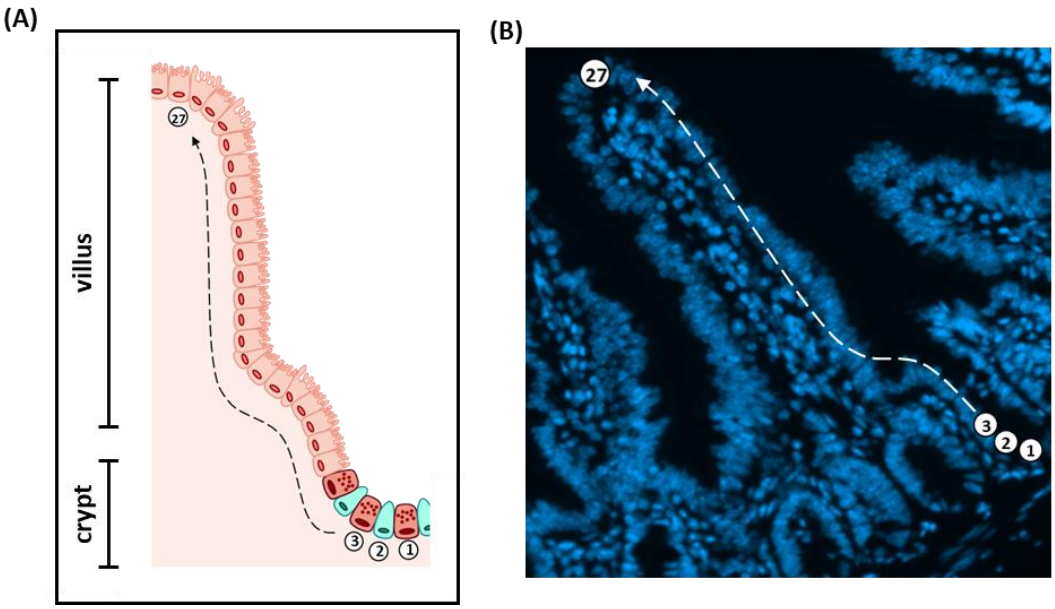

Figure 2.2 Determining epithelial cell and EEC numbers in sections of formalin fixed intestinal tissue. (A) Schematic and (B) photomicrograph illustrating the hemi-villus crypt with the dashed arrows depicting the region used to count epithelial cells in the intestine.

cultures, were used to assess intestinal EEC networks in these murine models (Fig. 2.1). Enumeration of specific subsets of EECs, including L, K and EC cells, was undertaken by quantifying immuno-positive cells within the hemi-villus crypt (HVC) region (Fig. 2.2) from a minimum of 20 hemi-villus crypts from each anatomically distinct region of the intestine (taken from <10 sections of each tissue sample group, ≤3 mice per group).

2.2.1. Global EEC numbers in the small intestine are increased upon absence of the gut microbiota

The acidic glycoprotein chromogranin A (ChrA) is considered a global marker of EECs expressed in the secretory granules stored in the majority of EEC populations (Facer, Bishop et al. 1985, Mouland, Bevan et al. 1994, Massironi, Zilli et al. 2016). Immunohistochemical staining with anti-ChrA of formalin fixed paraffin embedded (FFPE) intestinal tissue from GF (*n=11*) and SPF (*n=10*) mice (Fig. 2.3A), and subsequent cell counts (Fig. 2.5A) showed that GF mice have significantly increased numbers of immuno-positive ChrA ($ChrA^+$) EECs in the duodenum (0.55 ± 0.03 cells/HVC; $p<0.05$), jejunum (0.39 ± 0.04 cells/HVC; $p<0.01$) and ileum (0.34 ± 0.03 cells/HVC; $p<0.01$) compared to SPF (0.32 ± 0.05, 0.23 ± 0.02 and 0.21 ± 0.02 cells/HVC, respectively*;* Fig. 2.5A and Table 2.1). No significant differences in $ChrA^+$ cells between SPF and GF mice are observed in the proximal and distal colon (Fig. 2.5A and Table 2.1).

2.2.2. Regional variations in specific EEC populations are observed in SPF and GF mice

To determine whether the differences (in the small intestine) or similarities (in the colon) of ChrA-expressing EECs observed in SPF and GF mice are reflected in specific subsets of EECs, intestinal tissue sections were stained with antibodies against GLP-1, GIP and 5-HT, respectively (Fig. 2.3C and 2.4). For identification of the L cell EEC populations, two different GLP-1 antibodies were used (mouse and rabbit-derived). Initial testing of the antibodies on GF intestinal tissue showed specific staining of GLP-1-expressing ($GLP\text{-}1^+$) EECs. Non-specific background

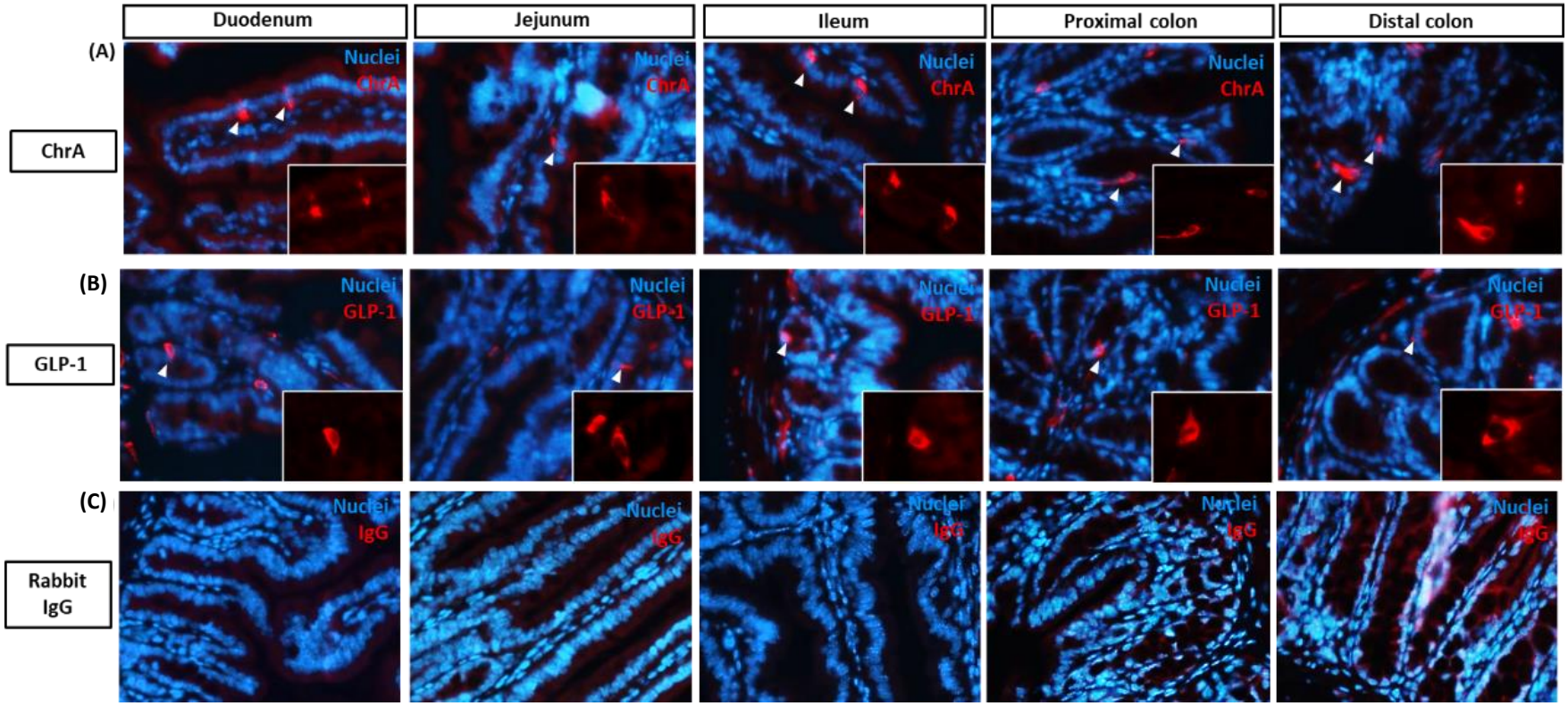

Figure 2.3 Distribution of ChrA$^+$ and GLP-1$^+$ enteroendocrine cells throughout the GIT of germfree mice. Photomicrographs depict representative images of sections of the mouse GIT stained with antibodies specific for (A) ChrA, (B) GLP-1 or (C) rabbit IgG. Stained cells are in red with cell nuclei visualised by Hoechst 33342 nuclear stain (blue). White arrowheads identify ChrA$^+$/GLP-1$^+$ EECs with the insets showing higher magnification images. Images taken on widefield fluorescence microscope (40/60x objective).

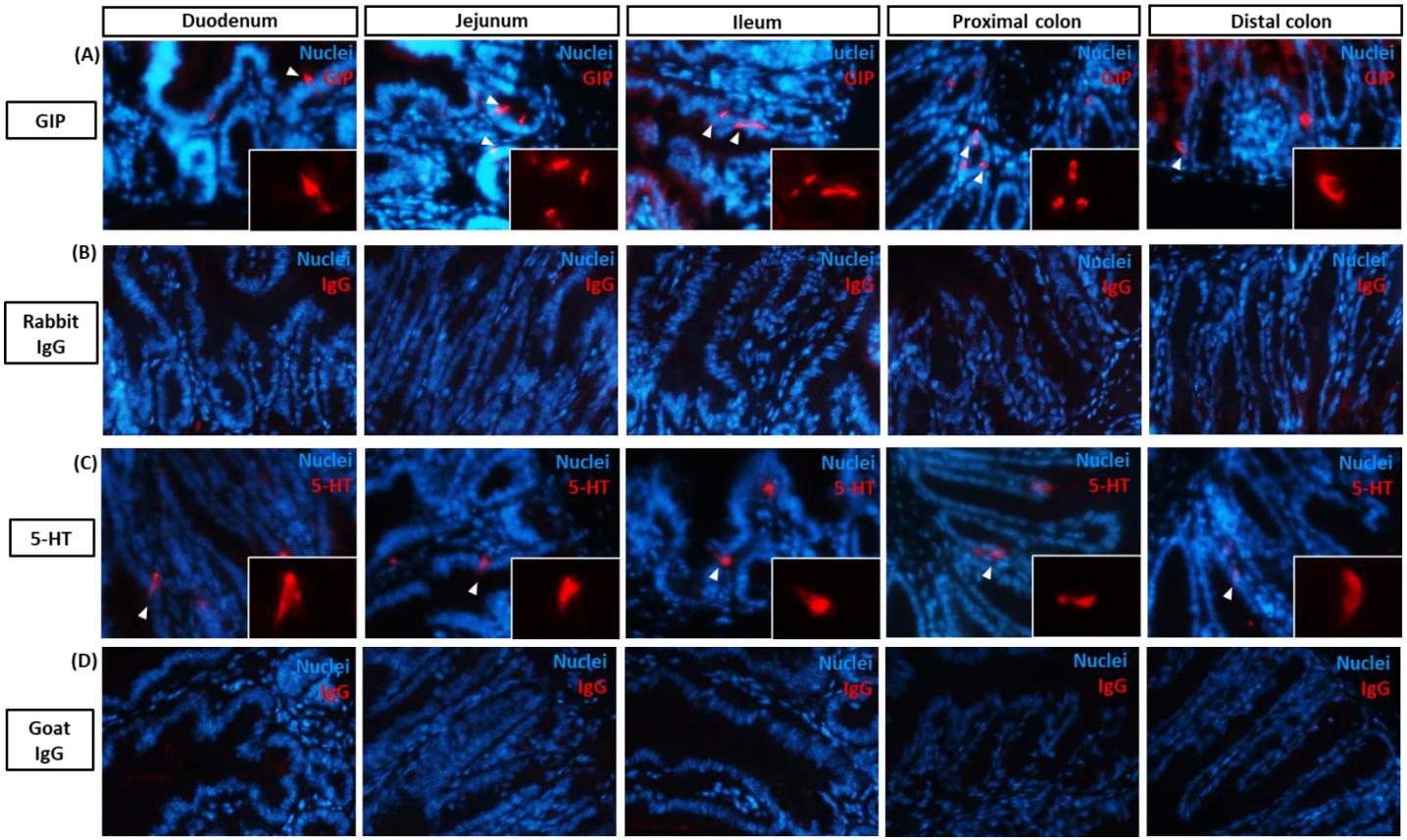

Figure 2.4 Distribution of GIP$^+$ and 5-HT$^+$ enteroendocrine cells throughout the GIT of germfree mice. Photomicrographs depict representative images of sections of the mouse GIT stained with antibodies specific for (A) GIP, (B) rabbit IgG, (C) 5-HT or (D) goat IgG. Stained cells are in red with cell nuclei visualised by Hoechst 33342 nuclear stain (blue). White arrowheads identify GIP$^+$/5-HT$^+$ EECs with the insets showing higher magnification images. Images taken on widefield fluorescence microscope (40/60x objective).

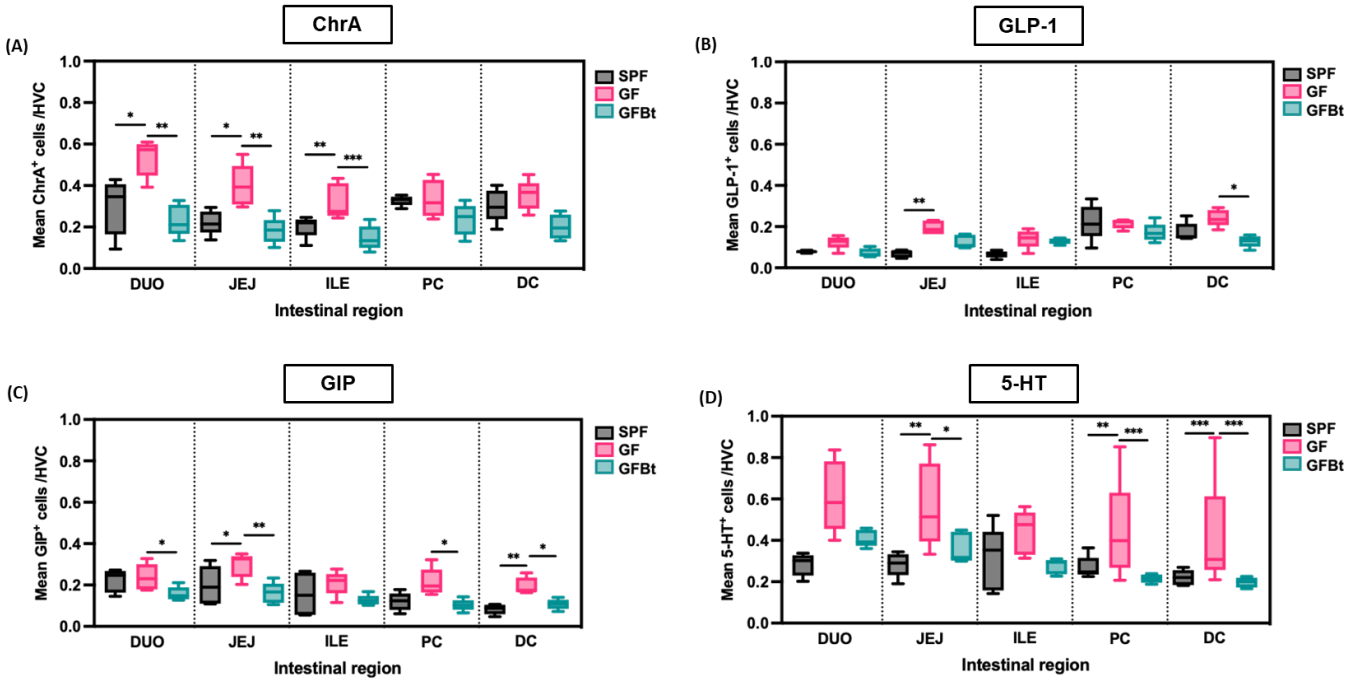

Figure 2.5 ChrA$^+$, GLP-1$^+$, GIP$^+$ and 5-HT$^+$ enteroendocrine cell numbers are higher in GF mice intestine and are normalised by mono-colonisation with Bt. Quantification of (A) ChrA$^+$, (B) GLP-1$^+$, (C) GIP$^+$ and (D) 5-HT$^+$, cells per hemi-villus crypt (HVC) in small and large intestine of SPF (*n=10*), GF (*n=11*) and GFBt (*n=10*) mice. Regional specificity is observed with significant increase in numbers of ChrA$^+$ cells in GF small intestine compared to SPF mice, higher numbers of GLP-1$^+$ cells in jejunum/distal colon of GF mice compared to SPF mice, significantly increased numbers of GIP$^+$ cells in GF jejunum and distal colon compared to SPF mice and significant increase in 5-HT$^+$ cells in jejunum, proximal colon and distal colon of GF mice compared to SPF which are all normalised by mono-colonisation with Bt (GFBt). The box plots represents first quartile, median and third quartile, with whiskers representing minimum and maximum. Statistical significance analysed using mixed-effects analysis (GraphPad Prism 9.2.0). *p<0.05, **p<0.01, ***p<0.001 were considered statistically significant.

Table 2.1 Mixed effects analysis to compare ChrA, GLP-1, GIP and 5-HT intestinal cell numbers in SPF, GF and GFBt mice.

		Duodenum		Jejunum		Ileum		Proximal colon		Distal colon	
		P value	**Significance**	**P value**	**Significance**	**P value**	**Significance**	**P value**	**Significance**	**P value**	**Significance**
ChrA	**GF/GFBt**	0.0031	**	0.0082	**	0.0001	***	0.064	ns	0.0985	ns
	GF/SPF	0.0111	*	0.0236	*	0.0049	**	0.8831	ns	0.6703	ns
GLP-1	**GF/GFBt**	0.071	ns	0.094	ns	0.9953	ns	0.2671	ns	0.028	*
	GF/SPF	0.0819	ns	0.0012	**	0.4302	ns	0.8382	ns	0.1196	ns
GIP	**GF/GFBt**	0.0458	*	0.0028	**	0.2034	ns	0.0357	*	0.0106	*
	GF/SPF	0.6889	ns	0.0299	*	0.4172	ns	0.0697	ns	0.0039	**
5-HT	**GF/GFBt**	0.1791	ns	0.0158	*	0.0644	ns	0.0001	***	<0.0001	***
	GF/SPF	0.095	ns	0.005	**	0.163	ns	0.0021	**	<0.0001	***

Two-Way ANOVA preformed on raw data to compare enteroendocrine cell numbers in SPF (*n=10*), GF (*n=11*) and GFBt (*n=10)* mice. Statistical significance conducted using mixed effects analysis, Geisser-Greenhouse correction was not used (GraphPad Prism 9.2.0). *p<0.05, **p<0.01 and ***p<0.001 were considered statistically significant.

staining was very low or absent from staining with isotype-matched controls (rabbit IgG and mouse IgG2a) (Fig. 2.6). Analysis of GLP-1$^+$ cells in GF (*n=11*) and SPF (*n=10*) mice using the two different (mouse and rabbit-derived) anti-GLP-1 antibodies revealed that GLP-1$^+$ cells were equivalent along the entire length of the small intestine in both groups of animals with the exception of the jejunum where they were present in significantly ($p<0.01$) higher numbers in GF mice (0.19 ± 0.03 cells/HVC) and SPF mice (0.07 ± 0.01 cells/HVC; Fig. 2.5B and Table 2.1). Furthermore, analysis of plasma levels of GLP-1 in fed mice showed that GF mice had higher levels than SPF mice, although this did not reach statistical significance (Fig. 2.7).

The distribution of GIP-expressing (GIP$^+$) EECs in the small intestine of SPF (*n=10*) and GF (*n=11*) animals was the same as that of GLP-1$^+$ EECs with significant ($p<0.05$) differences only evident in the jejunum (0.32 ± 0.03 versus 0.21 ± 0.03 cells/HVC of GF and SPF mice, respectively; Fig. 2.5C and Table 2.1). In the distal colon, and in contrast to GLP-1$^+$ EECs that were similar in SPF and GF animals, an approximately two-fold increase ($p<0.05$) in GIP$^+$ cells was observed in GF mice (0.19 ± 0.02 versus 0.08 ± 0.01 cells/HVC of GF and SPF mice, respectively; Fig. 2.5C and Table 2.1). Considering previous inconsistent findings regarding the presence of GIP-expressing EECs in the colon (Sjolund, Sanden et al. 1983, Jorsal, Rhee et al. 2017, Billing, Larraufie et al. 2019, Roberts, Larraufie et al. 2019), two different anti-GIP (mouse and rabbit-derived) antibodies was used to identify GIP$^+$ cells in the colon of both SPF and GF mice (Fig. 2.8A). No discrete staining with isotype matched anti-IgG1 control was detected (Fig. 2.8B), ruling out the possibility of non-specific staining of anti-GIP antibodies used.

5-HT expressing (5-HT$^+$) EEC also displayed region-specific differences in their distribution along the GIT of GF (*n=11*) versus SPF (*n=10*) mice with significantly higher numbers of positive cells ($p<0.01$ and $p<0.001$) in the jejunum (0.57 ± 0.06 versus 0.29 ± 0.03 cells/HVC, in GF and SPF mice, respectively; Fig. 2.5D and Table 2.1) and throughout the colon of GF mice (0.39 ± 0.06 and 0.34 ± 0.07 cells/HVC in proximal and distal colon respectively; Fig. 2.5D and Table 2.1).

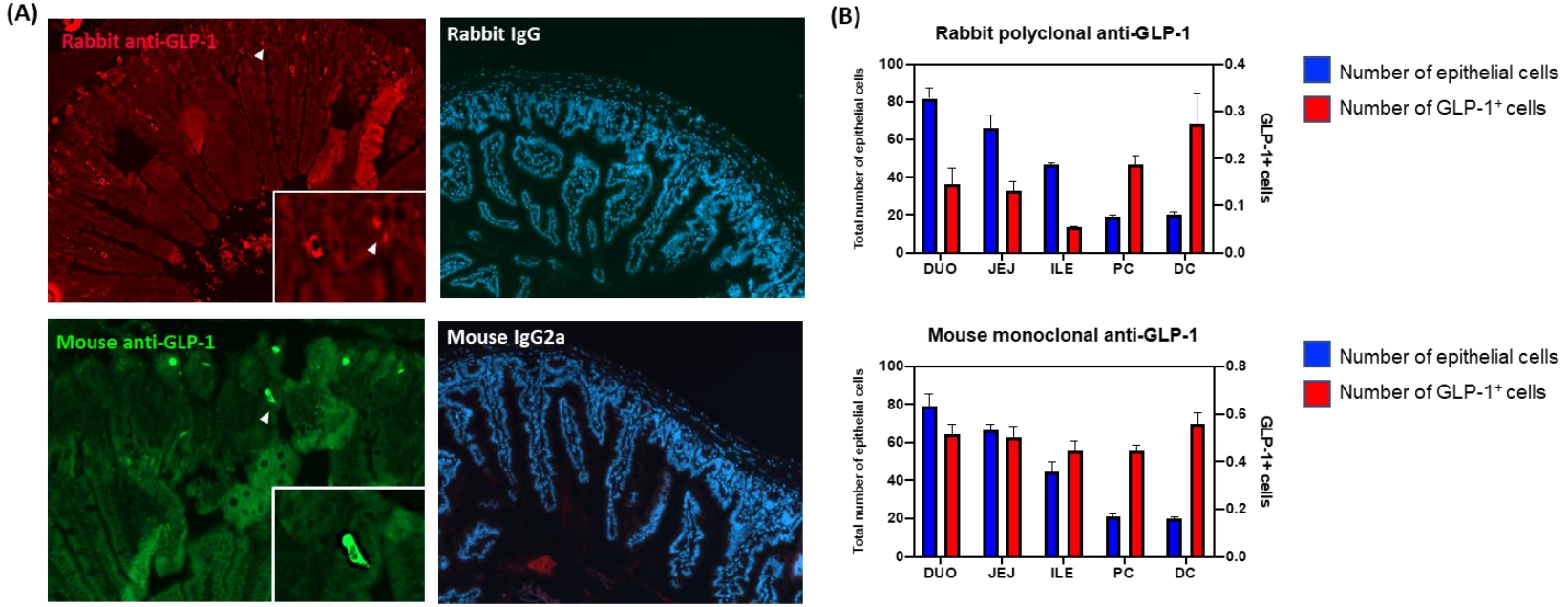

Figure 2.6 Distribution of GLP-1$^+$ enteroendocrine cells throughout the GIT of germfree mice. (A) Sections of ileum from germfree (GF) mice were stained with a rabbit polyclonal anti-GLP-1 antibody (red; Abcam, ab22625) or a mouse monoclonal anti-GLP-1 IgG2a antibody (green; Abcam, ab23468) and their isotype-matched controls rabbit IgG and mouse IgG2a, respectively. Cell nuclei visualised by Hoechst 33342 nuclear stain (blue) White arrowheads identify GLP-1$^+$ EECs. (B) The bar graphs depict the total number of epithelial cells and GLP-1$^+$ cells counted in hemi-villus crypts as detailed in Figure 2.2. Data is represented as mean ± SEM. Images taken on widefield fluorescence microscope (40/60x objective).

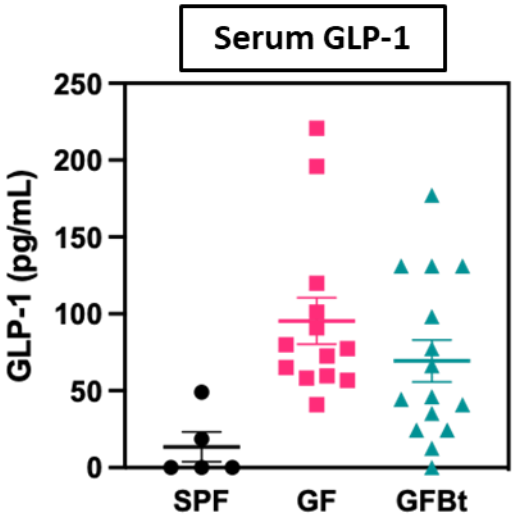

Figure 2.7 Serum GLP-1 is higher in GF mice. GLP-1 levels in serum of SPF (*n=5*), GF (*n=11*) and GFBt (*n=15*) mice. Data is presented as mean ± SEM.

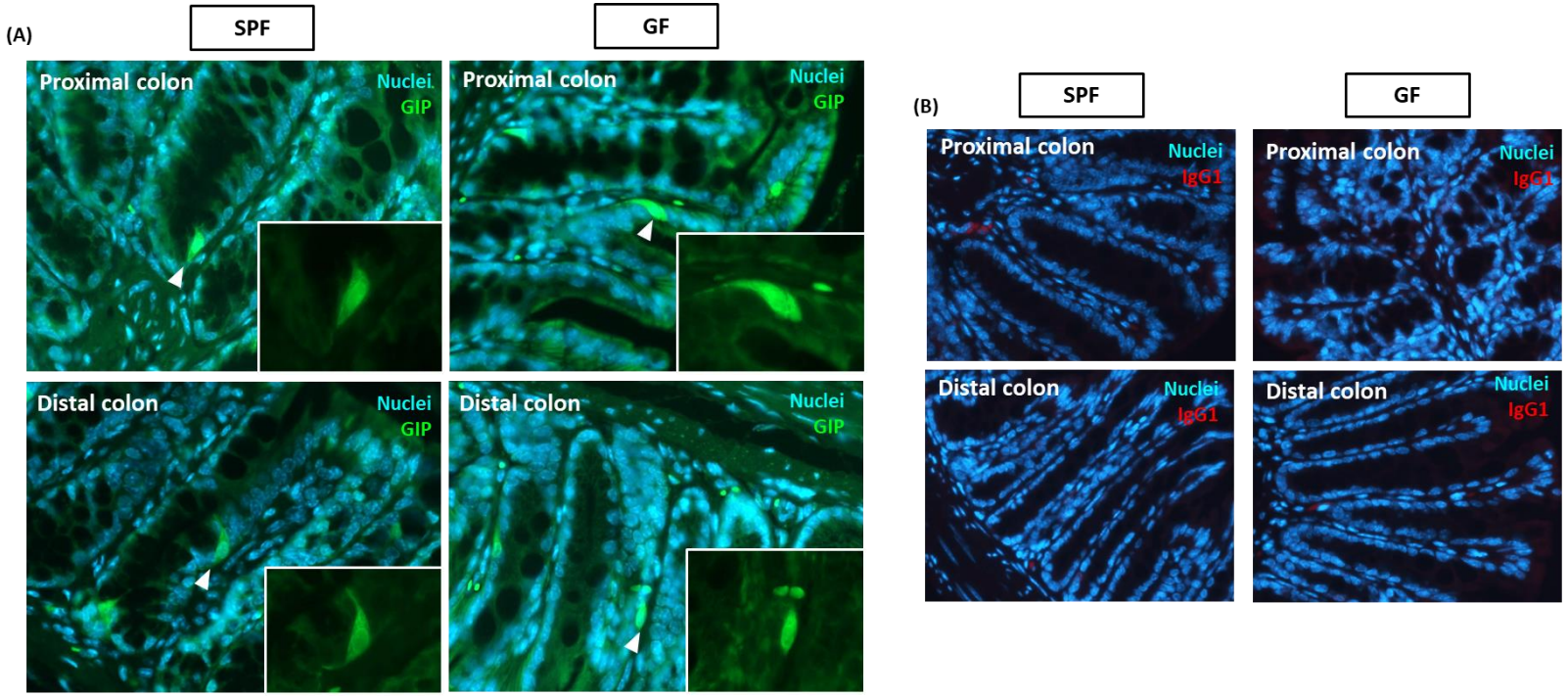

Figure 2.8 GIP-expressing cells in the mouse colon. Sections of proximal and distal colon from SPF and GF mice were stained with a (A) mouse monoclonal anti-GIP IgG1 antibody (green) or a (B) control mouse IgG1 antibody (red). White arrowheads identify GIP^{+} EECs with the insets showing higher magnification images. Cell nuclei visualised by Hoechst 33342 nuclear stain (blue). Images taken on widefield fluorescence microscope (40/60x objective).

The regional variations in EEC subset numbers are unlikely to be due to any bias in the sectioning or presentation of the tissues as the number of epithelial cells within the hemi-villous crypts of sections of the same regions of the GIT from different mouse strains were equivalent (Fig. 2.9).

2.2.3. Increased ND1-expressing cells in distal colon of GF mice

Gut microbiota, through their production of various SCFAs, have been shown to regulate EEC differentiation and expansion (Cani, Hoste et al. 2007, Petersen, Reimann et al. 2014, Larraufie, Martin-Gallausiaux et al. 2018). To determine if the effects of microbiota in SPF or lack of microbiota in GF mice on EEC numbers could be explained by alterations in EEC differentiation and maturation, sections of colonic tissue from SPF and GF mice were stained with NeuroD1 (ND1) (Fig. 2.10A and B), a pro-endocrine transcription factor involved in the maturation and subtype specificity of EECs along the crypt-villus axis (Mutoh, Fung et al. 1997).

Quantification of immuno-positive ND1 cells in the proximal colon revealed no significant differences between SPF (*n=5*) and GF (*n=10*) mice (Fig. 2.10C), although a trend towards increased ND1 fluorescent intensity was observed in GF mice (Fig. 2.10D). In the distal colon, a statistically significant ($p<0.01$) increase in $ND1^+$ cells was observed in GF mice (14.66 ± 0.52 cells/HVC) compared to SPF (12.14 ± 0.56 cells/HVC; Fig. 2.10C), but this was not reflected in the mean fluorescent intensity (Fig. 2.10D).

2.2.4. Normalisation of EEC populations in GF mice by Bt mono-conventionalisation

The ability of commensal gut microbes to directly influence the makeup of the EECs network was assessed by conventionalising GF mice with Bt (GFBt), a universal and prominent member of the mammalian intestinal microbiota (Salyers 1984, ScienceDirect Topics 2021) and comparing the distribution and numbers of EEC populations in the small and large intestine pre- and 5-days post-conventionalisation. Bt was found throughout the length of the GIT of conventionalised mice which individually showed considerable variation

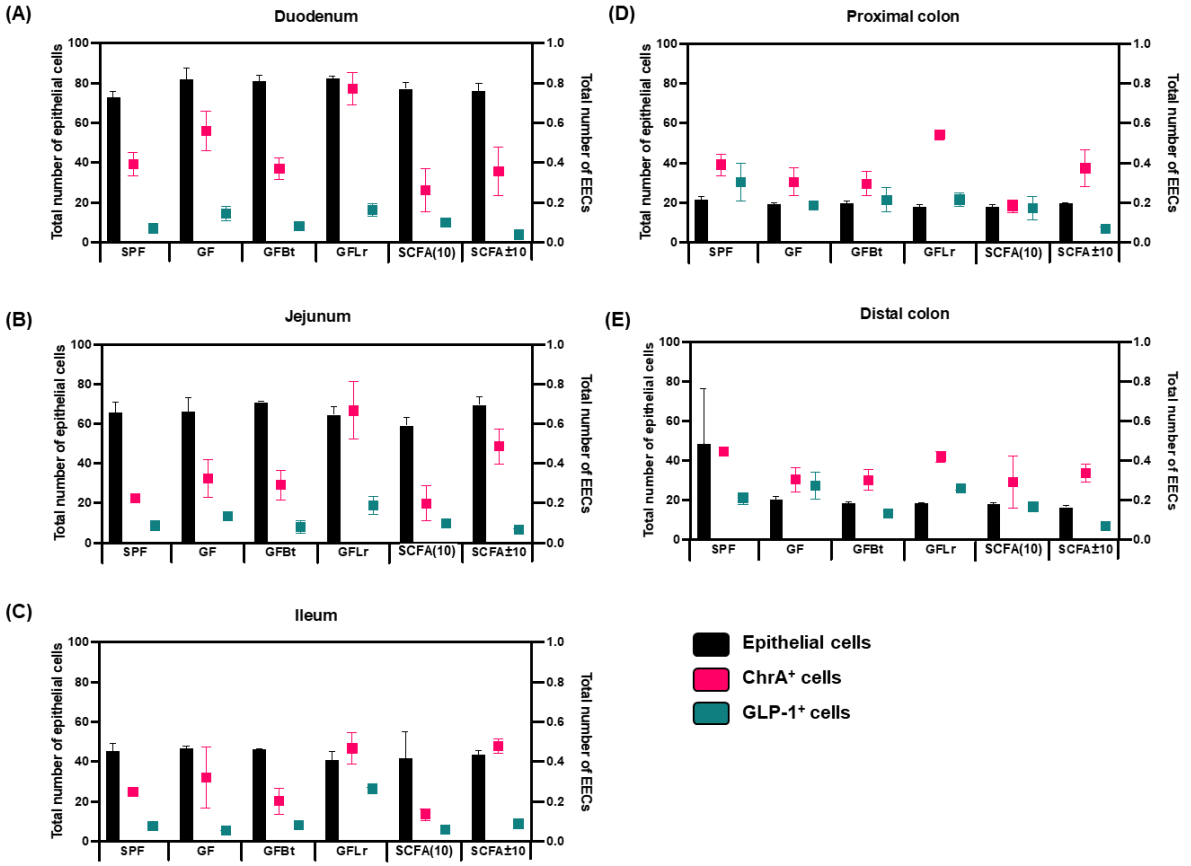

Figure 2.9 Epithelial cell and EEC numbers in different regions of the GIT in different groups of mice. Cell counts showing the numbers of ChrA- and GLP-1-expressing EECs (plotted on the right Y-axis) compared to the total number of epithelial cells (plotted on the left Y-axis) in the (A) duodenum, (B) jejunum, (C) ileum, (D) proximal colon and (E) distal colon, A minimum 30 hemi-villus crypts were counted for each section with at least 10 sections from each tissue sample/experimental group used 3 mice per group to obtain cell counts. Data represents mean ± SEM.

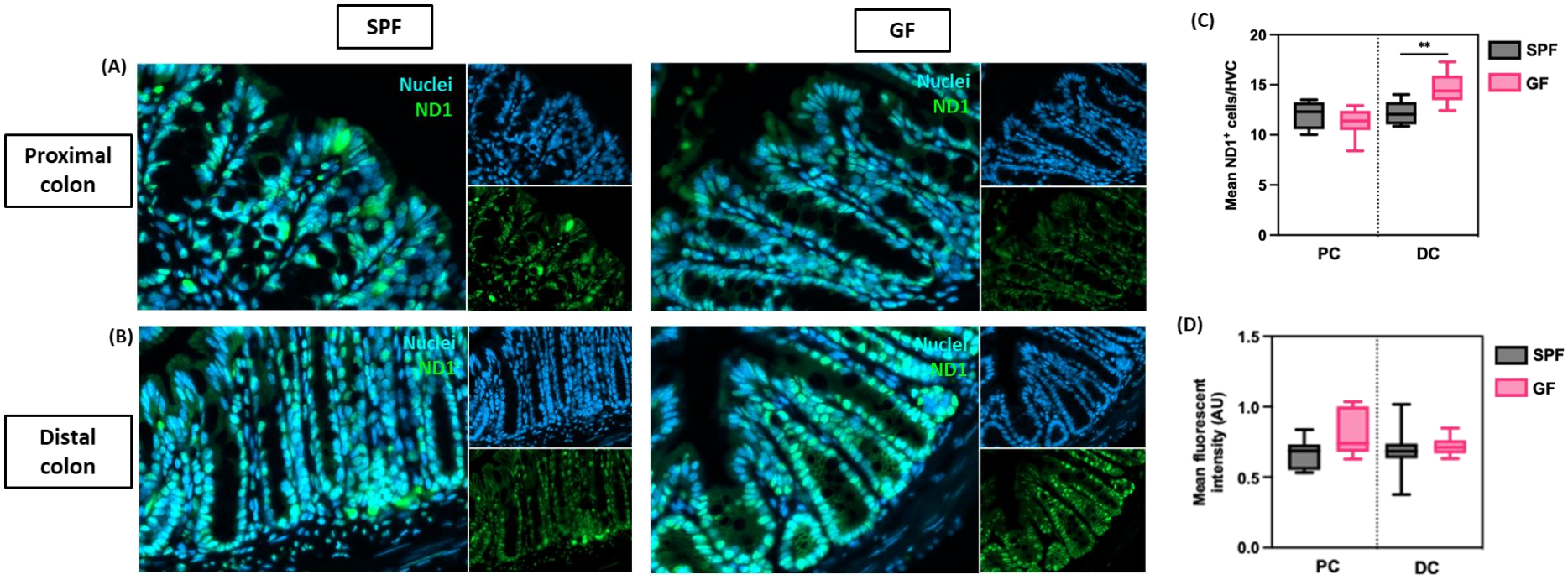

Figure 2.10 Number of ND1-expressing cells is increased in the distal colon of germfree mice. Sections of (A) proximal and (B) distal colonic tissue from SPF (*n=5*) and GF (*n=10*) mice were stained for an antibody against ND1. (C) the number of ND1-expressing cells along the hemi-villus crypt (HVC) were quantified in the proximal and distal colon from SPF and GF mice. (D) Mean fluorescent intensity of ND1 staining. The box plots represents first quartile, median and third quartile, with whiskers representing minimum and maximum values. Statistical significance was calculated using Mann-Whitney test (GraphPad Prism 9.2.0), $p<0.05$ were considered statistically significant; $^{**}p<0.01$.

in colonising density based on colony forming unit (CFU) determinations of the luminal contents of different regions of the GIT (Table 2.2). However, a consistent finding in all conventionalised animals was that the cecum and colon contained the highest levels of Bt as previously noted (Wrzosek, Miquel et al. 2013, Curtis, Hu et al. 2014), and Bt colonisation resulted in significant changes in EEC populations to the extent that they more closely resembled the profile and number of EEC seen in SPF mice. This was exemplified by the analysis of $ChrA^+$ EECs pre- and post-conventionalisation which showed a significant reduction ($p<0.01$ and $p<0.001$) in $ChrA^+$ EECs throughout the small intestine of Bt conventionalised mice (*n=10*) to levels comparable to that of SPF (*n=10*) mice (Fig 2.5A and Table 2.1). In the colon, Bt colonisation also reduced the number of ChrA-expressing cells compared to both SPF and GF mice although the differences were not statistically significant (Fig. 2.5A and Table 2.1).

Analysis of individual EEC populations revealed subtle differences in the impact of Bt colonization on their regional distribution and/or numbers. For GLP-1 expressing EECs the effect of Bt was most apparent in the distal colon where it significantly reduced ($p<0.05$) the number of positive cells (0.13 ± 0.01 versus 0.25 ± 0.02 cells/HVC in GFBt (*n=10*) and GF (*n=11*) mice, respectively; Fig. 2.5B and Table 2.1), comparable to $GLP\text{-}1^+$ EECs seen in the distal colon of SPF mice (0.18 ± 0.02 cells/HVC). By contrast, the impact of Bt conventionalisation on GIP^+ EECs was more profound with significant reductions in cell numbers seen in both the small (duodenum and jejunum; $p<0.05$ and $p<0.01$, respectively) and large intestine ($p<0.05$) post-Bt conventionalisation (*n=10*) making them comparable to that of SPF (*n=10*) mice (Fig. 2.5C and Table 2.1). A similar effect was noted for 5-HT-expressing EECs with significant reductions post-Bt conventionalisation seen in the jejunum ($p<0.05$) and particularly, throughout the colon ($p<0.001$; Fig. 2.5C and Table 2.1).

2.2.5. The effects of Bt on EEC networks are not seen with an unrelated gut commensal bacterium

To determine if the effects of Bt on EEC networks after colonising GF mice were specific to this bacterium, GF mice were conventionalised with a strain of

Table 2.2 Concentrations of Bt along the length of the intestinal tract in Bt mono-colonised germ-free mice (GFBt).

	Mouse ID	Duodenum	Jejunum	Ileum	Caecum	Proximal colon	Distal colon
cfu/g	**3517**	4.00×10^7	3.25×10^7	3.50×10^6	3.07 × 10^9	2.98×10^8	1.30×10^9
	3534	4.90×10^5	6.49×10^6	6.23×10^6	2.23×10^7	1.72×10^7	1.92×10^7
	3555	5.75×10^6	3.43×10^6	5.02×10^6	3.39×10^7	6.24×10^7	2.78×10^7
	3556	1.23×10^6	8.88×10^6	1.12×10^7	5.21×10^8	6.00×10^8	5.54×10^8
	3557	4.62×10^6	5.38×10^6	2.83×10^6	6.18×10^8	4.66×10^8	9.50×10^8

Estimated cfu/g of Bt in content from the different regions along the intestinal tract.

Lactobacillus reuteri (Lr) (100-23) isolated from the rat GIT that is able to stably colonise GF mice (Wesney and Tannock 1979). The data shown in Fig. 2.11 and Table 2.3 shows striking differences in the effects of Bt (*n=10*) and Lr (*n=5*) on EEC networks in the GIT post-conventionalisation. Whereas Bt generally reduces the number of EECs in GF mice, Lr either had no significant effect on GLP-1^+ cells (Fig. 2.11B and Table 2.3) or had the opposite effect and significantly increased numbers of EECs as seen in the jejunum ($p<0.05$) and proximal colon ($p<0.01$) for $ChrA^+$ cells (Fig. 2.11A and Table 2.3), in the duodenum for GIP^+ cells ($p<0.05$; Fig. 2.11C and Table 2.3), and throughout the colon for 5-HT^+ cells ($p<0.05$ and $p<0.01$ in proximal and distal colon, respectively; Fig. 2.11D and Table 2.3).

2.2.6. Acetate, propionate and succinate (APS) are the major fermentation products of Bt

To confirm that the principal fermentation products of *Bacteroides* polysaccharide metabolism (acetate, propionate and succinate (APS)) (Hooper, Midtvedt et al. 2002, Wrzosek, Miquel et al. 2013, Curtis, Hu et al. 2014) produced in GFBt mice, nuclear magnetic resonance (NMR) based analysis was used to quantify these metabolites in the luminal contents of the duodenum, cecum and distal colon, 5-days post-conventionalisation. As shown in Table 2.4, all of the metabolites were present at very low levels (0.01–0.09 mM) in the GIT of GF mice. Post Bt colonisation, the levels of acetate, succinate and propionate increased in the cecum and distal colon (0.26–2.35 mM). Strikingly, the levels of succinate in the cecum increased by >200-fold compared to those in both GF and SPF mice, as seen previously in C3H/HEJ mice post Bt colonisation (Curtis, Hu et al. 2014). As expected, Bt conventionalisation had no impact on butyrate levels (0.01 to 0.02 mM and 0.01 to 0.02 mM pre- and post-conventionalisation, respectively) which is consistent with Bt not being a butyrate producer.

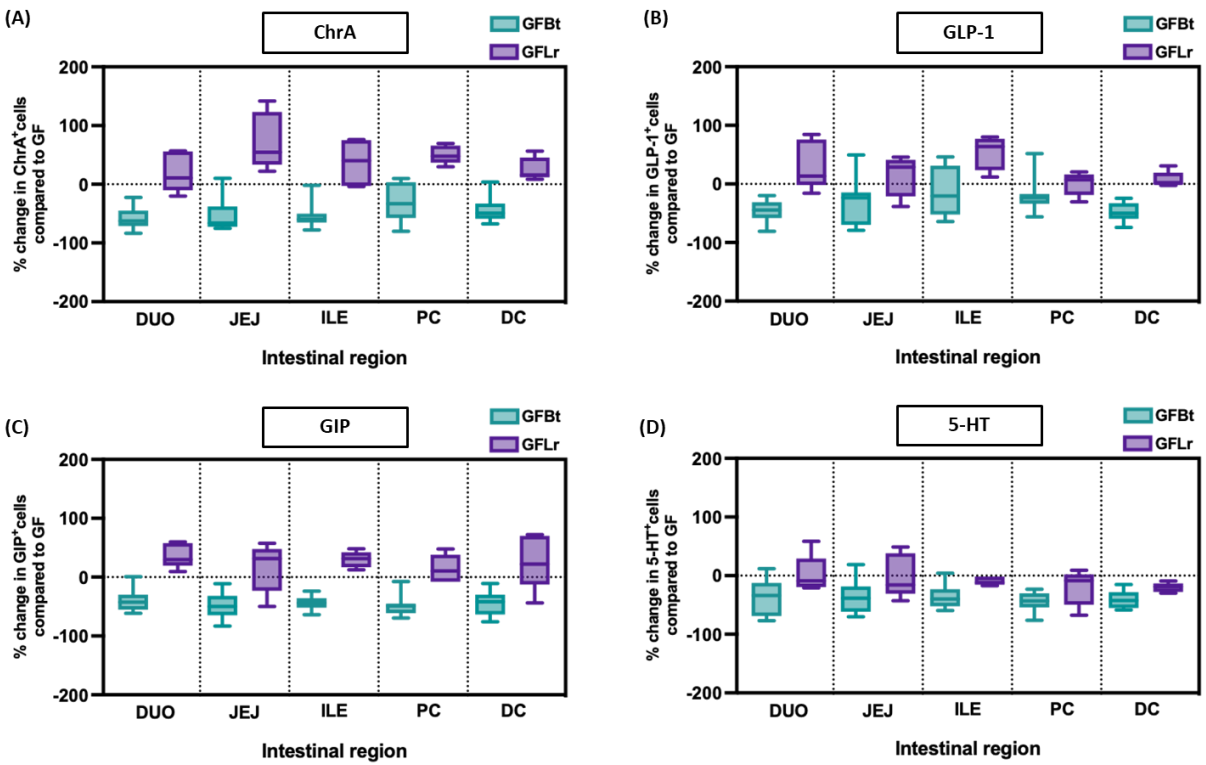

Figure 2.11 Bacterial effect on enteroendocrine cell numbers is specific to Bt. Percentage change of (A) ChrA$^+$, (B) GLP-1$^+$, (C) GIP$^+$ and (D) 5-HT$^+$ cells in GFBt (*n=10*) and GFLr (*n=5*) mice intestine compared to mean number of ChrA$^+$, GLP-1$^+$, GIP$^+$ and 5-HT$^+$ cells, respectively, in GF (*n=11*) mice intestine (used as baseline comparator), show that colonisation of GF mice with Bt (GFBt) results in an overall reduction in ChrA$^+$, GLP-1$^+$, GIP$^+$ and 5-HT$^+$ cells across the intestine whilst colonised with Lr (GFLr) has little/no effect on EEC numbers. The box plots represents first quartile, median and third quartile, with whiskers representing minimum and maximum values.

Table 2.3 Mixed effects analysis to compare ChrA, GLP-1, GIP and 5-HT intestinal cell numbers in GF, GFBt and GFLr mice.

		Duodenum		Jejunum		Ileum		Proximal colon		Distal colon	
		P value	**Significance**	**P value**	**Significance**	**P value**	**Significance**	**P value**	**Significance**	**P value**	**Significance**
ChrA	**GF/GFBt**	0.0104	*	0.0195	*	0.3243	ns	0.0431	*	0.0462	*
	GF/GFLr	0.1245	ns	0.0156	*	0.3926	ns	0.0059	**	0.287	ns
GLP-1	**GF/GFBt**	0.1161	ns	0.323	ns	0.9859	ns	0.167	ns	0.0108	*
	GF/GFLr	0.3863	ns	0.9612	ns	0.3492	ns	0.6908	ns	0.5014	ns
GIP	**GF/GFBt**	0.0642	ns	0.0061	**	0.062	ns	0.0741	ns	0.0196	*
	GF/GFLr	0.0208	*	0.1568	ns	0.1143	ns	0.6633	ns	0.2354	ns
5-HT	**GF/GFBt**	0.2321	ns	0.0255	*	0.0409	*	0.0007	***	<0.0001	***
	GF/GFLr	0.8772	ns	0.9927	ns	0.4338	ns	0.0463	*	0.003	**

Two-Way ANOVA preformed on raw data to compare enteroendocrine cell numbers among GF (*n=11*), GFBt (*n=10*) and GFLr (*n=5*) mice. Statistical significance conducted using mixed effects analysis, Geisser-Greenhouse correction was not used (GraphPad Prism 9.2.0). *p<0.05, **p<0.01 and ***p<0.001 were considered statistically significant.

Table 2.4 Amounts of Bacteroides fermentation products in the intestinal and caecal contents of SPF, GF and Bt- and Lr-conventionalised GF mice.

		Acetate	Butyrate	Propionate	Succinate
SPF	**Duodenum**	0.26 (0.08)	0.03 (0.01)	0.07 (0.03)	0.09 (0.04)
	Distal colon	1.59 (0.59)	0.38 (0.29)	0.47 (0.08)	0.06 (0.07)
	Caecum	9.61 (8.62)	2.89 (2.63)	1.69 (1.67)	0.04 (0.02)
GF	**Duodenum**	0.06 (0.02)*	0.01 (0)	0.03 (0.01)	0.02 (0)
	Distal colon	0.07 (0.01)	0.01 (0)	0.01 (0)	0.01 (0)
	Caecum	0.09 (0.01)	0.02 (0)	0.02 (0)	0.01 (0)
GFBt	**Duodenum**	0.09 (0.05)	0.02 (0.01)	0.04 (0.03)	0.03 (0.02)
	Distal colon	0.58 (0.2)	0.01 (0.01)	0.26 (0.17)	0.9 (0.49)
	Caecum	0.73 (0.17)	0.02 (0.01)	0.64 (0.32)	2.35 (1.52)
GFLr	**Caecum**	0.65 (0.22)	0.02 (0.01)	0.67 (0.35)	0.17 (0.11)

Data presented as mean (standard deviation) concentration (mM) ($n=5$).

2.2.7. APS reproduces the effect of Bt conventionalization on EECs cells *in vivo*

EECs are highly enriched in free fatty acid receptors that contribute to physiological responses to microbially produced metabolites and SCFA (Lu, Gribble et al. 2018). Considering this, Bt fermentation products (APS), were investigated to determine whether they could reproduce and provide an explanation for the effects of the bacterium itself on EEC populations seen *in vivo*. GF mice were administered APS via their drinking water for 10-days (APS10, *n=10*) in amounts corresponding to those present in the cecum of SPF rodents maintained on regular chow (95 μM acetate, 29 μM propionate and 5.6 μM succinate) (Mineo, Amano et al. 2006). Intestinal tissues were removed 10-days later and examined for EECs.

The impact of APS on $ChrA^+$ cells was comparable to that seen after Bt conventionalisation of GF mice with a reduction ($p<0.05$ and $p<0.001$) in the number of positive cells throughout the GIT and in particular in the small intestine which showed an approximate 50% reduction (0.21 ± 0.05, 0.20 ± 0.04 and 0.18 ± 0.03 cells/HVC in the duodenum, jejunum and ileum, respectively; Fig. 2.12 and Table 2.5), as seen with Bt conventionalisation (0.23 ± 0.04, 0.18 ± 0.03 and 0.15 ± 0.02 cells/HVC in the duodenum, jejunum and ileum, respectively). The impact of APS on $GLP\text{-}1^+$ cells was more variable with the most apparent reductions in positive cells seen in the duodenum, jejunum and proximal colon (0.10 ± 0.01, 0.15 ± 0.02 and 0.18 ± 0.02 cells/HVC, respectively; Fig. 2.12B and Table 2.5). APS administration for 10-days had a similar effect on GIP^+ (Fig. 2.12C) and $5\text{-}HT^+$ cells (Fig. 2.23D) as that of $ChrA^+$ cells with reductions in positive cells seen throughout the small intestine and the colon.

2.2.8. The reproducible effects of APS to Bt conventionalisation on EECs *in vivo* are not seen in shorter or withdrawal of APS administration

To determine if the effects of administering APS on EEC networks were dependent on longer exposure to APS (APS10, *n=10*), GF mice were treated with APS for 3-days (APS3, *n=3*) via drinking water and intestinal tissue excised to determine EEC distribution. With the exception of the duodenum, no significant differences were

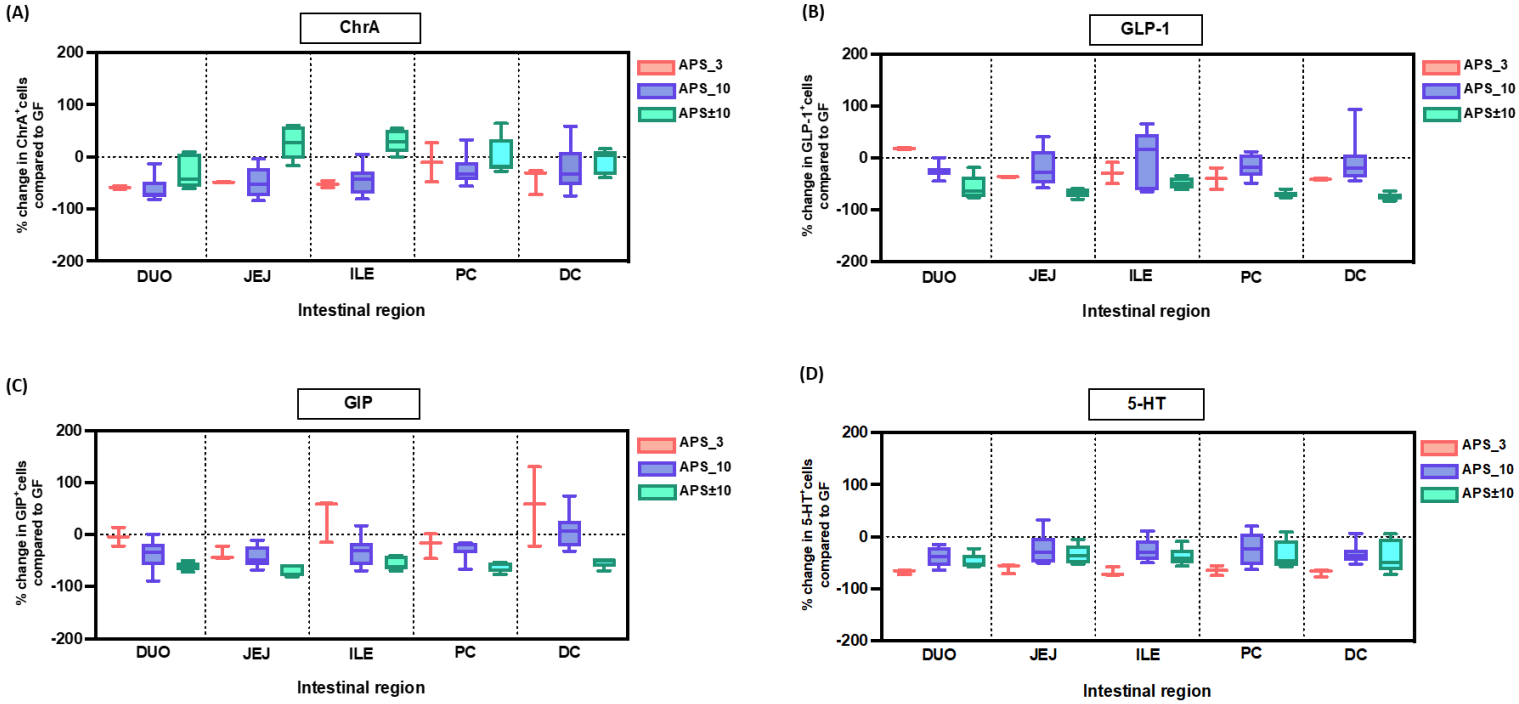

Figure 2.12 Administration of APS in GF mice replicates ChrA and GLP-1 cell numbers from GF colonisation with Bt, but the effects are not long lasting. SCFA mixture administered composed of SCFAs produced by Bt *in vitro* (APS; acetate, propionate and succinate) used at physiological levels. Percentage change of (A) $ChrA^+$, (B) $GLP\text{-}1^+$, (C) GIP^+ and (D) $5\text{-}HT^+$ cells in the intestine upon APS administration for 3-days (APS3, *n=3*) or 10-days (APS10, *n=10*) and/or removal (APS±10, *n=5*) compared to mean number of $ChrA^+$, $GLP\text{-}1^+$, GIP^+ and $5\text{-}HT^+$ cells, respectively, in GF (*n=11*) mice (used as a baseline comparator). Results show that administration of APS for 10-days replicate the results seen in GF mice colonised with Bt. For ChrA expressing cells, the removal of APS led to a rebound effect with increased number of positive cells along the intestine. Contrasting effects were observed upon APS removal in specific EEC subsets, leading to further reductions in numbers of GLP-1 and GIP expressing cells. 3-day administration of APS did not appear to effect ChrA, GLP-1 or GIP expressing cells, but significantly reduced 5-HT expressing cells in GF mice. The box plots represents first quartile, median and third quartile, with whiskers representing minimum and maximum values.

Table 2.5 Mixed effects analysis to compare ChrA, GLP-1, GIP and 5-HT intestinal cell numbers in GF, APS3, APS10 and APS±10 mice.

		Duodenum		Jejunum		Ileum		Proximal colon		Distal colon	
		P value	Significance	P value	Significance	P value	Significance	P value	Significance	P value	Significance
ChrA	GF/APS10	0.0228	*	0.0005	***	0.0172	*	0.192	ns	0.2656	ns
	GF/APS±10	0.1791	ns	0.0222	*	0.0342	*	0.9374	ns	0.6989	ns
	APS10/APS±10	0.1498	ns	<0.0001	***	0.0009	***	0.2507	ns	0.4315	ns
	GF/ASP3	0.0437	*	0.0655	ns	0.0681	ns	0.5959	ns	0.5955	ns
	ASP3/ASP10	0.8299	ns	0.8299	ns	0.699	ns	0.5777	ns	>0.9999	ns
GLP-1	GF/APS10	0.0584	ns	0.2878	ns	0.9352	ns	0.127	ns	0.741	ns
	GF/APS±10	0.0026	**	0.0006	***	0.015	*	<0.0001	***	<0.0001	***
	APS10/APS±10	0.0286	*	0.0065	**	0.0438	*	0.0005	***	0.0029	**
	GF/ASP3	0.5627	ns	0.472	ns	0.6609	ns	0.1269	ns	0.1095	ns
	ASP3/ASP10	0.1025	ns	0.8467	ns	0.7635	ns	0.4104	ns	0.2643	ns
GIP	GF/APS10	0.0207	*	0.0014	**	0.0754	ns	0.0303	*	0.6032	ns
	GF/APS±10	0.0006	***	<0.0001	***	0.0036	**	<0.0001	***	0.001	**
	APS10/APS±10	0.0756	ns	0.0219	*	0.0808	ns	0.0031	**	0.0009	***
	GF/ASP3	0.9009	ns	0.141	ns	0.2925	ns	0.549	ns	0.2493	ns
	ASP3/ASP10	0.0935	ns	0.5982	ns	0.0357	*	0.5365	ns	0.4257	ns
5-HT	GF/APS10	0.031	*	0.1555	ns	0.0269	*	0.2477	ns	0.1391	ns
	GF/APS±10	0.0125	*	0.0263	*	0.0051	**	0.1082	ns	0.1476	ns
	APS10/APS±10	0.3887	ns	0.4262	ns	0.2826	ns	0.5119	ns	0.7802	ns
	GF/ASP3	0.0006	***	0.0013	**	0.0005	***	0.004	**	0.002	**
	ASP3/ASP10	0.031	*	0.0247	*	0.033	*	0.0402	*	0.421	*

Two-Way ANOVA preformed on raw data to compare enteroendocrine cell numbers in GF (*n=11*), APS3 (*n=3*), APS10 (*n*=10) and APS±10 (*n=5*) mice. Statistical significance conducted using mixed effects analysis, Geisser-Greenhouse correction was not used (GraphPad Prism 9.2.0). *p<0.05, **p<0.01 and ***p<0.001 were considered statistically significant.

observed in ChrA$^+$ cells in the intestine of ASP3 compared to GF mice (Fig. 2.12 and Table 2.5). Furthermore, no significant alterations were observed in the GLP-1$^+$ and GIP$^+$ subsets of EECs following 3-day APS treatment (Fig. 2.12 and Table 2.5).

However, similar to the effects of 10-day APS administration, short-term APS exposure reduced the number of 5-HT-expressing cells along the entire intestine (0.21 ± 0.02, 0.23 ± 0.03, 0.14 ± 0.02, 0.14 ± 0.02 and 0.11 ± 0.01 cells/HVC, in the duodenum, jejunum, ileum, proximal and distal colon, respectively; Fig. 2.12 and Table 2.5) compared to GF (*n=11*) and to an extent that was significantly ($p<0.05$) lower compared to APS10 (Fig. 2.12D and Table 2.5). These results indicate that the intestinal epithelium in GF mice may rapidly respond to initial APS exposure, selectively reducing the number of 5-HT$^+$ cells.

To determine if the effects of administering APS on EEC were dependent upon constant exposure to APS, GF mice were treated with APS for 10-days followed by a 10-day wash out period (APS±10, *n=5*) prior to EEC analysis. For ChrA$^+$ cells the removal of APS led to a rebound effect and increase in the number of positive cells in the small intestine and in particular, in the jejunum (0.50 ± 0.06 cells/HVC) and ileum (0.44 ± 0.03 cells/HVC) where the levels significantly ($p<0.001$) exceeded that of non-treated GF mice (0.39 ± 0.03 and 0.34 ± 0.03 cells/HVC, respectively; Fig. 2.12A and Table 2.5). Amongst individual EEC subsets some interesting and contrasting effects were noted. For GLP-1$^+$ cells the removal of APS resulted in further and significant ($p<0.05$, $p<0.01$ and $p<0.001$) reductions in the proportion of positive cells throughout the small intestine and colon (Fig. 2.12B and Table 2.5) with the number of GLP-1 expressing cells in the small intestine (0.06 ± 0.01, 0.06 ± 0.01 and 0.08 ± 0.01 cells/HVC in the duodenum, jejunum and ileum, respectively) now being comparable to that of SPF mice (0.08 ± 0.00, 0.07 ± 0.01 and 0.07 ± 0.01 cells/HVC in the duodenum, jejunum and ileum, respectively). A similar albeit more regionalised effect was seen for GIP$^+$ cells with the withdrawal of APS resulting in further significant reductions (Fig. 2.12C and Table 2.5) in immuno-positive cells in the jejunum ($p<0.05$) and in the proximal ($p<0.01$) and distal colon ($p<0.001$). In contrast, the withdrawal of APS had no significant impact on 5-HT$^+$ cells (Fig. 2.12D and Table 2.5).

2.2.9. APS does not induce hormone secretion when added directly to colonic tissues from GF mice

To determine if APS could directly stimulate GLP-1 hormone secretion, colonic intestinal tissues were excised from non-treated GF mice (*n=10*) and incubated with either APS-containing or control buffer for 2 hr. Subsequent analysis of GLP-1 secretion in the supernatants showed no significant differences in levels of GLP-1 protein following incubation with control or APS-buffer treated colonic tissues (Fig. 20). However, one biological replicate did show ~6-fold increase in GLP-1 protein levels following incubation with APS-buffer (Fig. 2.13).

2.2.10. EEC in cultured intestinal crypt-derived epithelial monolayers do not accurately reflect EECs in vivo

The ability to establish cultures of the intestinal epithelium that reflect the architecture and distribution of differentiated cell types seen *in vivo* (Sato, Vries et al. 2009, Sato and Clevers 2013) provides a valuable and tractable *in vitro* system to interrogate microbe-host cell interactions at the molecular and cellular level. To determine if such culture systems can faithfully replicate the different profiles of EECs seen in sections of preserved tissues of SPF (*n=3*) and GF mice (*n=7*), EEC in monolayer cultures established from small intestinal crypts of SPF, GF, Bt conventionalized GF mice (GFBt, *n=5*), and GF mice treated with APS10 (*n=3*) was examined.

Representatives of the differentiated epithelial cell lineages including EEC and mucus-producing goblet cells were readily detected in cultured two-dimensional (2D) epithelial cell monolayers (Fig. 2.14). However, analysis of EEC populations in the cultured monolayers (Fig. 2.14C-F) revealed inconsistencies in the distribution of EEC subsets compared to tissues preserved and directly processed *ex vivo*. In particular, the epithelial monolayer cultures established from GF mice showed no differences in the number of GLP-1^+ or ChrA$^+$ cells compared to those from SPF mice. By contrast, a

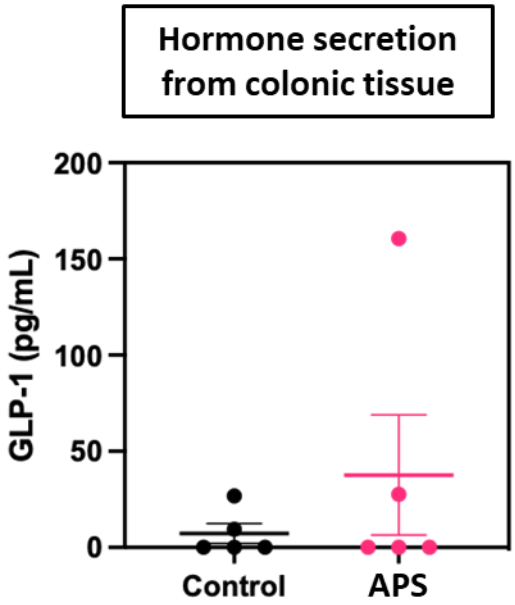

Figure 2.13. APS doesn't induce hormone secretion when added directly to luminal surface of colonic tissues from GF mice. Sections of colon were excised from GF mice (*n=10*), cut longitudinally and placed in a customised media containing DPPVI inhibitor. Tissues were then incubated with APS (95 μM sodium acetate, 29 μM sodium propionate and 5.6 μM sodium succinate) for 2 hours. Supernatant was collected and stored at -20℃, prior to measurement of GLP-1 levels (pg/ml) by a mouse multiplex GLP-1 assay (Merk Millipore, Milliplex MAP Multiplex assay). Data is represented as mean ± SEM.

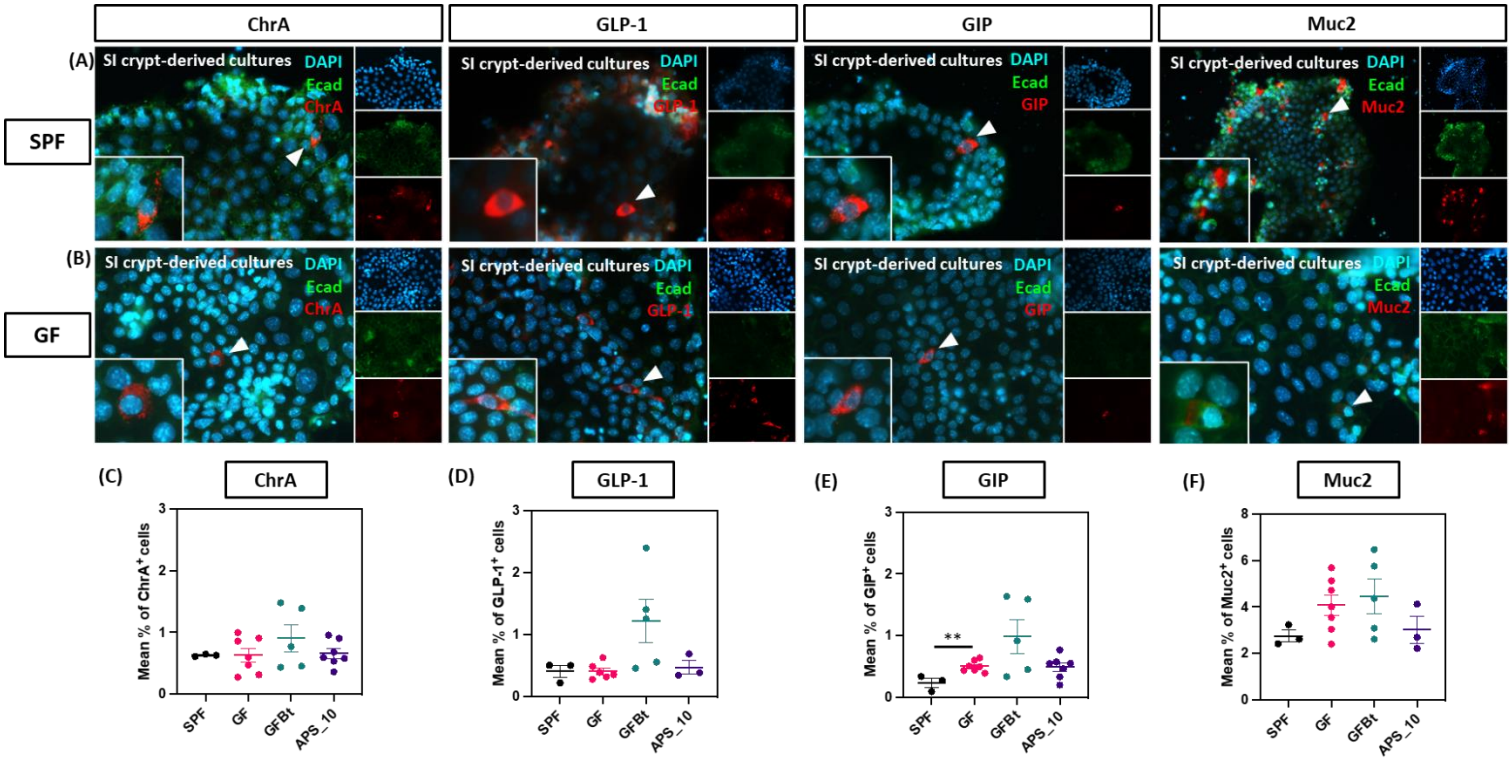

Figure 2.14. **Quantification of hormone secretory cells in murine small intestinal crypt-derived monolayers.** Epithelial monolayers were cultured from the small intestinal tissue SPF (*n=3*), GF (*n=7*), GF mice mono-conventionalised with *Bacteroides thetaiotaomicron* (GFBt/*n=5*), or GF mice administered APS for 10-days (APS10/*n=3*). Representative images of monolayers established from (A) SPF and (B) GF mice stained with antibodies against the peptides ChrA, GLP-1, GIP and Muc2 (red). Ecad (green) was used to identify cellular membranes and Hoechst 33342 nuclear stain (blue) to visualise nuclei. Images taken on fluorescent widefield microscope (40/60x objective). Immuno-positive cells (red) are identified by white arrowheads. The graphs depict quantification of (C) ChrA$^+$, (D) GLP-1$^+$, (E) GIP$^+$ and (F) Muc2$^+$ cells from monolayers cultured from SPF, GF, GFBt and ASP10 small intestinal tissues. Data is represented as mean ± SEM. Unpaired t-test preformed assuming Gaussian distribution was used to calculate statistical significance (GraphPad Prism 9.2.0). A value of $p<0.05$ was considered statistically significant; **$p<0.01$.

significant increase in GIP^+ cells ($p<0.01$) was seen in monolayer cultures established from GF mice ($n=7$) compared to SPF mice ($n=3$) small intestine (Fig. 2.14E) similar to that seen in intact tissue sections (Fig. 2.5C). Generally, therefore, our analysis of secretory cell cultures from whole small intestine showed that intestinal monolayers are variable and with the possible exclusion of GIP-expressing cells, do not accurately reflect the EEC cell makeup seen *in vivo* indicating that immunohistochemistry of intact tissues provides a more accurate enumeration of EECs.

2.3. Discussion

A major challenge to developing a more detailed understanding of the nature of microbiota-EEC interactions that underpin the development of new evidence-based treatments for disorders affecting the GIT and other connected organ systems is identifying which microbes are important and how they contribute to this crosstalk. The recent study describing the ability of Bt to promote neurogenesis within the enteric nervous system of Bt conventionalised GF mice with accompanying effects on L-cells and EC cells (Aktar, Parkar et al. 2020) prompted investigation in greater depth the mechanism and selectivity of the effect of Bt on EEC in the current study. The results presented in this Chapter show for the first time that Bt is directly involved in shaping EEC networks throughout the mouse GIT in a process that is related to, and may be dependent on, their metabolism and production of succinate and the SCFAs acetate and propionate. Using GF mice to investigate the role of the normal microbiota on EEC cell populations, revealed that in the absence of microbiota, there is significant disruption to EEC population and distribution across the intestine. This is normalised upon mono-conventionalisation with Bt or 10-day treatment with the major fermentation products of Bt. The results also suggest that the constant exposure to Bt fermentation products is required for the maintenance of EEC networks. Furthermore, upon culturing intestinal crypt-derived monolayers, it is established that EECs in these cultures do not provide an accurate reflection to their *in vivo* counterpart.

2.3.1. Microbial regulation of EEC networks

GF mice provide a useful tool when determining the extent to which the gut microbiota is responsible for shaping host physiology. In this Chapter, analysis between SPF and

GF mice indicated that gut microbiota regulates EEC distribution and population in a region- and subset-specific manner. In similar studies, increased colonic proglucagon mRNA expression and L cell numbers were reported in GF mice and conventionally raised (CONV-R) mice (Arantes and Nogueira 1997, Arantes and Nogueira 2001, Wichmann, Allahyar et al. 2013) which reflected in increased plasma GLP-1 levels being detected (~140 pg/ml and ~40 pg/ml in GF and CONV-R mice) (Wichmann, Allahyar et al. 2013). The lack of statistical significance in serum GLP-1 in GF mice reported in this Chapter (Fig. 2.7), could be due to the large variation between groups, and may partly be contributed to by GLP-1 degradation or a reflection on normal variations within mice models. Active GLP-1 is rapidly degraded *in vivo*, with a half-life of around 1-2 min (Holst 2007), and although dipeptidyl peptidase-4 (DPPIV) inhibitor was added to blood samples collected to minimise GLP-1 degradation, it cannot be excluded that some GLP-1 degradation did occur. A reduction in number of densely packed vesicles and intracellular GLP-1 content observed in ileal L cells from CONV-R mice (Arora, Akrami et al. 2018), could potentially explain the differences in plasma GLP-1 levels in GF compared to conventional mice (Wichmann, Allahyar et al. 2013). Furthermore, in the caecum, enteroglucagon expressing L cells are reported to be approximately 2.5 times more abundant in GF mice than CONV-R mice, although this may also be a result of an enlarged caecum in GF mice (Arantes and Nogueira 1997, Arantes and Nogueira 2001).

Discrepancies in quantification of GLP-1^+ cells in colonic tissue between the results from this Chapter (Fig. 2.5B and Table 2.1) and those observed by Wichmann et al., and Arantes and Nogueira could be due to inherent limitations of immunohistochemical methods used for detection of GLP-1^+ cells, including different antibodies used and potential bias arising from Visiopharm Integrator System used by Wichmann et al., to quantify GLP-1^+ cell abundance (Arantes and Nogueira 1997, Arantes and Nogueira 2001, Wichmann, Allahyar et al. 2013). Whereas all cell counts obtained from experiments in this Chapter were conducted manually from raw, unprocessed images (Fig. 2.3 and 2.4). Other confounding factors for GLP-1 secretion include circadian disruptors, with GLP-1 secretion demonstrated to have a circadian rhythm in rodents and humans (Brubaker and Gil-Lozano 2016). To minimise the effects of possible circadian disruptors, mice were housed in a 12 hr light/dark cycle and samples were collected at the same time of day for all experiments.

Transcriptomic profiling provides a more accurate method for determining cellular alterations at a gene transcript level. This method, in combination with the generation of transporter mouse strain have been recently used to show that the gut microbiota predominantly regulates L cell functions in the ileum, in particular, genes related to vesicle organisation and localisation in ileal L cells (Arora, Akrami et al. 2018). One possible explanation to regional-specificity could be a result of anatomical differences between the crypt-villus structure between the ileum and colon, that enable easier access of the gut microbiota and their products to either directly or indirectly regulate intestinal EECs.

To further determine the extent of microbial regulation of colonic proglucagon mRNA expression and L cell numbers, Wichmann et al., colonised GF mice with microbiota from CONV-R mice. They showed that plasma GLP-1 decreased gradually following colonisation, accompanied by a reduction in colonic proglucagon mRNA and L cell numbers in the proximal colon (Wichmann, Allahyar et al. 2013), indicating that the endocrine effects induced by a lack of gut microbiota are in part, reversible. Furthermore, colonisation in GF mice increases the energy availability, and in addition to its incretin effects, suppression of colonic GLP-1 results in increased intestinal transit, helps to prevent bacterial overgrowth (Kashyap, Marcobal et al. 2013, Wichmann, Allahyar et al. 2013).

In contrast to GLP-1, a more profound increase as a result of microbial absence was observed in 5-HT^{+} cells (Fig. 2.5D and Table 2.1). This contradicts findings from a previous study that reported no significant differences between 5-HT^{+} cells in the proximal colon of GF and CONV-R mice. Conversely, a significant reduction in Tph1 (a rate limiting enzyme involved in production of 5-HT in EC cells (Gershon 2013)) mRNA expression and protein content in GF mice has been reported, corresponding with reduced colonic 5-HT levels (Reigstad, Salmonson et al. 2015). This could potentially be explained by the method used for identifying EC cells, where antibodies against 5-HT used only detect 5-HT stored in secretory granules, prior to its secretion. Increased Tph1 mRNA expression and colonic 5-HT reported indicates increased 5-HT secretion from EC cells meaning less 5-HT stored in secretory granules. In addition, hormone content in secretory granules has been shown to differ between

regions, with ileal L cells having higher GLP-1 content, with more densely packed secretory vesicles (Arora, Akrami et al. 2018). This raises fundamental drawbacks to identification of EECs based on secretory granular content and would benefit from validation with protein and mRNA analysis.

A decrease in ChrA mRNA expression reported in the proximal colon of GF mice (Reigstad, Salmonson et al. 2015), is comparable to the results displayed in Fig. 2.5A. Interestingly, population-based metagenomics have revealed a strong correlation between faecal ChrA levels and an increasingly diverse microbiome, containing members from the Bacteroidetes phylum (Zhernakova, Kurilshikov et al. 2016).

Most EECs express ND1 (Ratineau, Petry et al. 2002), a transcription factor required for enteroendocrine progenitor differentiation and maturation . Evaluating the immunoreactivity of ND1 in SPF and GF mice revealed significantly increased $ND1^+$ cells in the GF distal colon (Fig. 2.10), in line with previous studies indicating microbial regulation of EEC differentiation (Cani, Hoste et al. 2007, Wichmann, Allahyar et al. 2013, Mazzawi, El-Salhy et al. 2021). ND1 is a transcriptional target for Neurogenin 3 (Ngn3) (Anderson, Torres et al. 2009) and a recent study investigating the effects of faecal microbiota transplant (FMT) on stem cell differentiation into EECs (detected by Ngn3) in irritable bowel syndrome (IBS) patients, revealed that in addition to improvements in IBS symptoms, FMT resulted in increased duodenal ChrA, cholecystokinin (CCK), GIP, 5-HT and somatostatin cell densities (Mazzawi, El-Salhy et al. 2021). These changes are thought to result from differentiation of stem cells into EECs, detected by increased Ngn3 cell densities (Mazzawi, El-Salhy et al. 2021). Interestingly, sodium butyrate has been shown to inhibit ND1 expression at RNA and protein levels in β-cells (Wang, Yuan et al. 2022). Studies looking at EEC differentiation have used traditional co-staining methods to determine the correlation of Ngn3/$ND1^+$ cells with markers for EECs, such as ChrA (El-Salhy and Gilja 2017, Mazzawi, El-Salhy et al. 2021). Determining whether the $ND1^+$ cells are also positive for the pan-enteroendocrine marker, ChrA, would help establish the importance of ND1 expression for driving the increase in EEC numbers observed in GF mice. Additional methods for quantifying ND1 expression, such as at the mRNA level, can help to minimise discrepancies caused by the short half-life of

ND1 (Lee, Cho et al. 2020) and lack of sensitivity of the antibodies used to detect low levels of ND1.

2.3.2. Probiotics and commensals

There are several cases reported in the literature of individual gut microbes which when administered exogenously, affects the host physiology, highlighting the importance of the microbiota in host homeostasis. These include alterations in brain function and behaviour upon microbial depletion (Hoban, Moloney et al. 2016), improvement in anxiety-like and depression-related behaviours following probiotic *L. rhamnosus* treatment (Bravo, Forsythe et al. 2011), and also improved motor deficits following *C. butyricum* treatment in a murine model of Parkinson's disease (Sun, Li et al. 2021). Such studies exemplify the ability of individual species to have potent influences at sites remote from the GIT. Their fundamental mechanisms of action, however, are not addressed.

In this Chapter, a single microbial species (Bt, a major constituent of the mammalian intestinal microbiota) was reinstated in mice that were otherwise GF from birth. Thus, the role of Bt in postnatal development of EEC could be determined without quorum or network mediated effects that could be responsible in antibiotic-depleted or exogenously supplemented normal animals. In a similar study, GF mice were mono-colonised with either *E. coli* or Bt for 4-weeks (Wichmann, Allahyar et al. 2013). This study reported that GLP-1 positive cells in the proximal colon are increased by colonisation with Bt but not by *E. coli*, although there were regional differences to the results presented in this chapter. This study also found serum GLP-1 increased in GF mice in line with findings presented in Fig. 2.7. Other studies conventionalising GF mice with specific microbiota agree with our findings. For example, Turnbaugh and colleagues showed that conventionalisation of GF mice with an obesity-associated mouse gut microbiome induces an increased capacity for energy harvest (Turnbaugh, Ley et al. 2006). Reigstad and co-workers, using GF and humanised mice, showed that gut microbiota are important determinants of enteric 5-HT production and homeostasis (Reigstad, Salmonson et al. 2015), as observed in the proximal and distal intestine of Bt conventionalised GF mice (Fig. 2.5D). The results here also demonstrated the selectivity of the effect of Bt on EEC networks as seen by the inability of another

unrelated rodent gut commensal bacterium, Lr, to replicate the effects of Bt on EECs in mono-conventionalised GF mice (Fig. 2.11 and Table 2.3). Lr, unlike Bt, is a gram-positive bacterium with a different metabolic profile and has been well studied as a host-health promoting anti-inflammatory probiotic (Mu, Tavella et al. 2018). Using Lr in this study therefore enabled the demonstration the specificity of Bt to singularly regulate murine EEC networks.

The ability of Bt to influence EEC throughout the GIT is perhaps not surprising considering *Bacteroides* species are found in close association with the mucus that coats intestinal epithelial cells (Bry, Falk et al. 1996) and are therefore juxtaposed with EEC. It is important to note, however, that the impact of Bt on EEC is not uniform throughout the GIT with some but not all EEC subsets being modulated to the same degree, suggesting both a regionalised and subset specific effect of Bt on EEC. Unlike Billing et al. and Roberts et al. who using "omics-based approaches were unable to detect GIP expression in the mouse colon", the results presented in this chapter confirm the presence of GIP-expressing cells in the proximal and distal colon of both GF and SPF mice (Billing, Larraufie et al. 2019, Roberts, Larraufie et al. 2019). Whilst it cannot be entirely excluded the possibility that this is the result of non-specific antibody reactivity, similar findings to ours have been reported in human studies using immunohistochemistry and mRNA analyses to detect GIP expression in the distal colon (Jorsal, Rhee et al. 2017). Discrepancies in detecting GIP expressing cells in the mouse colon may, in addition to experimental design and methodological differences, be related to variations in environmental conditions within different animal facilities. Each facility has their own unique combination of various and numerous attributes of animal husbandry that impact on the bacterial communities within each facility and on the microbiome of their occupants that can influence host physiology and phenotype (Rausch, Basic et al. 2016).

It is particularly noteworthy that Bt exerts effects on EEC in the small intestine, which is at odds with the conventional view of Bt being a resident of the anoxic cecum and colon. However, data presented in this Chapter demonstrates the ability of Bt to colonise both the small and large intestine of GF mice (Table 2.2), comparable with its presence in regions of the small intestine of healthy humans (Mallory, Savage et al. 1973). This could therefore provide a possible route for its global effect on EEC

networks. Alternatively, Bt might act via non-cognate interactions and through the production of metabolites or other mediators that are absorbed from the intestinal lumen and then disseminated throughout the body via the circulatory or nervous systems.

2.3.3. SCFAs as key mediators in microbial modulation of EECs

Among the various pathways and products that could be responsible for the effects of Bt on EEC we investigated their major products of polysaccharide fermentation, acetate, propionate, and succinate (Wrzosek, Miquel et al. 2013, Curtis, Hu et al. 2014). NMR metabolite analysis confirmed that all three metabolites are produced by and accumulate in the cecum and colon of Bt-conventionalised GF mice. High levels of succinate are particularly noteworthy and replicate prior studies of Bt colonised, antibiotic pre-treated, C3H/HeJ mice demonstrating a 200-fold increase in caecal succinate levels post-Bt colonisation (Curtis, Hu et al. 2014). Amongst SCFAs, acetate, propionate, and butyrate are the most abundant (≥95%) (Cook and Sellin 1998) and are present in an approximate molar ratio of 60:20:20 in the colon and stool (Cummings, Pomare et al. 1987, Hijova and Chmelarova 2007, Binder 2010). The prominence within the human colon of *Bacteroides* which make up ~25% of the total anaerobes (Salyers 1984), and are adept glycan metabolisers (Salyers, Vercellotti et al. 1977) as well as producers of high levels of acetate (Wrzosek, Miquel et al. 2013, Curtis, Hu et al. 2014), helps explain the prominence of acetate amongst SCFA in the colonic lumen. The importance of acetate and other SCFAs to the host is exemplified by the fact that they provide ~10% of the host daily caloric requirements (McNeil 1984, Bergman 1990). In addition to this, propionate stimulates intestinal gluconeogenesis (De Vadder, Kovatcheva-Datchary et al. 2014) and contributes to protecting the integrity of the blood-brain barrier (Hoyles, Snelling et al. 2018), whereas succinate is a key intermediary in several metabolic pathways, playing an important role in the elimination of reactive oxygen species (Tretter, Patocs et al. 2016).

What emerged from the data presented in this Chapter, is that a mixture of acetate, propionate and succinate administered in physiologically appropriate concentrations

and molar ratios (Mineo, Amano et al. 2006) was able to recapitulate the effect of Bt, with certain exceptions. For example, the regulation of EEC in the colon was weakly affected by APS compared to Bt, which may indicate there is reduced access of oral APS to the colon compared with the small intestine. Alternatively, there may be additional factors and metabolites to APS that convey the efficacy of Bt in the colon. The inability of Lr, which produces a similar profile of SCFA (including acetate and propionate but not succinate) to Bt (Kahouli, Malhotra et al. 2015) to replicate the effects of Bt on EEC supports this proposal. Surprisingly, in several cases, the effects of APS were greater in the colon after a 10-day washout period. This may be indicative of the effects of APS being gradual in onset, and/or their initial effect persisting and being amplified. A long-lasting effect could also arise as a consequence of influencing epithelial stem cells and driving production of EEC lineage cells as recently demonstrated in a SCFA (acetate, propionate, butyrate)-murine and human intestinal enteroid co-culture model system (Pearce, Weber et al. 2020). In this *in vitro* culture system, butyrate was shown to be the most effective SCFA in increasing ChrA expression (Pearce, Weber et al. 2020). This may explain the loss of $ChrA^+$ cells observed *in vivo* after washout of the butyrate-deficient APS cocktail. The absence of a long lasting effect of APS on ChrA cells might also reflect a separate population of EEC not otherwise labelled in our study that accounted for its transient effect, such as PYY containing L-cells (Aktar, Parkar et al. 2020) or tuft cells (Sutherland, Young et al. 2007).

In this study, APS was administered via drinking water. The main resaon behind this was the ease of APS delivery and maintenance of GF status. Other methods of SCFA delivery include oral gavage and intraveneous injection, but are generally considered more invasive. Another more invasive approach utilised surgical procedures involving luminal perfusion. Although useful for assessing direct systemic exposure to SCFAs and measuring intestinal motility, this approach is technically challenging (Wan Saudi and Sjöblom 2017). Since in this present chapter, the main assessment was focused on EEC populations, oral administration presented a suitible approach for exposure of APS along the GIT. Of course, the absorption of APS following intake via drinking water would likely differ along the GIT and can be confirmed by NMR metabolite analysis from intestinal and caecal contents, but it can be assumed that any

unmetabolised APS is carried on through the GIT, accumulating in the caecum and distal intestine.

2.3.4. Constraints in current EEC research

2.3.4.1. *In vivo* and *in vitro* models

Most of the research on endocrine cells has been undertaken using animal tissues, namely mice, as with the experiments conducted in this Chapter. These provide a relatively inexpensive and readily available samples to analyse. In contrast to in humans, the diet and microbiome of murine models differ greatly, and therefore the enteroendocrine nutrient sensing and hormone secretion also differ (Nguyen, Vieira-Silva et al. 2015). Studying EECs is made further challenging by their rarity, comprising less than 1% of the total intestinal epithelial cell population (Gribble and Reimann 2016). Immortalised cells lines, including GLUTag and STC-1, provide a promising high-throughput alternate to the native counterpart, but are highly simplified models that don't actually reflect the *in vivo* cellular heterogeneity and therefore raises questions regarding their physiological relevance (Lee, Asa et al. 1992, Kuhre, Wewer Albrechtsen et al. 2016). Hormone secretion from intestinal biopsies and tissues *ex vivo* in response to stimuli has also been reported (Symonds, Peiris et al. 2015, Sun, de Fontgalland et al. 2017), but in line with data from this Chapter (Fig. 2.13), the responses are relatively weak and unreliable.

2.3.4.2. Primary intestinal cultures

Over the last decade, the use of primary EECs in research has undergone profound development. Results from the experiments conducted in this Chapter indicate that epithelial cell monolayers (Fig. 2.14) generated from small intestinal crypts of GF mice do not accurately reflect the EEC makeup or response to Bt (with the exception of GIP^+ cells) and APS seen *in vivo.* This is at odds with their increasing use as a physiologic model of intestinal response to stimuli including microbes and nutrients (Leushacke and Barker 2014, Pearce, Coia et al. 2018, Yin, de Jonge et al. 2019). These contradictory findings may relate to the use of GF mice in this Chapter, as SPF

mice are the usual source of intestinal crypts (Petersen, Reimann et al. 2014, Roberts, Larraufie et al. 2019). The finding that the transcriptome and proteome of small intestinal stem cell-derived organoids from SPF and GF mice co-cluster (Hausmann, Russo et al. 2020), would argue against this possibility. However, in this study no account was made for any possible differences in EEC distribution or number. Indeed, whereas the current analysis relied on cellular comparisons of EEC in epithelial cell monolayer cultures versus intact tissue, other studies have used single or multi-omics-based approaches in comparative studies (Lindeboom, van Voorthuijsen et al. 2018, Beumer, Puschhof et al. 2020, Hausmann, Russo et al. 2020, Ohki, Sakashita et al. 2020). Other possible confounding factors include comparing EECs in two-dimensional (2D) epithelial cell monolayers versus stem cell-derived three-dimensional organoids (3D), methodological differences including the age of the mice used, where in the small intestine crypts are obtained from (Fuller, Faulk et al. 2012), the duration of culture, and the type and concentrations of growth and differentiation factors used.

Short-lived 2D intestinal cultures such as those used in this Chapter, was first developed to assess the electrophysiology, calcium signalling and hormone secretion of primary L cells (Reimann, Habib et al. 2008, Psichas, Tolhurst et al. 2017). They enable the study EECs close to their native counterpart but are limited due to the short-lived nature of the cultures, with experiments conducted withing 24-36 hr (Reimann, Habib et al. 2008), meaning that a constant supply of fresh tissue is required for each preparation. Furthermore, it was reported that the L_{pos} cells in the mixed intestinal cultures were non-proliferative, assessed by EdU incorporation, and no new EECs were found to be generated under these conditions (Reimann, Habib et al. 2008). It remains to be investigated whether the intestinal cultures derived from crypts contain active and proliferative stem cells and whether manipulation of Notch signalling can drive immature cells towards the secretory cell fate. By contrast, separation of EECs using Percoll density gradient has been demonstrated to yield high purity EEC populations that can be kept in culture for around 4-days (Raghupathi, Duffield et al. 2013), but like the short-lived primary intestinal cultures, the functional significance of using EECs that have been separated from their surrounding enterocytes hasn't been investigated (Goldspink, Reimann et al. 2018).

In comparison to short-lived cultures, the generation of intestinal organoids (3D structures derived from intestinal crypt-stem cells grown in specialised media) provide a promising tool for the study of EECs that more closely resembles their native environment, the stem cell niche (Sato, Vries et al. 2009). Manipulating levels of specific niche factors or differentiation-promoting factors, helps to disregard the need for a constant source of fresh intestinal tissue. These provide a replenishable *de novo* source of differentiated EECs (Barker, Huch et al. 2010, Sato, Stange et al. 2011, Huch, Dorrell et al. 2013, Fujii, Matano et al. 2018), that have been shown to retain their regional hormonal repertoire identity (Basak, Beumer et al. 2017). Recent comparative analysis of intestinal organoids generated from mice expressing Ngn3 enhanced green fluorescent protein (eGFP) (Ngn3-EGFP) revealed similar gene expression patterns and mRNA expression of keys hormones (Cck, GIP, glucagon and ghrelin) of intestinal organoids to EECs in native tissues (Ohki, Sakashita et al. 2020). However, murine small intestinal and colonic intestinal organoids were shown to not reflect the differences in 5-HT concentration or expression of ChrA or Tph1 mRNA in native tissues (Tsuruta, Saito et al. 2016). Additionally, the growth and functional differentiation of intestinal organoids have been shown to differ according to method of isolation and culture conditions (Miedzybrodzka, Foreman et al. 2020, Wilson, Mayo et al. 2020), reiterating the need for standardisation of protocols to minimise discrepancies between studies and to validate intestinal organoids as an accurate tool for recapitulating EECs *ex vivo*.

Cells generated in 3D organoid cultures have apico-basal polarity, with apical cell surfaces that are arranged towards the organoid core (luminal environment). This makes it challenging to administer stimuli, with there being reports organoids are "leaky" allowing stimuli added in the media to access apical membranes (Zietek, Rath et al. 2015). Progress has also been made in manipulating 3D organoid cultures to expose the apical membranes for transport studies that recapitulate many important aspects of the *in vivo* intestinal epithelium (Schweinlin, Wilhelm et al. 2016, Goldspink, Lu et al. 2018).

Whilst 2D crypt-derived intestinal cultures allow for easier identification and imaging of specific cell types, additional multidisciplinary studies incorporating both molecular and cellular methodologies are required in order to address these discrepancies, and to determine what aspects of EEC physiology can, and can't, be faithfully represented by crypt-derived epithelial cell monolayers from conventional versus GF mice.

2.3.4.3. Classification of EECs

Historically, EECs had been identified by their expression of a single distinct peptide hormone (Polak, Bloom et al. 1971, Larsson, Sundler et al. 1977, Larsson and Rehfeld 1978), but it is becoming increasingly apparent that EECs co-express a broad spectrum of peptide hormones and display heterogeneity, that may reflect regional specificity along the GIT and exposure to dietary nutrients.

Classical immunohistochemical methods, such as those used in this Chapter, remain critical to confirm the co-expression and identification of hormones at a peptide level. Many earlier studies used this method to reveal a complex co-localisation pattern of peptide hormones in EECs (Roth and Gordon 1990, Roth, Kim et al. 1992, Aiken, Kisslinger et al. 1994, Hörsch, Fink et al. 1994). It has long been established that crypt-based EECs can expressed multiple hormones (Roth, Kim et al. 1992), but recent generation of a transgenic reporter mice expressing enhanced eGFP under the control of the CCK promotor was used to show that CCK^+ cells co-express several functionally related hormones (GLP-1, GIP, PYY and secretin) and were not restricted to crypt-based CCK^+ cells, but also mature EECs further along the length of the villus (Egerod, Engelstoft et al. 2012). This was further validated by immunohistochemical methods used to co-stain EECs in both murine and human intestine (Egerod, Engelstoft et al. 2012). Fluorescent-activated cell sorting (FACs) analysis of murine L and K cell populations have revealed that the majority of isolated colonic L cells contained GLP-1 and PYY, whereas in the upper intestine, the majority of L_{pos} cell populations were CCK^+, and PYY was expressed at relatively low levels (Habib, Richards et al. 2012). Further demonstration of the extent of peptide hormone overlap revealed that upper intestinal K_{pos} cells contained approximately 10% overlap with GLP-1 (Habib, Richards et al. 2012). FACs based approaches are limited by the efficacy and

specificity of the fluorescently tagged reporter gene, which can be overcome by single-cell RNA sequencing on pools of isolated EECs. Using this method, Haber et al., identified eight different clusters of mature EECs all expressing secretin. Two of the eight clusters expressed Tph1, five co-expressed CCK and another co-expressed ghrelin, GIP and somatostatin (Sst) (Haber, Biton et al. 2017).

The heterogeneity of EECs along the crypt-villus axis is believed to be influenced by the stem cell niche. During maturation and differentiation, EECs migrate upwards along the villi, but it was recently noted that a small subpopulation of EECs also migrate downwards, residing in close proximity to $Lgr5^{+}$ stem cells and Paneth cells (Aiken, Kisslinger et al. 1994, Sei, Lu et al. 2011). Upward, villus migrating EECs have also been shown to switch hormone expression, regulated by the bone morphogenetic protein (BMP) gradient (Beumer, Puschhof et al. 2020). The complexity of EECs regarding their hormone expression patterns highlights the need for the development of methods to enrich the full diversity of EECs *in vitro*. Indeed, inhibiting Notch, Wnt and BMP signalling in murine derived intestinal organoids has been shown to drive EEC differentiation and recapitulate EEC subtype distribution (Basak, van de Born et al. 2014, Beumer, Artegiani et al. 2018, Beumer, Puschhof et al. 2020). Identification of EEC regulators, such as Rfx6 and Tox3, and their conditional knockout in mice, has also been shown to yield specific EEC subtypes (Gehart, van Es et al. 2019).

These results demonstrate that the intestinal enteroendocrine system is in fact highly adaptive, displaying large regional diversity and plasticity in their hormonal repertoire. It remains to be determined whether the pattern of co-expression in individual EECs confers any physiological significance. Indeed, 5-HT cells from the duodenum and colon have been shown to express different repertoires of nutrient receptors and mechanosensory receptors, possibly reflecting different regional requirements (Martin, Lumsden et al. 2017, Alcaino, Knutson et al. 2018). It is becoming increasingly apparent that an updated EEC taxonomy is required, one that recognises the diversity and plasticity of EEC networks. A combination of classical

immunohistochemical methods and flow cytometric, proteomic, and transcriptomic techniques will be needed to address these issues.

2.4. Concluding remarks

In this chapter, SPF and GF mice were used to demonstrate that the intestinal microbiota is required for regulation of EEC networks, and that a single microbe, Bt, can recapitulate its role in a process that may be dependent on their metabolism and production of APS. Since Bt is a major human symbiont, these findings have implications for novel interventions for the maintenance of human health via the microbiome and modulation of the gut endocrine system.

2.5. Methods

2.5.1. Bacterial strain and culturing

Bacteroides thetaiotaomicron (Bt; VPI 5482, ATCC) was grown anaerobically at 37°C in brain heart infusion (BHI) medium (Oxoid) supplemented with 15 μM hemin. *Lactobacillus reuteri* (Lr; 100-23, DSMZ) was grown anaerobically at 37°C in MRS medium (Difco Laboratories).

2.5.2. Animal handling

C57BL/6 mice of 8-12 weeks of age were housed in a specific pathogen free (SPF) Disease Modelling Unit (DMU) at the University of East Anglia (UEA), Norwich, United Kingdom. All mice were housed in ventilated cages and exposed to 12 hr light/dark cycle with free access to water and standard chow at all times throughout the study. All experiments were conducted in accordance with the Home Office Animals (Scientific procedures) Act 1986 under the licence number PPL180/2545 at the UEA.

Germ-free mice. C57BL/6 germ-free (GF) mice were maintained in sterile isolators in the Quadram Institute Germ Free Facility within the DMU with GF status being continuously monitored by microscopy, aerobic and anaerobic culturing, and PCR for bacterial contamination.

Conventionalisation of GF mice. C57BL/6 GF mice were conventionalised by administering 0.1 ml ($1.4x10^9$ cells/ml) of Bt or Lr in sterile PBS by oral gavage and maintained in individual ventilated cages for up to 5-days. To assess extent of colonisation, contents of the GIT were cultured under anaerobic conditions and colony count determined. Additional aerobic and anaerobic cultures were preformed to exclude contamination.

SCFA experiments. C57BL/6 GF mice were administered via their drinking water a cocktail of APS at levels comparable to those in the gut lumen consisting of sodium acetate (95 μM, Sigma-Aldrich, S2889), sodium propionate (29 μM, Sigma-Aldrich, P1880) and sodium succinate (5.6 μM, Sigma-Aldrich, 14160) (Mineo, Amano et al. 2006) for 3- or 10-days. Additional GF mice were administered APS-containing drinking water for 10-days after which the drinking water was replaced with regular drinking water (wash out) for a further 10-days.

2.5.3. Intestinal cfu

Content was obtained from all regions of the intestine (duodenum, jejunum, ileum, caecum, proximal colon and distal colon) from GF mice 5-days post-conventionalisation with Bt. Content was weighed prior to addition of 400 μl sterile PBS to each sample. Samples were then vortexed briefly, centrifuged at 1000 rpm for 10 min at 20-22°C and sterile two-fold dilutions carried out and plated on BHI agar plates. Following incubation in an anaerobic cabinet for 48 hrs (37°C, 5% CO_2), colonies were counted and used to calculate the CFU/g of contents at 5-days post-colonisation with Bt in GF mice.

2.5.4. Blood and tissue sampling

Sampling was carried out at the same time of day for all experiments.

Serum. Blood samples were taken by cardiac puncture following euthanasia with 0.1 ml of Dipeptidyl peptidase IV (DPPIV) inhibitor (TOCRIS BIOSCIENCE, 6019) per ml of blood, centrifuged at 1000-3000x*g* for 10 min, serum removed, aliquoted and stored at -20°C prior to analysis. GLP-1 protein levels quantified using a mouse multiplex kit according to manufacturer's instructions (Merk Millipore, Milliplex MAP Multiplex assay).

Tissue. The entire intestinal tract was excised, the contents removed by flushing with sterile Dulbecco's Phosphate Buffered Saline (DPBS), prior to dividing into anatomically distinct segments (duodenum, jejunum, ileum, proximal colon and distal colon) that were fixed in 10% neutral buffered formalin (Sigma-Aldrich, HT501128) for 24 hr at 20-22°C followed by 24 hr in 70% ethanol at 4°C. Tissues were then processed through xylene/alcohol dehydration and

clearing series followed by wax infiltration. Segments of tissues were embedded in paraffin wax prior to sectioning (5 μm) and mounting on SuperFrost® Plus glass slides (VWR, 631-0108).

2.5.5. Immunohistochemistry

Tissue sections were rehydrated through Histoclear and a graded ethanol series. Following washing in dH_20, slides were heated in citric acid buffer (10 mM, pH 6) (Sigma-Aldrich, C9999) for antigen retrieval, washed further in Tris-buffered saline with Tween-20 (TBS-T) and incubated for 16 h at 4°C with either a rabbit polyclonal anti-GLP-1 (Abcam, ab22625), mouse monoclonal anti-GLP-1 antibody (Abcam, ab23468), rabbit monoclonal anti-GIP antibody (Abcam, ab209792), mouse monoclonal anti-GIP (021-04, Santa Cruz), rabbit polyclonal anti-Chromogranin A (ChrA) antibody (Santa Cruz Biotechnology, sc-13090), goat polyclonal anti-5-HT antibody (Abcam, ab66047) and Hoechst nuclear stain (Thermo Fisher, H1399). Unless specified, control antibodies were obtained from Abcam; rabbit IgG (Abcam, ab37415) and monoclonal IgG (Abcam, ab172730), mouse IgG2a (Abcam, ab18415), mouse IgG_1 (Miltenyi Biotech, IS5-21F5,), and goat IgG (Abcam, ab37373). Tissues were washed in TBS-T and incubated with Alexa Fluor®594 goat anti-rabbit Ig (Invitrogen, 27117), Alexa Flour®488 anti-mouse IgG (Thermo Fisher, A11001), or Alexa Fluor®594 donkey anti-goat Ig (Invitrogen, A11057) for 30 min at 20–22°C. Tissues were mounted using ProLong™ Diamond Antifade mountant (Thermo Fisher, P36961).

Cells were imaged using a Zeiss Axio Imager M2 microscope equipped with 40x/air objective and Zen blue software (Zeiss). In addition, Zeiss LSM880 confocal microscope equipped with 63x/1.4 oil DIC objective and Zen black software (Zeiss) was used to obtain higher resolution images. Fluorescence was recorded at 405 nm (blue), 488 nm (green), 594 nm (red) and 647 nm (far-red). Fluorescent intensity of ND1 staining was quantified using sum fluorescent pixel intensity of the field of view (FOV) using a macro written in Image J/FIJI v1.52p. Quantification of cell numbers were performed manually on ImageJ/FIJI v1.52p software using raw, unprocessed images.

2.5.6. Intestinal crypt isolation and culture

The intact small intestine was flushed with ice-cold DPBS, opened longitudinally and villi removed by gentle scraping using a glass coverslip. Tissues were then cut into 5–8 mm pieces, vigorously washed 5 times in ice-cold DPBS and transferred to 50 ml tubes containing 15 ml Gentle Cell Dissociation Reagent (Stem Cell Technologies, 07174) and incubated at 20–22°C for 15 min on a rolling platform. Tissues were then washed in ice-cold DPBS to release the

crypts and filtered to remove excess debris using a 70 μm cell strainer (Corning, CLS431751). The crypt suspensions were then centrifuged at 300×*g* for 3 min at 20–22°C. Supernatant was removed, and crypt pellets were resuspended in IntestiCult Organoid Growth Medium Mouse (Stem Cell Technologies, 06005) supplemented with Penicillin/Streptomycin, and Y-27632 ROCK inhibitor (TOCRIS BIOSCIENCE, 1254) to prevent anoikis. The crypts were then plated onto glass coverslips (Agar Scientific Ltd.) in 24 well cell culture plates (Greiner Bio-One Ltd.) coated with 1:20 dilution (in DPBS) of Matrigel Basement Membrane Matrix (Scientific Laboratory Supplies, 356231) for 20–24 hr to form semi-confluent monolayers. Monolayers were fixed in 10% neutral buffered formalin, washed with DPBS and permeabilized with 0.25% Triton X100 in DPBS, then incubated in blocking buffer (DPBS containing 10% goat serum (Sigma-Aldrich, G9023). Cultures were then incubated with rabbit polyclonal anti-GLP-1 antibody (Abcam, ab22625), rabbit monoclonal anti-GIP antibody (Abcam, ab209792), or rabbit polyclonal anti-ChrA antibody (Santa Cruz Biotechnology, sc-13090) and mouse anti-E-cadherin antibody (BD Transduction Laboratories, 610181) for 2 hr at 20–22°C. Following further washes in DPBS, monolayers were incubated for 30 min with the secondary antibodies, Alexa Fluor®594 goat anti-rabbit (Invitrogen, 37117) and Alexa Fluor®488 goat anti-mouse (Invitrogen, A11001), followed by Hoechst (Thermo Fisher, H1399). The monolayers were washed with H_20, coverslips carefully removed and mounted on glass slides using ProLong™ Diamond Antifade mountant (Thermo Fisher, P36961).

2.5.7. Hormone secretion assay

Colonic tissues from non-treated GF mice were excised and cut longitudinally to expose luminal interface. Tissues were then incubated for 2 hrs with either 400 μl control or nutrient-supplemented customised tissue culture medium consisting of carbonated solutions of Krebs buffer (Sigma-Aldrich, K4002) supplemented with 4.4 nM L-glutamine (Gibco, 25030-081). For nutrient-supplemented buffers, 95 μM sodium acetate (Sigma-Aldrich, S2889), 29 μM sodium propionate (Sigma-Aldrich, P1880) and 5.6 μM sodium succinate (Sigma-Aldrich, 14160) were used. To prevent breakdown of GLP-1, 50 nM dipeptidyl peptidase inhibitor IV (Sigma-Aldrich, D3572) was added to both control and nutrient-supplemented medium. Following incubation, supernatants were collected and stored at -20°C and GLP-1 protein levels quantified using a mouse multiplex kit according to manufacturer's instructions (Merk Millipore, Milliplex MAP Multiplex assay).

2.5.8. Metabolite analysis by nuclear magnetic resonance (NMR)

Acetate, butyrate, propionate and succinate were quantified in the contents of the duodenum, distal colon (SPF, GF, and GFBt), and cecum (SPF, GF, GFBt, and GFLr) ($n = 5$ ea.) using

1H NMR spectroscopy. Samples were prepared by mixing ~50 mg of the sample with 12 times the volume of phosphate buffer-D_2O (0.1 M K_2HPO_4, 0.1 M NaH_2PO_4, 145.1 μM TSP-d4 mixed 1:1 with deuterium oxide [D_2O]). The 1H NMR spectra were recorded on a 600 MHz Bruker Advance spectrometer (Bruker BioSpin GmbH, Germany). Each 1H NMR spectrum was acquired with 64 scans, a spectral width of 12,500 Hz, and an acquisition time of 2.62 s. The "noesygppr1d" pre-saturation sequence was used to suppress the residual water signal with low power selective irradiation at the water frequency during the recycle delay (D1 = 2 s) and mixing time (D8 = 0.15 s). A 90_ pulse length of 8.8 μs was set for all samples. Spectra were transformed with a 0.1 Hz line broadening and manually phased in TopSpin 3.9.1, and the chemical shift scale referenced to TSP. The spectra were then baseline corrected, removing the broad envelope between 0.7 and 4.5 ppm using AMIX 3.9.15 (underground removal tool, filter width = 20 Hz). Acetate, butyrate, propionate, and succinate were quantified using the Chenomx NMR Suite 8.12.

2.5.9. Statistical analysis

For box and whisker plots, the box extends from the 25th to 75th percentiles with the horizontal line within the box representing the median and the whiskers representing the minimum and maximum values. Other graphed date sets are expressed as mean ± SEM. For immunohistochemistry, data was analysed by fitting a mixed model (GraphPad Prism 9.2). Statistical analysis was performed using a two-way ANOVA mixed effects analysis with $p<0.05$ defined as significant with Geisser-Greenhouse correction not used.

3. Uptake of bacterial extracellular vesicles derived from the gut commensal *Bacteroides thetaiotaomicron* and their transport across an *in vitro* cellular model of the gut-brain axis

3.1. Introduction

Bacterial extracellular vesicles (BEVs) produced by both gram-negative and gram-positive bacteria were in the past believed to be a mechanism of cellular waste removal that held no significant biological relevance in the gastrointestinal tract (GIT) (Kulp and Kuehn 2010, Schwechheimer and Kuehn 2015). However, research now demonstrates that BEVs have an array of important biological functions including delivery of biomolecules such as virulence factors and toxins (Bomberger, Maceachran et al. 2009, Stentz, Carvalho et al. 2018), immunomodulation (Ellis and Kuehn 2010, Vidakovics, Jendholm et al. 2010, Fábrega, Aguilera et al. 2016), bacterial pathogenesis (Baumgarten, Sperling et al. 2012) and have also been utilised for vaccine development (Kulp and Kuehn 2010, Schwechheimer and Kuehn 2015). The encapsulation of cargo, including various polysaccharides, proteins, nucleic acids, metabolites and toxins, facilitates their functions by protecting cargo from degradation and signal amplification (O'Donoghue and Krachler 2016, Stentz, Carvalho et al. 2018).

In the GIT, BEVs are released into the lumen where the first host-interface they are in contact with is the intestinal epithelium. BEVs have been suggested to be transported across gut epithelial barriers via several transcellular and paracellular routes, including macropinocytosis, clathrin-mediated endocytosis and caveolin-mediated endocytosis (Irving, Mimuro et al. 2014, O'Donoghue, Sirisaengtaksin et al. 2017). Which transport routes are utilised by BEVs can differ amongst bacterial species and can be dependent on their size, protein content and virulence factors (Jones, Booth et al. 2020).

There is compelling evidence that indicates the transport of BEVs in the blood circulation (Jang, Kim et al. 2015, Park, Choi et al. 2017, Jones, Stentz et al. 2021) and together with recent published work carried out by the Carding group (Jones et al.,) demonstrate the *in vivo* biodistribution of BEVs across the intestinal epithelium and

accumulation in the liver and lungs following oral administration in mice (Jones, Booth et al. 2020), indicate the ability of BEVs to target tissues outside the GIT.

Indeed, recent studies have shed light onto the potential role of BEVs as long distance mediators in gut-brain axis (Kulp and Kuehn 2010, Schwechheimer and Kuehn 2015), with evidence not only suggesting BEV interactions with peripheral pathways involved in gut-brain communication (Al-Nedawi, Mian et al. 2015, Lee, Kim et al. 2020), but also direct influence on the central nervous system (CNS) (Han, Choi et al. 2019, Ha, Choi et al. 2020). For example, oral administration of the psychoactive *Lactobacillus rhamnosus* (*L. rhamnosus*) JB-1 derived BEVs have been demonstrated to replicate the psychoactive effects of the parent bacterium, by infiltrating murine Peyer's patches and inducing functional regulatory $CD4^{+}25^{+}Foxp3^{+}$ T cells (Bravo, Forsythe et al. 2011, Perez-Burgos, Wang et al. 2013, Al-Nedawi, Mian et al. 2015). The vagus nerve has been suggested to play a vital signalling route for gut bacteria (Bharwani, West et al. 2020) with vagotomy shown to inhibit cognitive impairment and hippocampal infiltration of BEVs derived from *Paenalcaligens hominis* (*P. hominis*) following oral administration (Lee, Kim et al. 2020).

Until recently there was little evidence supporting potential transport of BEVs across the blood brain barrier (BBB). An early study undertaken demonstrated increased BBB permeability in rats induced by *Haemophilus influenza* (*H. influenza*) type B derived BEVs (Wispelwey, Hansen et al. 1989). BEVs derived from the periodontal pathogen *Aggregatibacter actinomycetemcomitans* (*A. actinomycetemcomitans*) were used to show BEV transport to the brain following intracardiac or intravenous administration (Han, Choi et al. 2019, Ha, Choi et al. 2020). Furthermore, *A. actinomycetemcomitans* BEVs were shown to localised to brain microglia cells, activating the proinflammatory nuclear factor kappa beta (NFκB) signal transduction pathways and downstream tumour necrosis factor-α (TNF-α) and interleukin (IL)-6 production (Han, Choi et al. 2019, Ha, Choi et al. 2020).

There is also promising evidence indicating the biological relevance of the intestinal microbiota derived BEVs in host physiology. In a pathological setting for example, the metabolic profile of BEVs in Alzheimer's disease (AD) patients were shown to have significant alteration in levels of aspartate, L-aspartate, imidazole-4-acetate and L-

glutamate compared to healthy controls (Wei, Wei et al. 2019). Moreover, BEVs isolated from faecal microbiota from AD patients and administered to mice daily for 8 weeks, resulted in disruption of the BBB accompanied by increased activation of microglia and astrocytes, secretion of inflammatory cytokines (TNF-α and IL-1β) and tau phosphorylation resulting in cognitive impairment (Wei, Peng et al. 2020) This study demonstrates the potential downstream effects of microbial dysbiosis on cognitive function, mediated in part at least by BEVs. A summary of key studies indicating potential transport and signalling routes used by BEVs in the gut-brain axis, and their interactions with cells in the CNS are depicted in Fig. 3.1.

Whilst many of these studies have focused on BEVs derived from pathogenic-bacterial species, the role of BEVs in a non-pathological setting remain to be fully determined. The aim of the experiments undertaken in this chapter was to extend previous work undertaken by the Carding group (Jones et al.,) by developing *in vitro* cell culture methods. Using BEVs derived from the abundant gut commensal *Bacteroides thetaiotaomicron* (Bt), the aim was to determine the uptake and migration of Bt-BEVs across gut epithelial and blood vessel barrier cells and their subsequent interaction with CNS-derived microglia and neuronal cells (Fig. 3.2), in order to provide a better understanding of the role of BEVs derived from commensal gut bacteria on host gut-brain signalling.

3.2. Experimental outline

An *in vitro* single- and multi-cellular ThinCert™ cell culture system was developed and used to assess Bt-BEV uptake and transport across intestinal-epithelial and brain-endothelial barriers and interactions with cells in the CNS (Fig. 3.3).

The single-cell system was used to assess uptake, intracellular fate and immunomodulatory effects of Bt-BEVs in hCMEC/D3 and BV-2 cells (Fig. 3.3A). Transport of Bt-BEVs across the intestinal epithelium and uptake by CNS blood vessel endothelial cells was assessed using the ThinCert™ culture inserts in a two-cell culture system with Caco-2 and hCMEC/D3 cells (Fig. 3.3B). Subsequent Bt-BEV transport across BBB model and uptake by microglia cells was assessed by using hCMEC/D3 and BV-2 cells in a two-cell ThinCert™ cell culture system (Fig. 3.3B). A three -cell system with Caco-2 and hCMEC/D3 cells cultured on opposite sides of ThinCert™ insert membrane was used to assess Bt-BEV transport across intestinal epithelial and brain blood vessel endothelial cell barriers and uptake and immunomodulatory

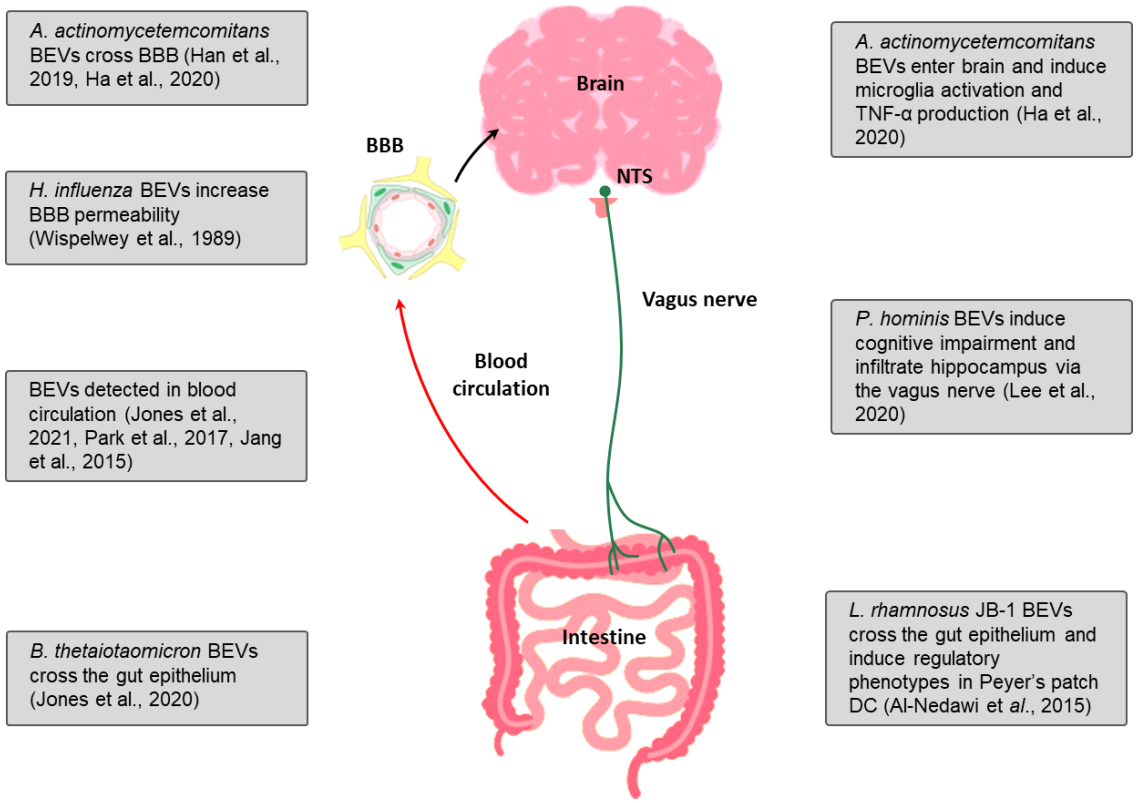

Figure 3.1 Summary of key studies identifying possible routes utilised by gut-derived BEVs in communication with the host gut-brain axis. Schematic depicts communication pathways from the gut to the brain that BEVs have been shown to use. They include peripheral pathways (involving the ENS and the vagus nerve) or direct pathways (including uptake into the blood circulation, transport across the BBB and entry into the CNS).

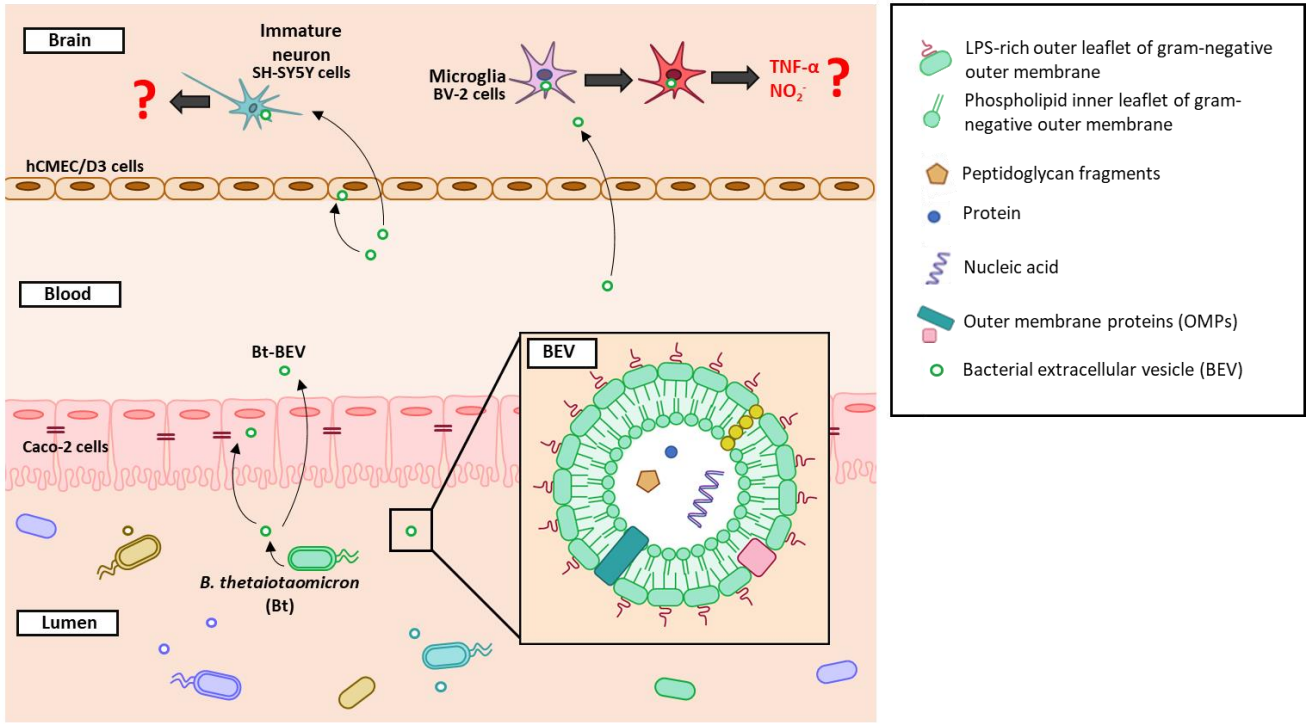

Figure 3.2 Hypothesised uptake and transport of Bt-BEVs across the gut-brain axis and uptake by CNS microglia and neurons. Schematic diagram depicts production of BEVs from the dominant gut gram-negative symbiont, *B. thetaiotaomicron* (Bt) in the intestinal lumen and their uptake and transport across the intestinal epithelium and BBB. BEVs are spherical phospholipid bilayer vesicles, produced from budding of the outer membrane of the gram-negative bacterium, composed of a LPS-rich outer leaflet and phospholipid inner leaflet. BEVs can contain a variety of bacterial products such as nucleic acids, proteins and peptidoglycan fragments. In this study, *in vitro* cell culture systems were utilised to model the gut-brain axis and show that Bt-BEVs are able to transmigrate across gut epithelial and CNS blood vessel endothelial barriers, modelled by Caco-2 and hCMEC/D3 cell monolayers, respectively, where they are taken up by immature neurons (modelled by non-differentiated SH-SY5Y cells) and induce immunomodulatory effects in microglia (modelled by BV-2 cells).

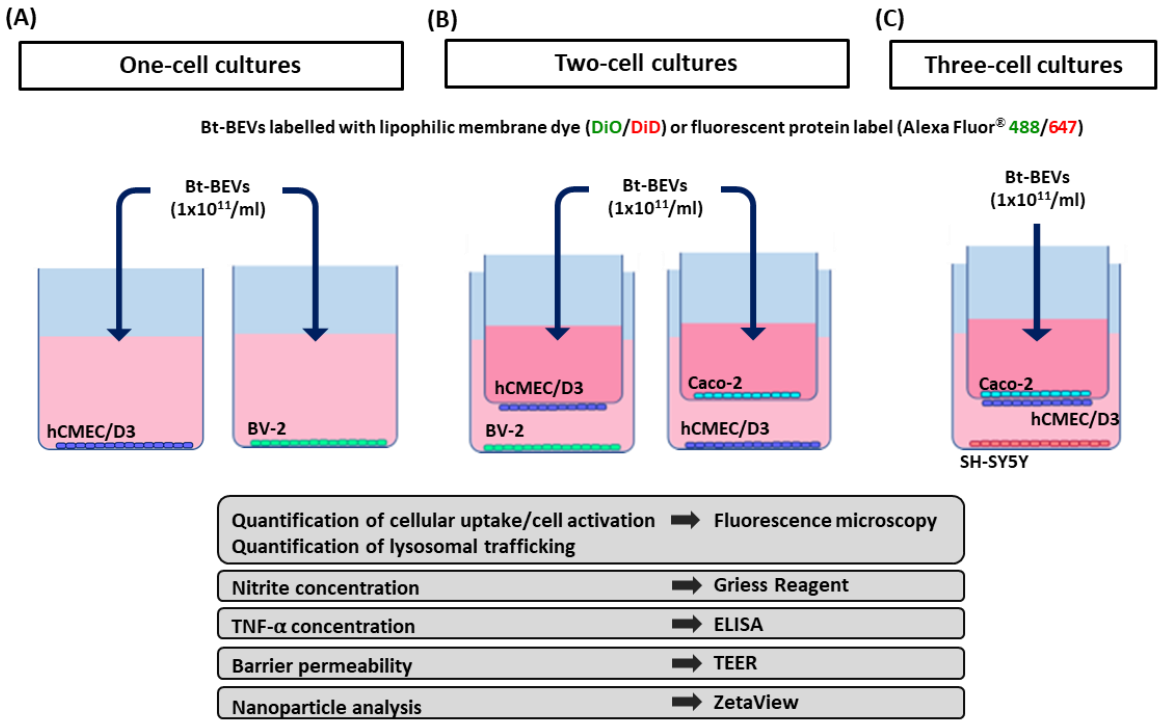

Figure 3.3 Experimental outline of *in vitro* one-, two- and three-cell culture systems to assess uptake and transport of Bt-BEVs across the gut-brain axis. (A) One-cell cultures with hCMEC/D3 modelling CNS blood vessel endothelium and mouse microglia BV-2 cells. (B) Two-cell ThinCert™ insert culture systems using Caco-2 cells and hCMEC/D3 cells modelling the gut-brain axis, and hCMEC/D3 and BV-2 cells to model transport across the BBB and interaction with cells in the CNS. (C) Three-cell culture ThinCert™ systems with Caco-2, hCMEC/D3 and SH-SY5Y cells to model transport across the gut-brain axis and interaction with CNS neurons. Bt-BEVs ($1x10^{11}$/ml) labelled with lipophilic dyes (DiO/DiD) or Alexa Fluor® fluorescent protein labels (AF488/647) were added to cell culture systems for 24 hrs (37°C, 5% CO_2). To assess cellular responses to Bt-BEVs, fluorescent microscopy, Griess Reagent, ELISA, TEER measurements and ZetaView analysis were conducted to quantify cellular uptake/activation, oxidative stress, inflammatory cytokine secretion (TNF-α), barrier permeability and nanoparticle analysis, respectively.

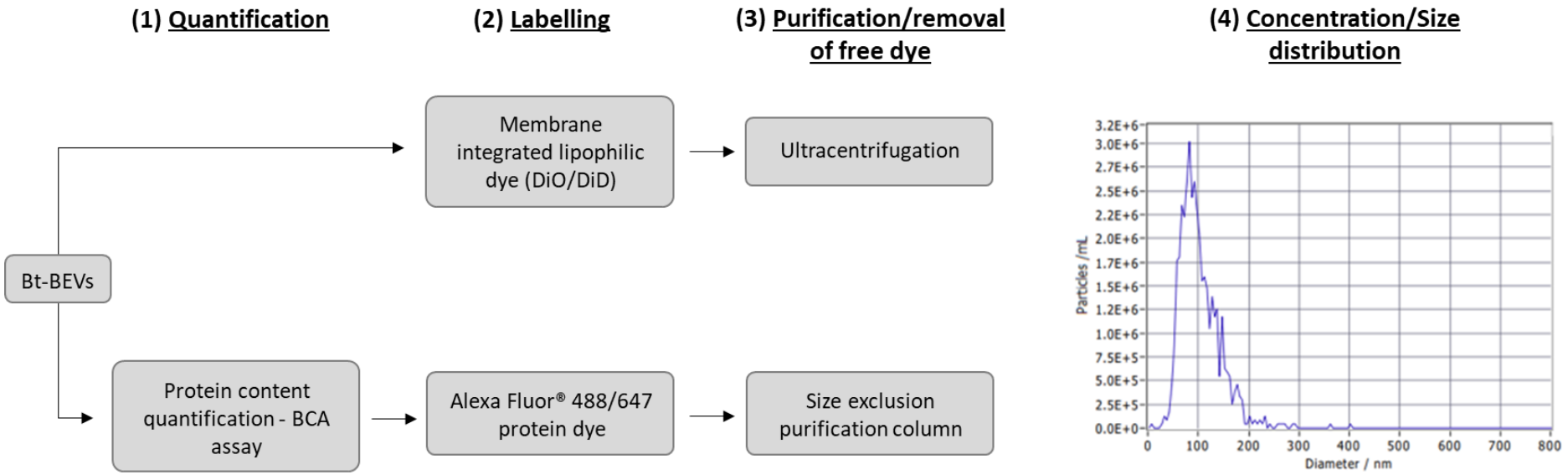

Figure 3.4 Bt-BEV labelling workflow. Typical workflow of labelling with lipophilic membrane integrated dyes (DiO/DiD) and Alexa Fluor® 488/647 protein label covers the following steps: (1) Prior to fluorescent protein labelling, quantification of total protein content of Bt-BEV sample and dilution to 2 mg/ml. (2) Incubation with either lipophilic dye (DiO/DiD, 37°C, 30 min) or Alexa Fluor® 488/647 reactive protein dye (21°C, 1 hr). (3) Removal of free dye by ultracentrifugation (100 kDa, Merck UFC510024) or through size exclusion purification (SEP) column (Bio-Rad BioGel P-30 fine SEP resin, MW > 40,000) for lipophilic and protein labelling, respectively. (4) ZetaView nanoparticle tracking for concentration and size distribution analysis. Labelled BEVs, with the majority of vesicles ranging between 50-200 nm in size. Bt-BEVs were adjusted to $1x10^{11}$/ml accordingly prior to use in experimental assays. Histogram from a ZetaView analysis report represents a typical outcome of the concentration-weight distribution of Bt-BEVs in samples.

effects on immature neurons, modelled by non-differentiated SH-SY5Y cells (Fig. 3.3C). Experimental parameters such as cellular uptake, oxidative stress, inflammatory responses, barrier permeability and nanoparticle analysis were conducted using fluorescence microscopy, Greiss Reagent, ELISA, transepithelial electrical resistance (TEER) and ZetaView analysis, respectively.

3.3. Results

For the studies conducted in this Chapter, Bt-BEVs were isolated from cultures, using protocols developed by Stentz et al., (Stentz, Osborne et al. 2014) collected at a single time point, yielding OD_{600} between 1.5-2.5. Here, all Bt-BEVs presented at a range of 50-200 nm, and where concentrated to $1x10^{12}$/ml prior to labelling with lipophilic membrane integrated dyes (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiD) and 3,3-dioctadecyloxacarbocyanine perchlorate (DiO)). Prior to labelling with Alexa Fluor® (AF488/AF647) protein dye, the total protein content of Bt-BEVs was first determined and diluted to 2 mg/ml (Fig 3.4).

3.3.1. Bt-BEVs are internalised by CNS blood vessel endothelial cells

To explore whether BBB endothelial cells can acquire Bt-BEVs, human cerebromicrovascular endothelial cells (hCMEC/D3) were used to model CNS blood vessel endothelial cells. Cells seeded at a density of approximately $6.0x10^4$ cells/ml formed confluent monolayers within 4-5 days on collagen coated 12-well chamber slides. Cells were then incubated with DiD- or AF647-labelled Bt-BEVs ($1x10^{11}$/ml) or PBS for 24 hrs prior to counterstaining with phalloidin-488 and Hoechst 33342 to visualise intracellular membranes and nuclei, respectively. Following incubation, both DiD- and AF647-labelled Bt-BEVs were observed in the cytoplasm of hCMEC/D3 cells by confocal microscopy, indicating that Bt-BEVs were internalised by hCMEC/D3 cells (Fig. 3.5A, B). No 647 nm fluorescence signal was observed in PBS-treated cells (Fig. 3.5C). To assess differences in BEV uptake when labelled with either the lipophilic dye ($n=5$) or fluorescent protein dye ($n=5$), the number of total Bt-BEV$^+$ cells were counted from a minimum of 20 field of view (FOV) images. The total percentage of Bt-BEV$^+$ cells per FOV was significantly ($p<0.01$) higher when incubating cells with AF647-labelled Bt-BEVs compared to DiD-labelled Bt-BEVs (Fig. 3.5D; 36.24 ± 2.08 % and 21.37 ± 1.41 %, respectively).

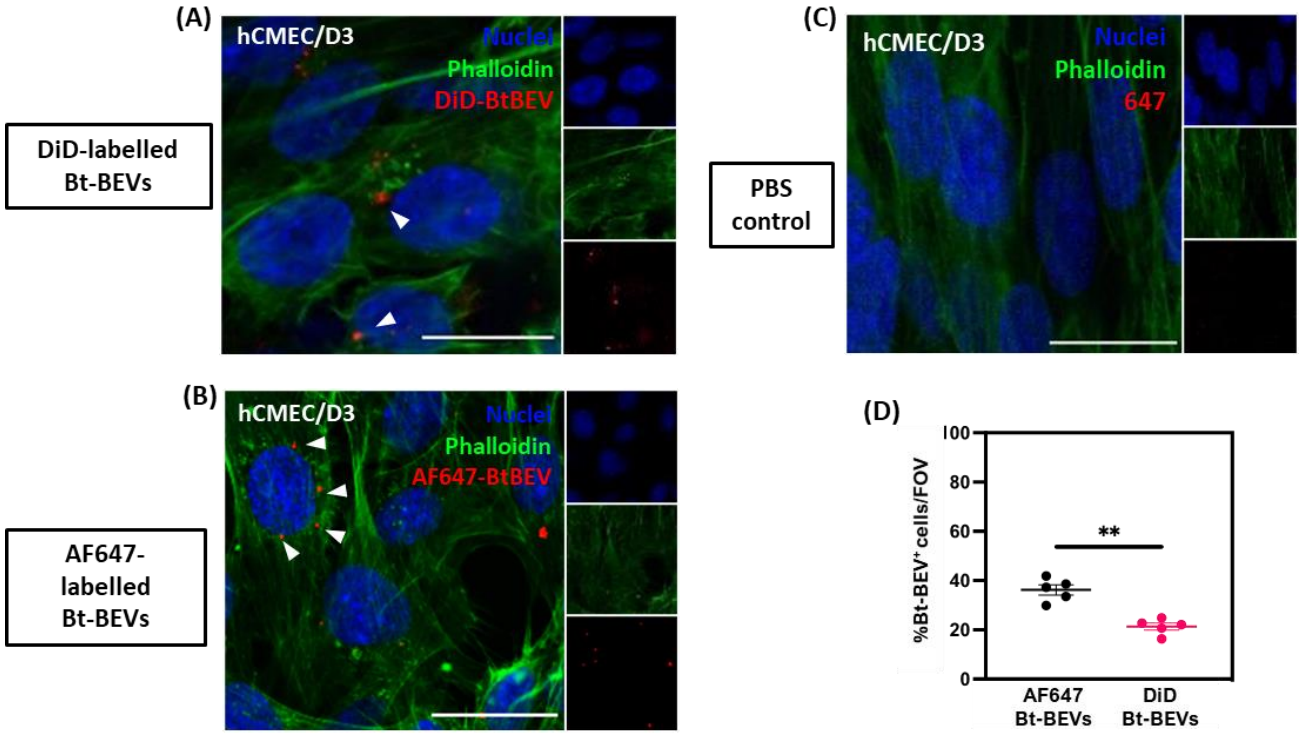

Figure 3.5 Bt-BEVs are acquired by CNS blood vessel endothelial cells. hCMEC/D3 cell monolayers were incubated with Bt-BEVs ($1x10^{11}$/ml) labelled with either (A) DiD (red), (B) AF647 (red) or (C) PBS for 24 hrs (37°C, 5% CO_2). Cells were fixed and stained with phalloidin-488 (green) to visualise intracellular membranes and nuclear stain Hoechst 33342 (blue) prior to imaging. White arrowheads indicate Bt-BEV internalisation. (D) Quantitative analysis of Bt-BEV$^+$ hCMEC/D3 cells following 24 hr incubation with AF647-labelled (*n=5*) or DiD-labelled (*n=5*) Bt-BEVs. Images taken on confocal microscope (63x/1.4 oil DIC objective). Scale bars = 25 µm. Data is shown as percentage of Bt-BEV$^+$ cells per field of view (FOV) and is represented as mean ± SEM. A minimum of 20 FOV images each were used for analysis. Statistical significance was analysed using Mann-Whitney test (GraphPad Prism 9.2.0), with $p<0.05$ considered statistically significant; **$p<0.01$.

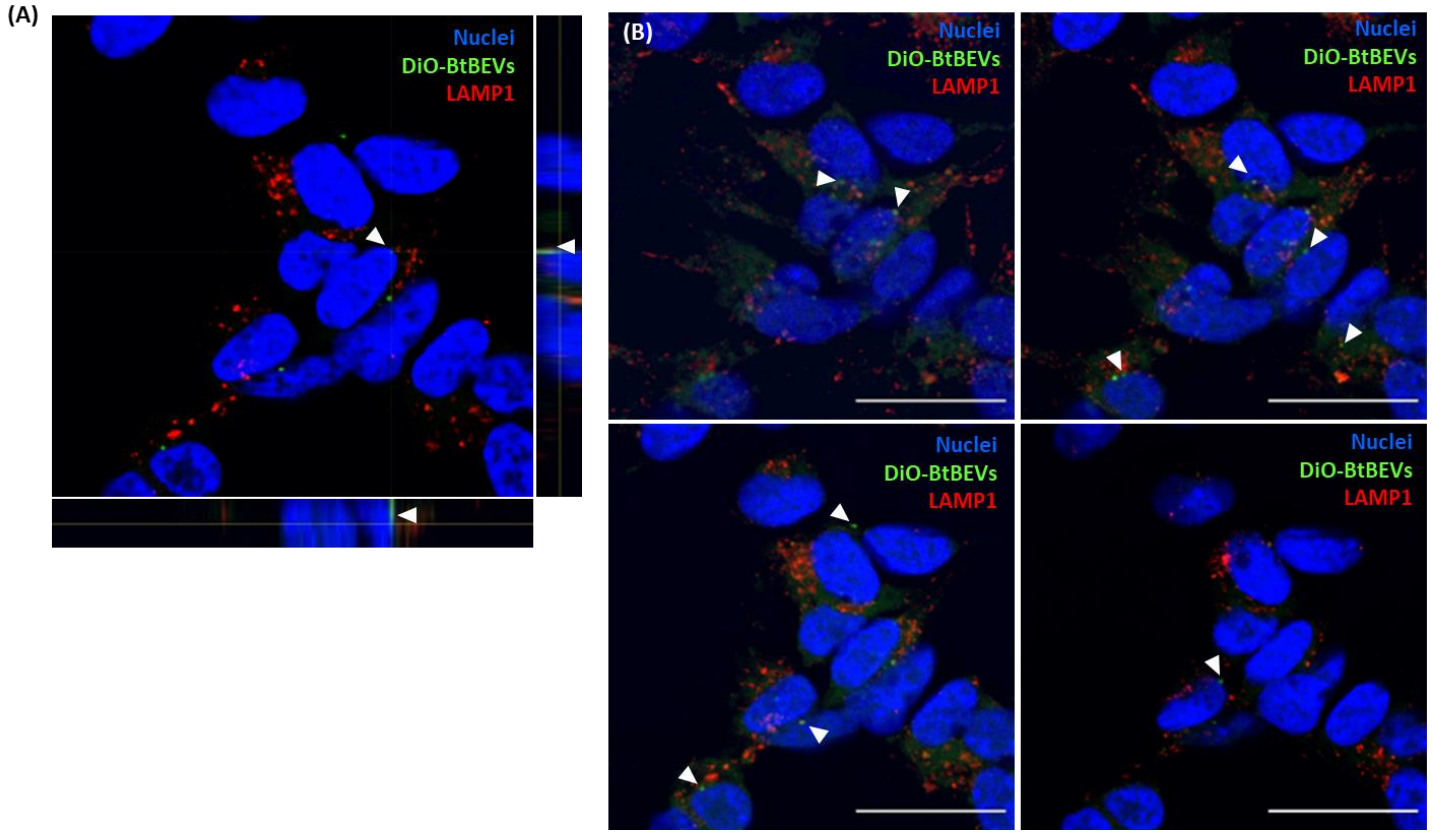

Figure 3.6 Acquired Bt-BEVs are detected in various focal planes in cells in CNS blood vessel endothelial cells. hCMEC/D3 monolayers incubated with DiO Bt-BEVs ($1x10^{11}$/ml) for 24 hrs (37°C, 5% CO_2) then fixed and co-stained with anti-LAMP1 (red) and nuclear stain Hoechst 33342 (blue). (A) Z-stack demonstrating the wide intracellular distribution of acquired Bt-BEVs. Arrowheads indicate acquired Bt-BEV (green) in different focal planes. (B-E) Photomicrographs demonstrating different number of identifiable Bt-BEVs (green, indicated by white arrowheads) at different singular focal planes, as used for field of view (FOV) imaging and subsequent quantification. Images taken on confocal microscope (63x/1.4 oil DIC objective). Scale bars = 25 μm.

Another point to consider when quantifying cellular internalisation of BEVs, is the method used to detect intracellular uptake. Confocal microscopy of hCMEC/D3 cells incubated with DiO-labelled Bt-BEVs for 24 hrs, revealed that the acquired Bt-BEVs were detected in various focal planes consistent with a wide intracellular distribution (Fig. 3.6). Z-stacks revealed underlying Bt-BEVs "hidden" from view in the current focal plane (Fig. 3.6A). This is important to consider when quantifying BEV uptake from images taken at a single focal plane, as they may not accurately reflect the "true" *in vitro* situation (Fig. 3.6B).

Proximity of Bt-BEVs to the nucleus were observed in hCMEC/D3 endothelial cells (Fig. 3.5A and B and Fig. 3.7), although this was not quantitatively analysed. To determine the intracellular trafficking of internalised Bt-BEVs in BBB endothelial cells, hCMEC/D3 cells were incubated with AF488-labelled Bt-BEVs ($1x10^{11}$/ml) for 24 hr and counterstained with nuclear stain Hoechst 33342 and anti-LAMP1, a marker of intracellular lysosomes. Imaging and quantification of colocalised Bt-BEVs revealed that 36.70 ± 3.47% of internalised Bt-BEVs localised to lysosomes in hCMEC/D3 (Fig. 3.7) suggesting that some Bt-BEVs are sequestered by the endo-lysosomal pathway.

3.3.2. Activation of microglia cells by Bt-BEVs

The murine microglia cell line (BV-2) was used to model CNS microglia cells and were plated at a seeding density of approximately $1x10^5$ cells/ml. AF488-labelled Bt-BEVs ($1x10^{11}$/ml) or PBS were added to cell cultures and incubated for 24 hrs. Imaging by confocal microscopy showed that BV-2 cells acquired Bt-BEVs (Fig. 3.8A) and were trafficked to intracellular lysosomes, as demonstrated by co-staining with anti-LAMP1, a marker for lysosomal compartments (Fig. 3.8B).

Upon detection of stimuli, microglia change phenotypes, differentiating to activated microglia and in doing so undergo morphological changes involving reorganisation of F-actin cytoskeleton that facilitates membrane ruffling and migration in microglia

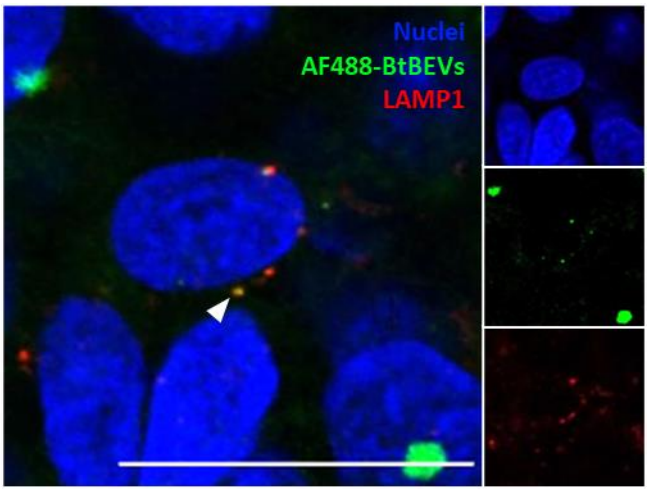

Figure 3.7 Bt-BEVs co-localised to lysosomes in CNS blood vessel endothelial cells. Following 24 hr incubation with AF488-labelled Bt-BEVs (green) (37°C, 5% CO_2), hCMEC/D3 cells were fixed and co-stained with anti-LAMP1 (red) and Hoechst 33342 (blue) to show intracellular trafficking of Bt-BEVs to hCMEC/D3 cell lysosomes (white arrowhead). Images taken on confocal microscope (63x/1.4 oil DIC objective). Scale bars = 25 µm. Data shown shows the percentage of acquired Bt-BEVs co-localised to intracellular lysosomes and is represented as mean ± SEM. A minimum of 20 FOV images each were used for analysis.

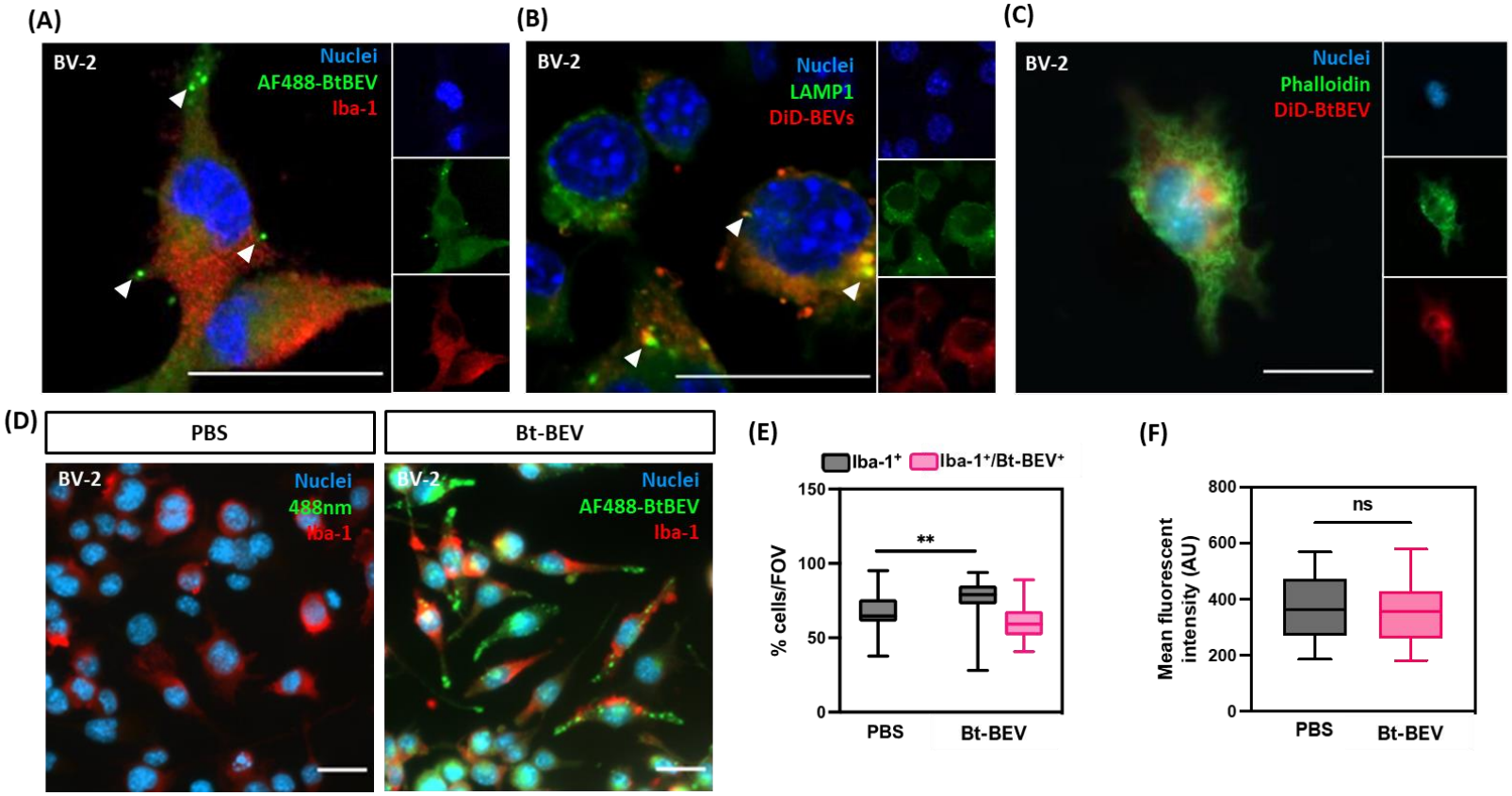

Figure 3.8 Bt-BEVs are acquired by BV-2 cells and induce microglia activation. AF488-labelled or DiD-labelled Bt-BEVs ($1x10^{11}$/ml) were added to BV-2 cell cultures for 24 hrs (37°C, 5% CO_2). Cells were then fixed and co-stained with anti-Iba-1, anti-LAMP1 and phalloidin-488 prior to imaging. (A) AF488-labelled Bt-BEVs (green) are internalised by BV-2 cells (white arrowheads) counterstained with anti-Iba-1 (red) and nuclear stain Hoechst 33342 (blue). Image obtained from confocal microscopy (63x/1.4 oil DIC objective). (B) Co-localisation of acquired DiD-labelled Bt-BEV (red) with lysosomes (anti-LAMP1, green), indicated by white arrowheads and nuclear stain Hoechst 33342 (blue). Image obtained from confocal microscopy (63x/1.4 oil DIC objective). (C)

Microglia membrane ruffling in DiD-labelled Bt-BEV (red) incubated BV-2 cells identified by counterstaining with phalloidin-488 (green), nuclear stain Hoechst 33342 (blue) prior to imaging by widefield fluorescence microscope (40x/objective). (D) Microglia activation from incubation with AF488-labelled Bt-BEVs or PBS assessed by co-staining with anti-Iba-1 (red). Image taken by widefield fluorescence microscope (40x/objective). (E) Quantitative analysis showing percentage of total Iba-1^+ cells and Iba-1^+ Bt-BEV^+ cells from field of view (FOV) images from PBS and Bt-BEV treated BV-2 cells. (F) Mean fluorescent intensity of Iba-1^+ staining in BV-2 cells incubated with PBS or Bt-BEVs. Analysis conducted from minimum of 20 FOV images. The box plots represents first quartile, median and third quartile, with whiskers representing minimum and maximum. Statistical significance analysed using Mann-Whitney test (GraphPad Prism 9.2.0), with $p<0.05$ considered statistically significant;**$p<0.01$, ns – not significant. Scale bars = 25 μm.

(Kanazawa, Ohsawa et al. 2002, Colton and Wilcock 2010, Lannes, Eppler et al. 2017, Gheorghe, Deftu et al. 2020). Here membrane ruffling was visualised by counterstaining BV-2 cells exposed to DiD-labelled Bt-BEVs ($1x10^{11}$/ml) for 24 hrs with phalloidin-488, a marker for intracellular membranes. This morphological phenotype is characterised by retraction of long multidirectional cellular projections and "cupping" of the membrane, as visualised by widefield fluorescent microscopy (Fig. 3.8C).

To assess the activation status of microglia cells, BV-2 cells were incubated with AF488-labelled Bt-BEVs (*n=5*) or PBS (*n=3*) for 24 hrs and counterstained with an anti-Iba-1 antibody (Fig. 3.8D). Quantification of Iba-1^+ cells per FOV showed that Bt-BEVs induced a significant ($p<0.01$)increase in Iba-1^+ cells compared to PBS-treatment (Fig. 3.8E; Bt-BEV 79.82 ± 1.56% compared to PBS 66.6 ± 2.65%). However, when measuring mean fluorescent intensity of Iba-1 immunostaining, no significant differences were observed in levels of Iba-1 staining between Bt-BEVs (374.92 ± 15.3 AU) and PBS (358.74 ± 17.67 AU) treated BV-2 cells (Fig. 3.8F), indicating increased number of Iba-1 cells but no change in levels of cellular Iba-1 expression.

3.3.3. Bt-BEVs induce proinflammatory responses in microglia

The immunomodulatory effects of Bt-BEVs on BV-2 cells were further explored by measuring extracellular secretion of TNF-α in the cell culture media following 24 hr incubation. The results showed that Bt-BEVs (*n=5*) significantly ($p<0.01$) increased TNF-α secretion in BV-2 cells compared to PBS (*n=3*) (Fig. 3.9A; 530.13 ± 63.9 pg/ml compared to 38.76 ± 9.86 pg/ml, respectively). Nitrite (NO_2^-) is the terminal product of NO oxidation and is used as a measure for NO production. Measurement of nitrite in the cell culture media using the Greiss Reagent revealed significantly ($p<0.001$) increased nitrite concentration in Bt-BEV (*n=9*) treated BV-2 cells compared to PBS (*n=7*) control (Fig. 3.9B; 6.67 ± 1.85 μM compared to 1.26 ± 0.28 μM, respectively). These results indicate that Bt-BEVs induced an inflammatory response in BV-2 cells following 24 hr exposure.

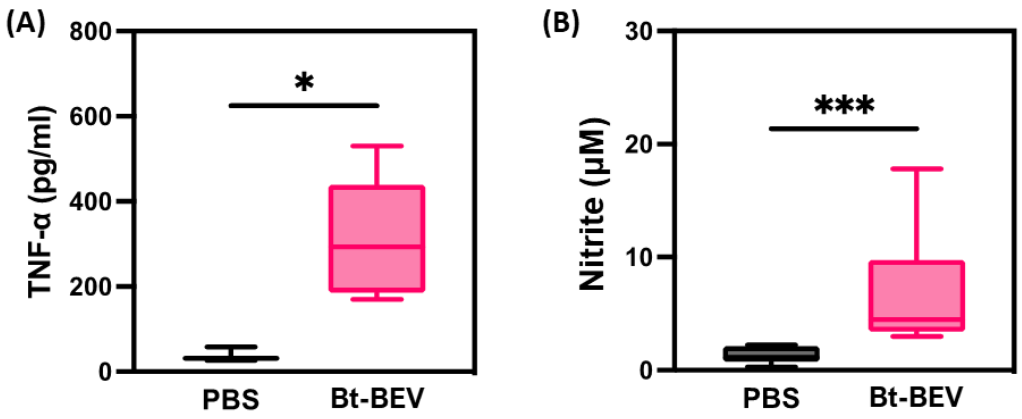

Figure 3.9 Bt-BEVs induce inflammatory responses in microglia. (A) TNF-α concentrations (pg/ml) in BV-2 culture media following 24 hr incubation with Bt-BEVs ($1x10^{11}$/ml) (*n=5*) or PBS (*n=3*) determined by ELISA. (B) Nitrite concentrations (μM) in BV-2 cell culture media following 24 hr incubation with Bt-BEVs (*n=9*) or PBS (*n=7*) measured by Griess Reagent. The box plots represents first quartile, median and third quartile, with whiskers representing minimum and maximum. Data is from two separate experiments. Statistical significance analysed using Mann-Whitney test (GraphPad Prism 9.2.0), with a value of $p<0.05$ considered statistically significant; *$p<0.05$, ***$p<0.001$.

3.3.4. Bt-BEVs cross the gut epithelial barrier and are acquired by CNS blood vessel endothelial cells

BEVs can cross the host intestinal epithelium and reach target cells in systemic tissue (Stentz, Carvalho et al. 2018, Jones, Booth et al. 2020). To explore this further in a simple reductionist *in vitro* system, a two -cell culture system using ThinCert ™ cell culture inserts to model the gut-brain axis was used. Caco-2 cells, modelling the gut epithelial barrier, were cultured to confluence on polyethylene terephthalate (PET) membrane of the ThinCert ™ inserts and assembled with confluent hCMEC/D3 monolayers cultured on collagen-coated glass coverslips (Fig. 3.3B). The apical compartment and basal compartment modelled the *in vivo* gut lumen and mucosal/blood circulatory system (Fig. 3.3B). The porous PET membrane of the ThinCert ™ cell culture inserts (pore diameter 0.4 μM) provided the structural support for Caco-2 cells yet allowed diffusion of Bt-BEVs (>400 nm) across the membrane. Upon addition of Bt-BEVs in the apical (luminal) compartment, the epithelial barrier function was assessed by measuring the TEER. The permeability of Caco-2 cell monolayers was significantly and transiently increased upon exposure to Bt-BEVs (*n=6*) demonstrated by reduced TEER measurements compared to PBS (*n=3*) treated cells within 1 hr ($p<0.001$) and subsequent restoration of baseline levels by 24 hrs (Fig. 3.10A). Following incubation, media from the apical and basal compartment were analysed for nano-particles. Using the nano-particle tracking analysis (ZetaView), higher numbers of particles were detected in the apical compartment from Bt-BEV treated two-cell culture system compared to controls composed of a blank ThinCert™ insert, indicating robust barrier integrity of Caco-2 cells (Fig. 3.10B). No particles were observed in the PBS group (Fig. 3.10B).

Having established Bt-BEVs can transmigrate gut epithelial cell monolayers, the uptake of Bt-BEVs by hCMEC/D3 CNS blood vessel endothelial cells was assessed by co-staining with anti-LAMP1. Fluorescent microscopy revealed uptake and co-localisation of Bt-BEVs with LAMP1 in hCMEC/D3 cells and further quantification demonstrated that 43.13 ± 3.15% of acquired Bt-BEVs were colocalised with LAMP1 in hCMEC/D3 cells (Fig. 3.10C)

3.3.5. Transepithelial resistance of CNS blood vessel endothelial cells is improved by culturing on Matrigel®

TEER values of the BBB *in vivo* have been estimated to exceed 1000 Ωcm^2 (Crone and Olesen 1982, Butt, Jones et al. 1990). In comparison, *in vitro* BBB model cell-cultures typically have TEER values ranging between 20-200 Ωcm^2, and obtaining TEER values above 1000 Ωcm^2 is difficult to achieve due to high barrier permeability of CNS blood vessel endothelial cell cultures (Eigenmann, Xue et al. 2013).

Collagen, a naturally occurring component of the *in vivo* BBB (Robert and Robert 1998), or the artificial basement matrix, Matrigel® (Kleinman and Martin 2005), can be used as a physical base for culturing hCMEC/D3 cells (Eigenmann, Xue et al. 2013). To improve barrier integrity and confluency of cultured hCMEC/D3 cells, they were seeded at a seeding density of 6.0x10^4/ml on either collagen-coated or Matrigel®-coated ThinCert™ inserts and cultured for 9 days. TEER measurements revealed that hCMEC/D3 cells cultured on Matrigel® (*n=2*) improved barrier permeability in comparison to collagen-coated (*n=2*) and non-coated (*n=2*) ThinCert™. This was particularly apparent at day 3 and 4, in which TEER reached values of approximately 300 Ωcm^2 in Matrigel®-coated cultures (Fig. 3.11A). A reduction in TEER was observed in both Matrigel®-coated and collagen-coated cultures at day-5 (Fig. 3.11A), indicative of hCMEC/D3 cells reaching maximum confluence, despite some detaching from the ThinCert™, most remained attached and high TEER values were sustained. Experiments conducted using hCMEC/D3 cultures were carried out on day 4/5 of culture where maximum confluence had been reached. Of note increased TEER values of cells grown on non-coated matrices were seen from day 7, reaching values comparable with Matrigel®-coated and collagen-coated cultures (Fig. 3.11A).

It has been reported that CNS blood vessel endothelial cells require other cells to achieve maximum barrier integrity BBB (Nakagawa, Deli et al. 2009, Eigenmann, Xue et al. 2013). Therefore, to assess whether mixed-cell cultures can improve BBB integrity, mixed cultures containing hCMEC/D3 and SH-SYSH cells were seeded at equal densities (6x10^4 cells/ml) on non-coated ThinCert™ inserts and TEER measured over 7-days. No significant improvements in permeability were seen during the first 3

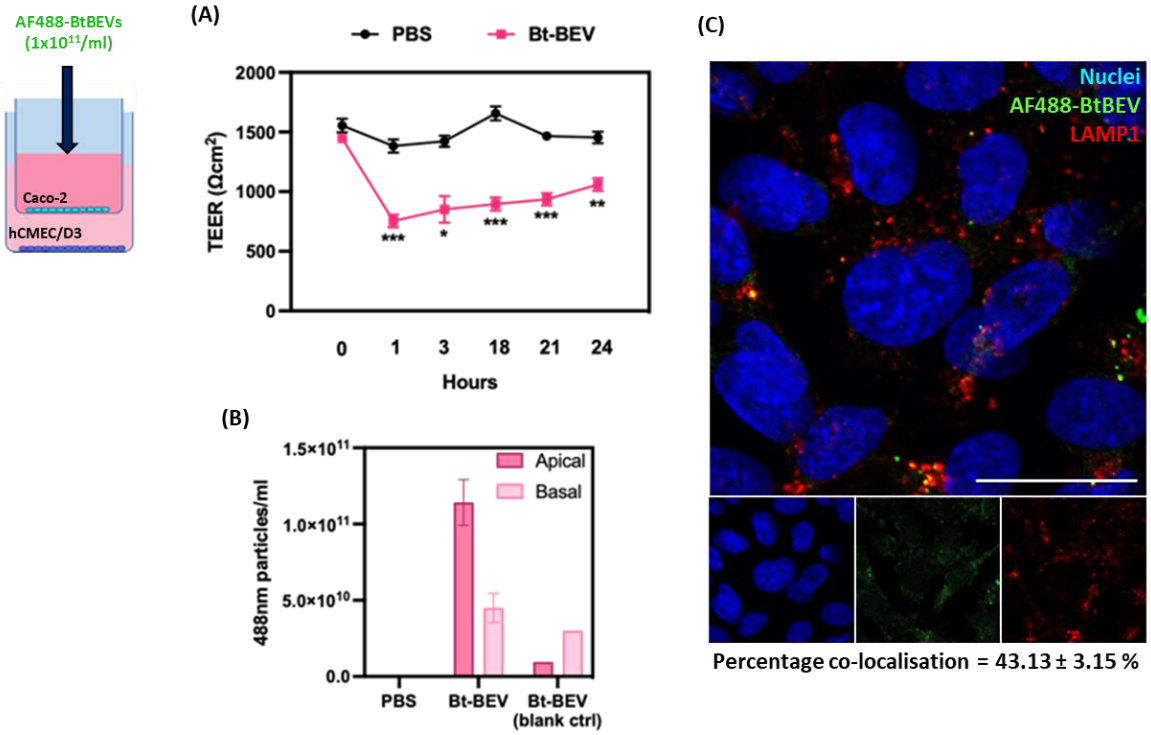

Figure 3.10 Bt-BEVs cross the gut-epithelial cell barrier and are acquired by CNS blood vessel endothelial cells. Caco-2 cells were cultured on ThinCert™ inserts (PET membrane, 0.4 µm) and hCMEC/D3 cells cultured on collagen-coated glass coverslips in basal compartment. AF488-labelled Bt-BEVs ($1x10^{11}$/ml) or PBS were added to co-culture system for 24 hrs (37°C, 5% CO_2). (A) TEER measurements for Caco-2 cell monolayer taken during 24 hrs following Bt-BEV (*n=6*) or PBS (*n=3*) addition. (B) ZetaView particle analysis of 488 nm^+ particles/ml in apical and basal compartments following 24 hr incubation with AF488-labelled Bt-BEVs. (C) Representative photomicrograph of hCMEC/D3 cells following 24 hr Bt-BEV (green) incubation in gut-BBB two-cell culture system. Cells co-stained with anti-LAMP1 (red) and nuclear stain Hoechst 33342 (blue). Image taken on confocal microscope (63x/1.4 DIC oil objective). Scale bar = 25 µm. (D) Quantitative analysis of percentage of total Bt-BEV^+ hCMEC/D3 cells (left Y-axis) and localisation with intracellular lysosomes (right Y-axis). Data are represented as mean ± SEM. A minimum for 20 field of view (FOV) images from 5 biological replicates were used. Statistical significance calculated using the Mann-Whitney test, (GraphPad Prism 9.2.0), with a value of $p<0.05$ considered statistically significant; *$p<0.05$, **$p<0.01$, ***$p<0.001$

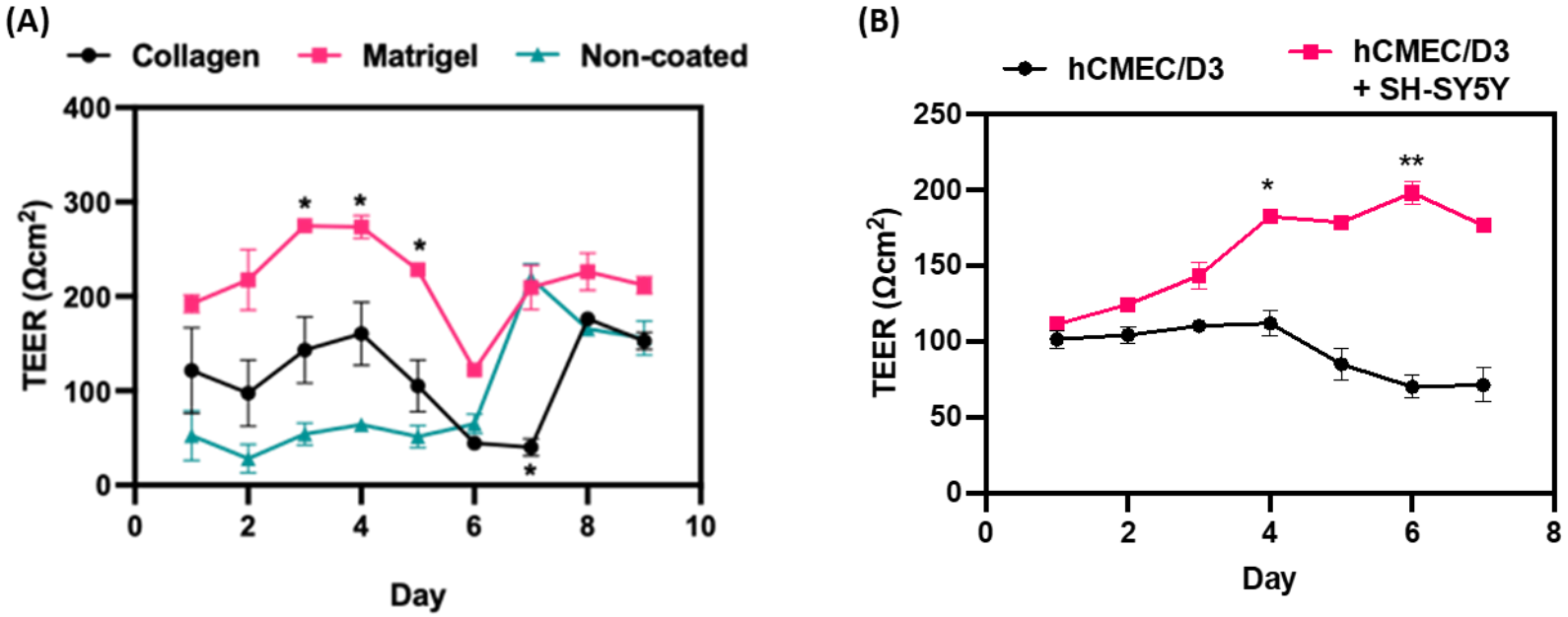

Figure 3.11 Optimisation of transepithelial permeability in an *in vitro* model of BBB. (A) hCMEC/D3 cells were cultured on ThinCert™ inserts (PET membrane, 0.4 µm), coated with either 1:20 collagen (*n=2*), 1:20 Matrigel® (*n=2*) or non-coated (*n=2*) for 9 days. TEER was measured daily. (B) Single-cell hCMEC/D3 cultures (*n=3*) and mixed-cell cultures containing hCMEC/D3 and SH-SY5Y cells (*n=3*) were cultured on non-coated ThinCert™ inserts (PET membrane, 0.4 µm), and TEER measured daily for 7-days. The graphs depicts mean ± SEM. Statistical significance calculated using multiple comparisons from mixed-model analysis (GraphPad Prism 9.2.0), with a value of $p<0.05$ considered statistically significant; *$p<0.05$, **$p<0.01$.

days, but from day-4 higher TEER values were recorded from the mixed-cell (*n=3*) cultures compared to single-cell hCMEC/D3 (*n=3*) cultures, reaching significance at day-4 ($p<0.01$) and day-6 ($p<0.05$) (Fig. 3.11B), indicating that SH-SY5Y cells promoted growth of hCMEC/D3 cells to a higher degree of confluency.

3.3.6. Bt-BEVs cross CNS blood vessel endothelial cells and are acquired by microglia cells

Single-cell cultures of hCMEC/D3 cells represent a simplified BBB model and do not fully recapitulate the *in vivo* conditions and it has been suggested that in order to attain a mature phenotype of BBB endothelial cells, other cells in the neurovascular unit (NVU) are required to better mimic the BBB (Nakagawa, Deli et al. 2009, Eigenmann, Xue et al. 2013). Therefore, other cell types of the NVU were incorporated into two-cell culture systems with hCMEC/D3 cells (Fig. 3.3B).

Firstly, hCMEC/D3 cells seeded at a density of 6.0×10^4 cells/ml on the underside of collagen-coated, Matrigel®-coated, or non-coated ThinCert™ inserts reached confluence within 4-days. The cell culture inserts were then placed in 24-well culture plates with BV-2 cells cultured on glass coverslips in basal compartment. DiO-labelled Bt-BEVs were added to the apical compartment for 24 hr, and BV-2 cells fixed, and counterstained with microglia activation (anti-Iba-1/Fig. 3.12A and anti-CD45/Fig. 3.12C) and microglia cell specific markers (anti-TREM119/Fig. 3.12B). Counterstaining with anti-IgG was used as the isotype control (Fig. 3.12D). Observations indicate that Bt-BEVs in the apical compartment (vascular) migrated

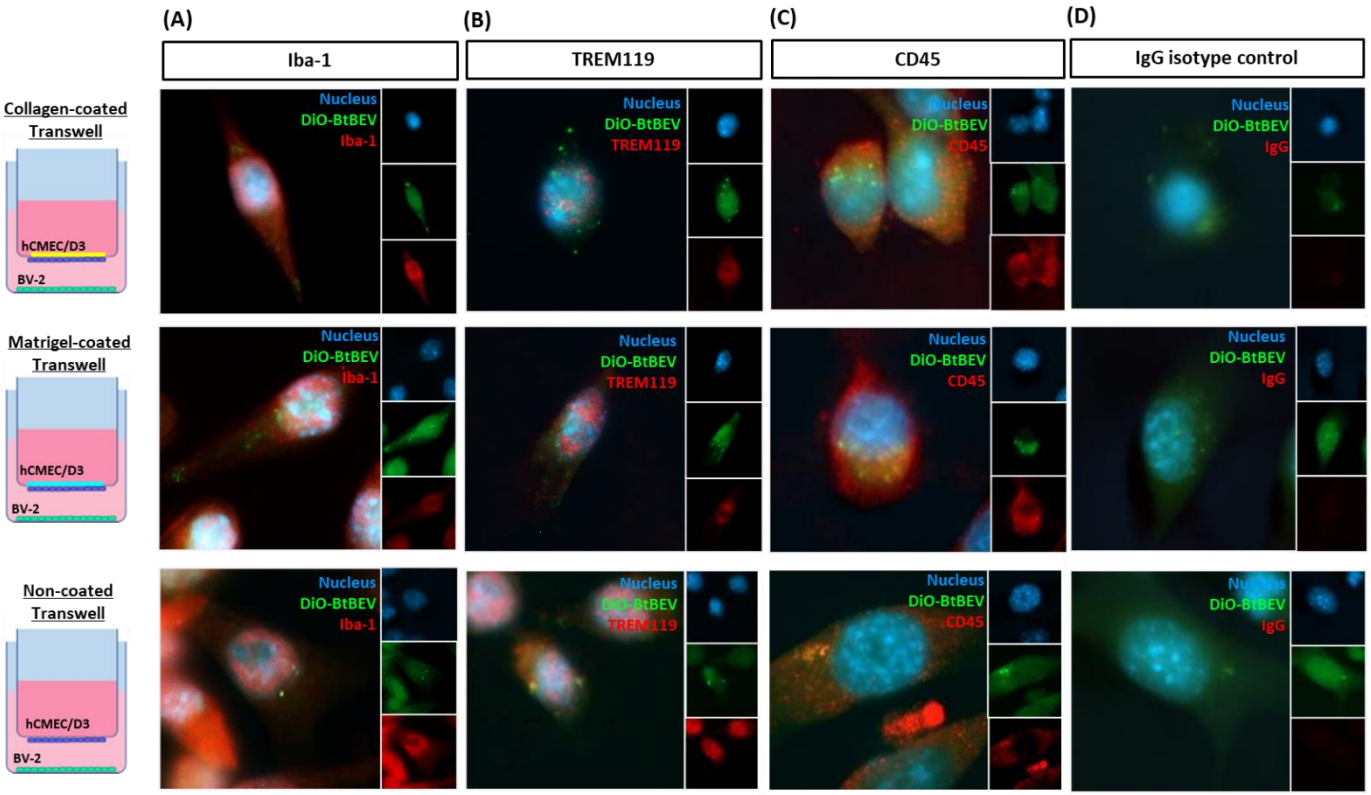

Figure 3.12 Bt-BEVs cross the BBB and are acquired by microglia cells. hCMEC/D3 cells were cultured on the underside of either 1:20 collagen-coated, 1:20 Matrigel®-coated or non-coated ThinCert™ inserts (PET membrane, 0.4 μm) and BV-2 cells cultured on glass coverslips in basal compartment. DiO-labelled Bt-BEVs ($1x10^{11}$/ml) added to co-culture system for 24 hrs (37°C, 5% CO_2). Representative images of BV-2 cells fixed and co-stained with (A) anti-Iba-1 (red), (B) anti-TREM119 (red), (C) anti-CD45 (red) or (D) anti-IgG isotype control (red) and nuclear stain Hoechst 33342 (blue). Images taken on widefield fluorescence microscope (40x/objective).

across hCMEC/D3 monolayer, where they were acquired by BV-2 cells in the basal compartment (parenchyma).

3.3.7. Phenotype of microglia cells acquiring Bt-BEVs

There are some reports that Iba-1 expression is not always associated with activated microglia (Park, Chen et al. 2016, Zhu, Liu et al. 2017). CD45, another marker for activated microglia (Ford, Goodsall et al. 1995, Townsend, Vendrame et al. 2004) was used to confirm microglia activation (Fig. 3.12 and 3.13).

Using TREM119, CD45 and Iba-1 to phenotype microglia cells, the number of $TREM119^{+}$ $Bt\text{-}BEV^{+}$ cells was higher in collagen and Matrigel®-coated ThinCert™ cell culture inserts (Fig. 3.13A; 21.79 ± 2.03% and 24.44 ± 2.19%, respectively) compared to non-coated ThinCert™ (Fig. 3.13A; 14.38 ± 1.35%). The opposite was observed in quantification of $CD45^{+}$ $Bt\text{-}BEV^{+}$ cells, with higher numbers seen in non-coated ThinCert™ cell culture inserts (30.05 ± 3.21%). The number of $Iba\text{-}1^{+}$ $Bt\text{-}BEV^{+}$ were higher in collagen-coated ThinCert™ cell culture inserts (42.98 ± 2.91%).

3.3.8. Increased CNS blood vessel endothelial cell barrier permeability results in elevated microglia inflammatory responses

Analysis of nano-particles in the apical and basal compartments following 24 hr incubation with AF488-labelled Bt-BEVs provided further evidence of BBB barrier permeability and transendothelial migration of Bt-BEVs. Fewer particles were present in the basal compartment of Matrigel®-coated two -cell system ($4.13 \times 10^{10} \pm 1.43 \times 10^{10}$ particles/ml), compared to collagen-coated, (Fig. 3.13B; $8.67 \times 10^{10} \pm 7.88 \times 10^{10}$ particles/ml), indicating hCMEC/D3 monolayers cultured on Matrigel® have lower permeability and higher barrier function in line with the TEER measurements (Fig. 3.11A). Furthermore, reduced number of nano-particles were detected in apical compartment of non-coated two -cell cultures ($6.15 \times 10^{10} \pm 3.23 \times 10^{10}$ particles/ml) compared to basal compartment ($7.00 \times 10^{10} \pm 0.00$ particles/ml) indicating increased hCMEC/D3 monolayer permeability. A blank ThinCert™ insert was used as a control to measure the maximum level of Bt-BEV diffusion across the PET membrane (Fig. 3.13B).

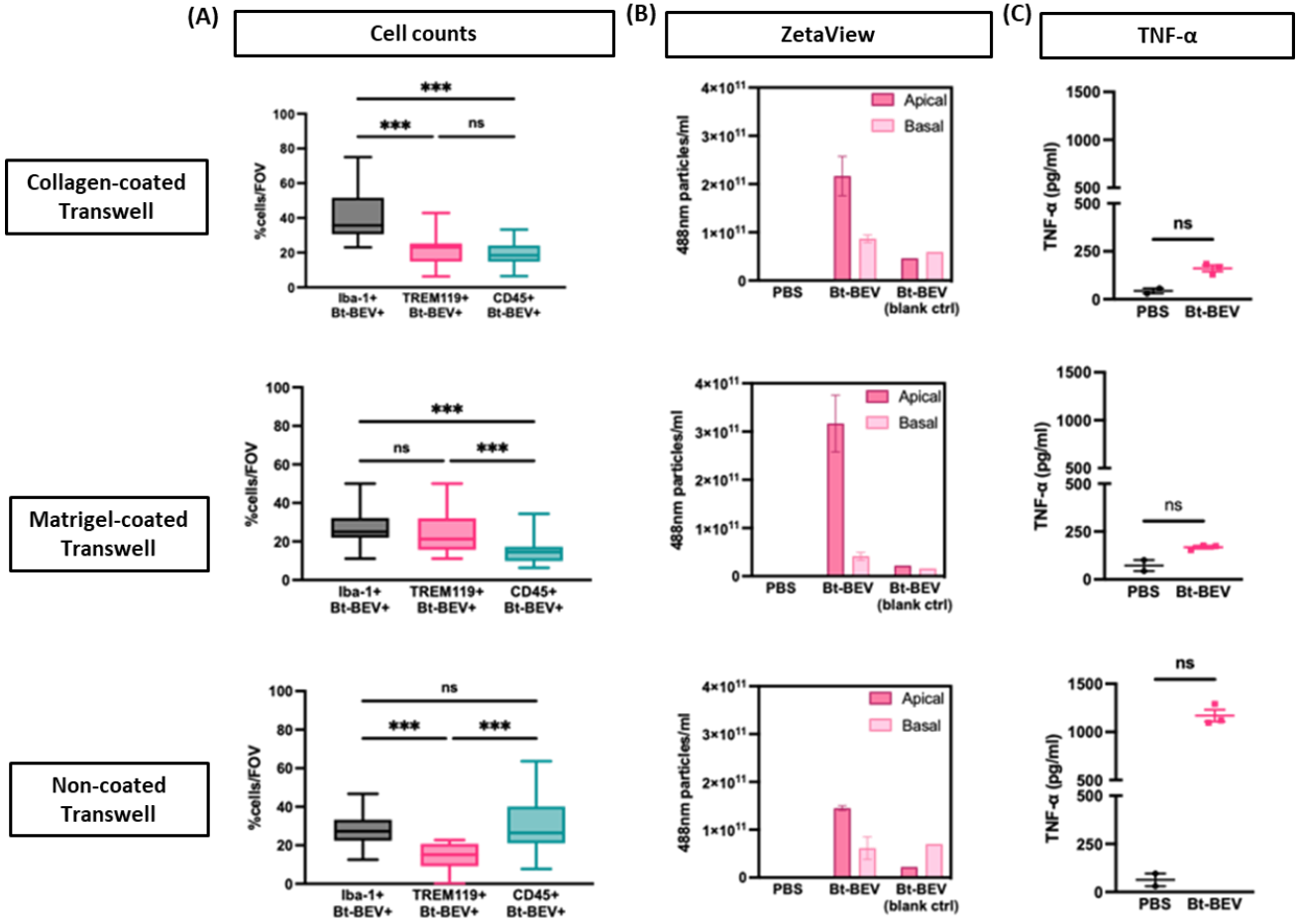

Figure 3.13 Increased transport of Bt-BEVs across brain blood endothelial cells results in increased microglia activation upon exposure to Bt-BEVs. hCMEC/D3 cells were cultured on the underside of either 1:20 collagen-coated, 1:20 Matrigel®-coated or non-coated ThinCert™ insert (PET membrane, 0.4

µm) and BV-2 cells cultured on glass coverslips in basal compartment. DiO-labelled Bt-BEVs ($1x10^{11}$/ml) (*n=2/3*) or PBS (*n=1*) were added to the two-cell culture system for 24 hrs (37°C, 5% CO_2). (A) Quantification of Bt-BEV$^+$ Iba-1$^+$, TREM119$^+$ and CD45$^+$ BV-2 cells following Bt-BEV incubation. Data presented as percentage of total cells per field of view (FOV) from a minimum of 20 images. The box plots represents first quartile, median and third quartile, with whiskers representing minimum and maximum. (B) ZetaView particle analysis of 488 nm$^+$ particles in apical and basal compartment following 24 hr incubation with Bt-BEVs. Data represented as mean ± SEM. (C) TNF-α concentrations (pg/ml) in basal compartment culture media following 24 hr incubation with Bt-BEVs (*n=3*) or PBS (*n=1*) determined by ELISA. Data represented as mean ± SEM. Statistical significance for cell counts (A) determined using Kruskal-Walis test with Dunn's multiple comparison and for TNF-α concentrations (C) the Mann-Whitney test, (GraphPad Prism 9.2.0), a value of $p<0.05$ was considered statistically significant; ***$p<0.0001$, n.s. not significant

Next, TNF-α production in the two -cell culture systems following 24 hr exposure to Bt-BEVs was measured. A 10-fold increase TNF-α production by BV-2 cells was measured in the non-coated ThinCert™ system compared to collagen-coated and Matrigel®-coated transwell system (Fig. 3.13C; 1169 ± 61.2 pg/ml 161 ± 16.3 pg/ml and 168 ± 7.5 pg/ml, in non-coated, collagen-coated and Matrigel®-coated ThinCert™, respectively), and although these differences did not reach statistical significance, the results indicate that increased BBB permeability can result in increased pro-inflammatory responses upon exposure to Bt-BEVs.

3.3.9. Translocation of Bt-BEVs across the gut epithelium and CNS blood vessel endothelial cell barriers and subsequent uptake in non-differentiated neurons

To explore Bt-BEV translocation to the CNS, a three -cell culture system was established utilising Caco-2 and hCMEC/D3 cells cultured on the upper-side and underside of a ThinCert™ PET 0.4 μm membrane, respectively with non-differentiated SH-SY5Y cells cultured on glass coverslips in the basal compartment of the three-cell system (Fig. 3.3C). In addition to incubation for 24 hrs with DiO/DiD-labelled Bt-BEVs, PBS (control) or LPS (50 ng/ml, positive control) were added to the apical compartments representing the luminal interface of the three-cell culture system.

Bt-BEVs were acquired by SH-SY5Y cells and as seen in hCMEC/D3 and BV-2 cells, were sequestered to endo-lysosomal pathways, identified by co-staining with anti-LAMP1 (Fig. 3.14A). No equivalent fluorescence signal was observed in PBS or LPS treated culture (Fig. 3.14B, C). In comparison to translocation across gut-epithelium and intracellular uptake in hCMEC/D3 cells (Fig. 3.10C; 43.13 ± 3.15 %), a greater proportion of internalised Bt-BEVs were co-localised with lysosomes (Fig. 3.14A; 85.19 ± 41.61 %).

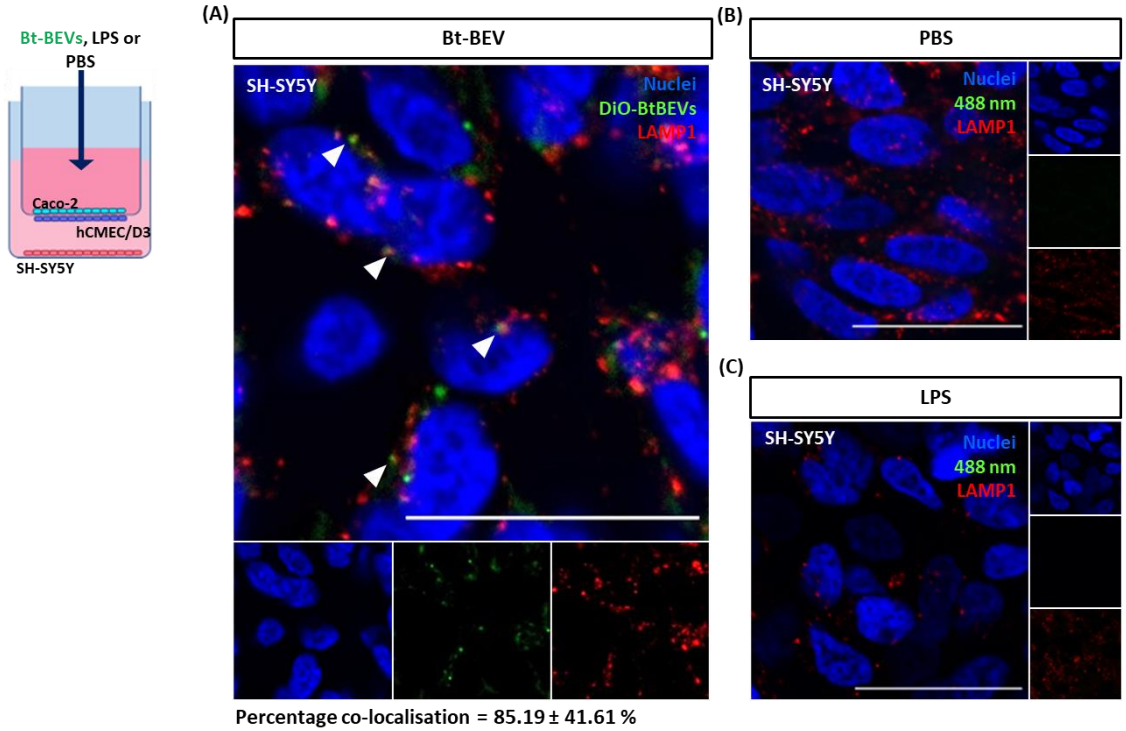

Figure 3.14. Bt-BEVs cross the gut-epithelial and brain endothelial barriers and are trafficked to intracellular lysosomal pathways in non-differentiated neurons. A three-cell culture system was set up with Caco-2 and hCMEC/D3 cells modelling the gut-brain barriers cultured on ThinCert™ inserts (PET membrane, 0.4 µm), and non-differentiated SH-SY5Y cells cultured on fibronectin coated glass-coverslips in the basal compartment. DiO-labelled Bt-BEVs ($1x10^{11}$/ml), PBS or LPS (50 ng/ml) were added to the apical compartment of the three-cell culture system for 24 hrs (37°C, 5% CO_2). SH-SY5Y cells were fixed and co-stained with anti-LAMP1 (lysosomes, red) and nuclear stain Hoechst 33342 (blue). (A) Co-localisation of Bt-BEVs (green) with intracellular lysosomes (red) in SH-SY5Y cells indicated by white arrowheads. (B) PBS and (C) LPS controls showing intracellular lysosomes (red) and absence of fluorescent signal in 488 nm channel. Images taken on confocal microscope (63x/1.4 oil DIC objective). Scale bars = 25 µm.

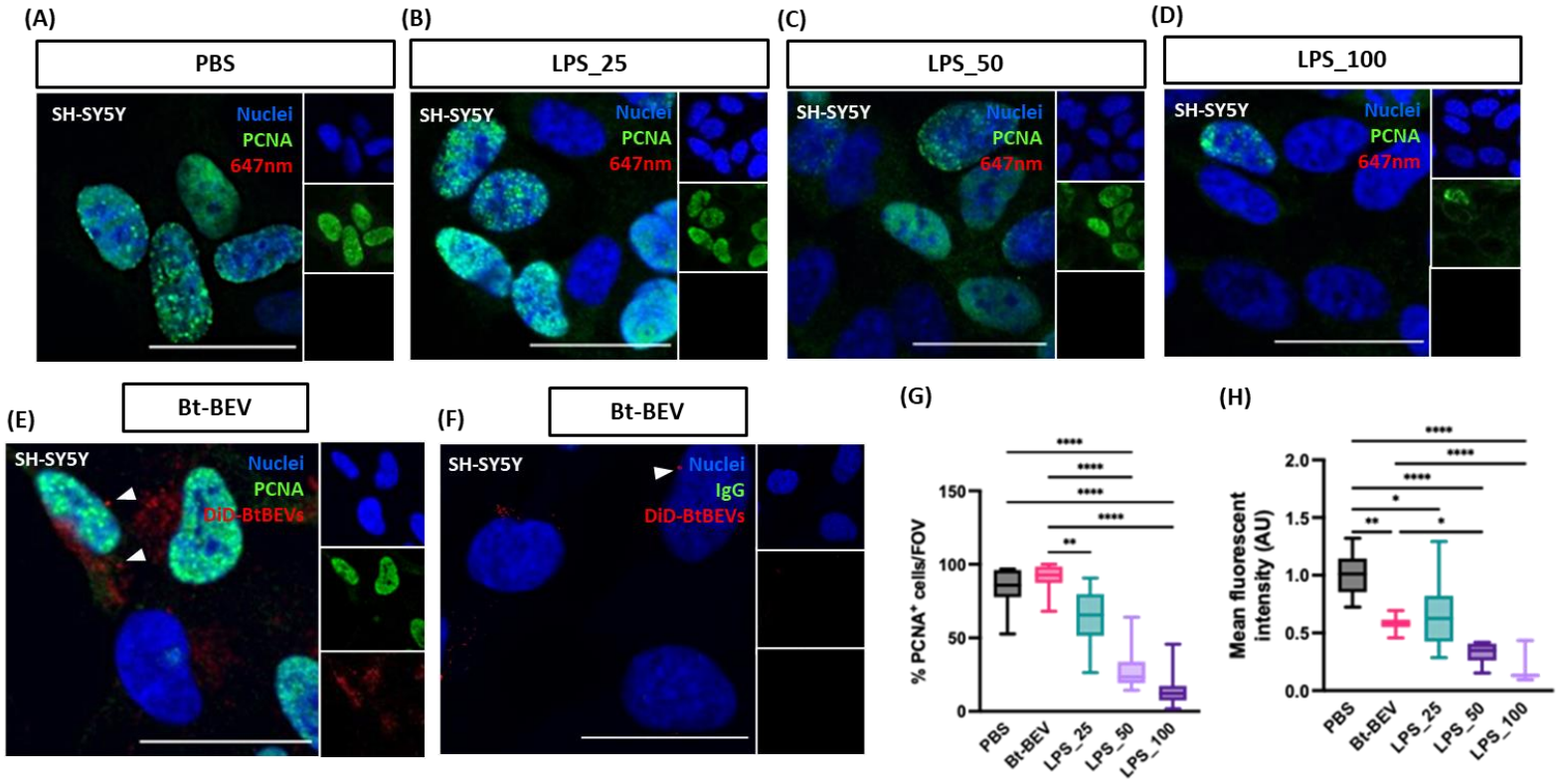

Figure 3.15 LPS, but not Bt-BEVs, alters the proliferative activity of non-differentiated neuronal cells. A three-cell culture system was set up with Caco-2 and hCMEC/D3 cells modelling the gut-brain barriers cultured on ThinCert™ inserts (PET membrane, 0.4 µm) and non-differentiated SH-SY5Y cells cultured on fibronectin coated glass-coverslips in the basal compartment. DiD-labelled Bt-BEVs (*n=2*; $1x10^{11}$/ml), PBS (*n=2*) or LPS (*n=2*; 25 ng/ml, 50 ng/ml, 100 ng/ml) were added to the apical compartment of the three-cell culture system for 24 hrs (37°C, 5% CO_2). SH-SY5Y cells were then fixed and co-stained with anti-PCNA (green), a marker for cell proliferation or anti-IgG (green) and nuclear stain Hoechst 33342 (blue). Representative images of SH-SY5Y cells from (A) PBS, (B) 25 ng/ml LPS, (C) 50 ng/ml LPS, (D) 100 ng/ml LPS and (E-F) SH-SY5Y cells treated with Bt-BEVs (red) in the three-cell culture system and co-stained with anti-PCNA or isotype control IgG antibody (green). (G) Percentage of total $PCNA^+$ SH-SY5Y cells from PBS, Bt-BEV and LPS treated three-

cell culture systems. (H) Mean fluorescent intensities of anti-PCNA staining in SH-SY5Y cells. Analysis conducted from minimum of 20 FOV images. The box plots represent first quartile, median and third quartile, with whiskers representing minimum and maximum. Statistical significance for cell counts (A) determined using Kruskal-Walis test with Dunn's multiple comparison (GraphPad Prism 9.2.0). A value of $p<0.05$ was considered statistically significant; *$p<0.05$, **$p<0.01$, $p<0.0001$. Images taken on confocal microscope (63x/1.4 oil DIC objective). Scale bars = 25 μm.

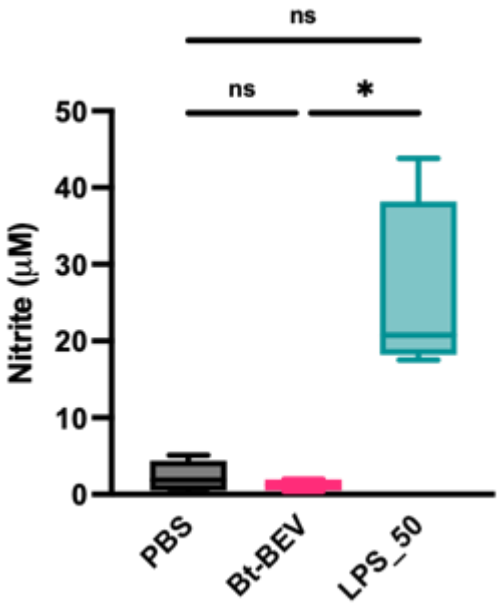

Figure 3.16 LPS induces nitrite production in a three-cell culture system modelling the gut-brain axis. A three-cell culture system was set up with Caco-2 and hCMEC/D3 cells modelling the gut-brain barriers cultured on ThinCert™ inserts (PET membrane, 0.4 µm) and non-differentiated SH-SY5Y cells cultured on fibronectin coated glass-coverslips in the basal compartment. DiD-labelled Bt-BEVs ($1x10^{11}$/ml), PBS or LPS (50 ng/ml) were added to the apical compartment of the three-cell culture system for 24 hrs (37°C, 5% CO_2). Nitrite (µM) was measured in media from the basal compartment following incubation with PBS (*n=4*), Bt-BEV (*n=4*) or LPS (*n=4*) using Griess Test. The box plots represent first quartile, median and third quartile, with whiskers representing minimum and maximum. Statistical significance determined using Kruskal-Walis test with Dunn's multiple comparison (GraphPad Prism 9.2.0). A value of $p<0.05$ was considered statistically significant; *$p<0.05$, ns. not significant.

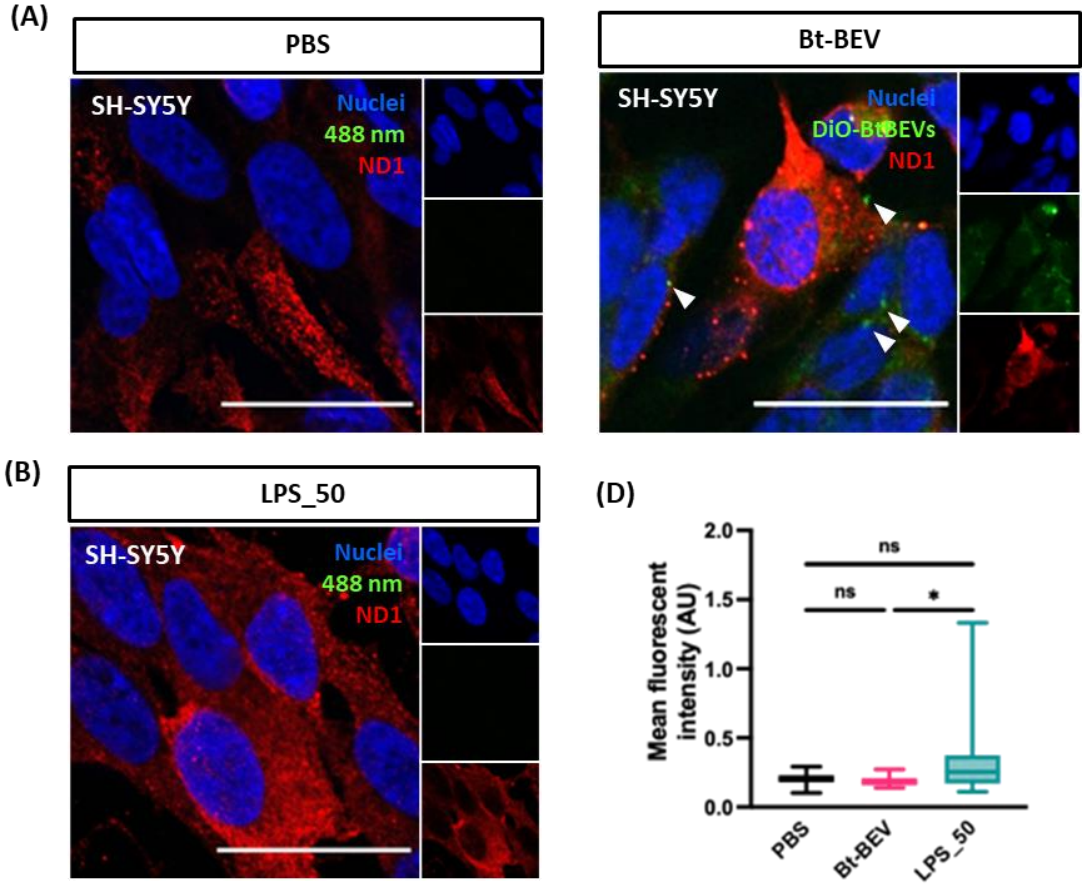

Figure 3.17 LPS induces a small increase in ND1 cellular expression. A three-cell culture system was set up with Caco-2 and hCMEC/D3 cells modelling the gut-brain barriers cultured on ThinCert™ inserts (PET membrane, 0.4 µm) and non-differentiated SH-SY5Y cells cultured on fibronectin-coated glass-coverslips in the basal compartment. DiO-labelled Bt-BEVs ($1x10^{11}$/ml), PBS or LPS (50 ng/ml) were added to the apical compartment of the three-cell culture system for 24 hrs (37°C, 5% CO_2). SH-SY5Y cells were fixed and co-stained with anti-ND1 (red) and nuclear stain Hoechst 33342 (blue). Representative images of SH-SY5Y cells from (A) Bt-BEV, (B) PBS and (C) LPS treated three-cell cultures. White arrowheads indicate Bt-BEV (green) uptake. (D) Mean fluorescent intensity of ND1 staining in SH-SY5Y cells from Bt-BEV, PBS or LPS treated cultures. Analysis conducted from minimum of 20 FOV images. The box plots represent first quartile, median and third quartile, with whiskers representing minimum and maximum. Images taken on confocal microscope (63x/1.4 oil DIC objective). Scale bars = 25 µm. Statistical significance determined using Kruskal-Wallis multiple comparisons, a value $p<0.05$ was considered statistically significant (GraphPad Prism 9.2.0), *$p<0.05$, ns. not significant.

3.3.10. Bt-BEVs do not induce changes in neuronal cell proliferative activity following translocation across the gut-epithelium and CNS blood vessel endothelial cell barriers

To determine the effects of Bt-BEVs on cell proliferation, Bt-BEV (*n=2*) treated SH-SY5Y cells were fixed and co-stained with anti-PCNA or isotype matched control antibodies and Hoechst 33342 nuclear stain and compared with cells from PBS (*n=2*) and LPS (*n=2*; 25 ng/ml, 50 ng/ml or 100 ng/ml) treated cultures (Fig. 3.15A-F). The number of $PCNA^+$ cells from each group were counted and total percentage of $PCNA^+$ cells per FOV calculated. No significant difference in percentage of $PCNA^+$ cells between PBS and Bt-BEV treated cultures were observed consistent with Bt-BEVs and their subsequent translocation across the epithelial and endothelial cell barriers having no adverse effects on SH-SY5Y cell proliferation (Fig. 3.15G). Interestingly, when looking at mean fluorescent intensities of $PCNA^+$ staining, there was a significant reduction ($p<0.01$) in fluorescent intensity of PCNA staining of PBS and Bt-BEV treated cells (Fig. 3.15H; 1.01 ± 0.04 and 0.58 ± 0.01, respectively;). LPS treatment (25 ng/ml, 50 ng/ml and 100 ng/ml) resulted in significantly ($p<0.001$) reduced $PCNA^+$ cells (63.19 ± 3.84 %, 27.79 ± 2.69% and 14.98 ± 2.40 %, respectively) compared to Bt-BEV treatment (Fig. 3.15G; 91.81 ± 1.71%). This effect of LPS was confirmed from measurements of mean fluorescent intensity of $PCNA^+$ staining (Fig. 3.15H), with significant reductions in fluorescent intensity observed in 50 ng/ml ($p<0.05$) and 100 ng/ml ($p<0.001$) LPS treated cultures (0.33 ± 0.02 AU and 0.15 ± 0.02, respectively) compared to Bt-BEV treated cultures (0.58 ± 0.01 AU).

3.3.11. Bt-BEVs do not elicit an inflammatory response in neuronal cells following translocation across gut-epithelial and CNS blood vessel endothelial cells

To determine whether Bt-BEVs induced an oxidative stress response in non-differentiated neuronal cells following translocation across epithelial and CNS blood vessel endothelial barriers, nitric oxide production was assessed by measurement of nitrite levels in the basal supernatant using the Greiss Reagent (Fig. 3.16). Under PBS (*n=4*) treatment, nitrite levels in the basal supernatant were 2.24 ± 1.05 μM with no significant differences observed in Bt-BEV (*n=4*) treated cultures (1.20 ± 0.35 μM).

This contrasted with LPS (*n=4*; 50 ng/ml) treatment that resulted in significant ($p<0.05$) increases in nitrite production (25.72 ± 4.96 μM).

3.3.12. Bt-BEVs do not promote differentiation of SH-SY5Y cells

Neuro D1 (ND1) is a transcription factor involved in neurogenesis and is important in the maturation and survival of neurons in the hippocampus and olfactory bulb (Gao, Ure et al. 2009).To determine if Bt-BEVs can promote differentiation of model neuronal cells, SH-SY5Y cells were fixed and co-stained with anti-ND1, following 24 hr incubation with PBS (*n=2*), Bt-BEVs (*n=2;* $1x10^{11}$/ml) or LPS (*n=2;* 50 ng/ml) (Fig. 3.17A-C). Quantification of mean fluorescent intensity of ND1 positive staining (Fig. 3.17D) revealed no significant alterations between PBS and Bt-BEV treated cultures (0.20 ± 0.01 AU and 0.19 ± 0.01 AU, respectively). However, a significant ($p<0.05$) increase was seen in comparing Bt-BEV and LPS treated cultures (Fig. 3.17D; 0.35 ± 0.06 AU), with LPS causing a reduction in ND1 expression.

3.4. Discussion

BEVs have been implicated as effective microbial mediators in the gut-brain axis Whilst BEVs in bacterial pathogenesis have been extensively studied, the role of BEVs from probiotic and commensal bacteria in the gut-brain communication pathways, and they're effects on CNS function, remain to be elucidated. Therefore, the results presented in this chapter aim to determine the translocation and modulatory effects of BEVs isolated from the dominant gut symbiont, Bt, across *in vitro* cell-models of gut-brain cellular barriers. Bt-BEV incubation in single-cell cultures demonstrated uptake and intracellular trafficking to endo-lysosomal pathways in hCMEC/D3 and BV-2 cells, with significant induction of downstream pro-inflammatory responses in microglia cells. Furthermore, it was shown that Bt-BEVs can cross CNS blood vessel endothelial cells, to be acquired by and induce inflammatory responses in microglia cells. After establishing Bt-BEVs transmigrated across gut-epithelial and CNS blood vessel brain endothelial cells, a three -cell culture system was established using Caco-2 and hCMEC/D3 cells, modelling the gut-epithelium and CNS blood vessel endothelium, respectively, and non-differentiated SH-SY5Y neurons. Identification of acquired Bt-BEVs and co-localisation with intracellular lysosomes in SH-SY5Y cells following incubation with Bt-BEVs, indicated translocation of Bt-BEVs across the

gut-epithelium and CNS blood vessel endothelium cell barriers. Comparing downstream effects of Bt-BEV acquisition in SH-SY5Y cells with LPS, indicated that Bt-BEVs did not induce significant changes in proliferative activity, differentiation or inflammatory responses in non-differentiated SH-SY5Y cells. This is in comparison to LPS which effectively altered the cellular and inflammatory activity of SH-SY5Y cells.

3.4.1. Uptake of BEVs by epithelial, endothelial and CNS cells

DiD-labelled and AF647-labelled Bt-BEVs were used to assess uptake into hCMEC/D3 cells, an *in vitro* cell model for CNS blood vessel endothelium. Fluorescent microscopy revealed cellular uptake of Bt-BEVs and their perinuclear accumulation in an *in vitro* CNS blood vessel endothelial immortalised cell model, hCMEC/D3 (Fig. 3.5A and B). To date, few studies have used BEVs derived from commensal or probiotic bacteria to study interactions with host cells (Durant, Stentz et al. 2020, Hu, Lin et al. 2020, Jones, Booth et al. 2020). Recently, Jones *et al*., used BEVs derived from Bt, to show uptake in Caco-2 cells and primary intestinal and caecal organoid monolayers (Jones, Booth et al. 2020). They further went on to demonstrate *in vivo*, transport of Bt-BEVs across the intestinal epithelium and migration to systemic tissues (Bhar, Edelmann et al. 2021) and combined with other studies demonstrating transport of pathogenic BEVs across the BBB and induction of negative effects on cognitive function (Wei, Peng et al. 2020), the possibility of BEVs to serve as long-distance mediators in microbiota communication in host gut-brain axis has been raised. The initial aim for the studies undertaken in this chapter was to determine uptake of Bt-BEVs by CNS blood vessel endothelial cells, that together with extracellular matrix, astrocytes and pericytes, make up the backbone of the BBB.

3.4.2. Intracellular fate of BEVs following acquisition in CNS blood vessel endothelial cells

The intracellular fate of BEVs is an important aspect to consider when exploring the effects of BEVs on host-cell physiology but can be challenging due to drawbacks associated with conventional fluorescence microscopy, which is limited in its ability to provide ultrastructural details that robustly identify intracellular localisation. The

data presented here demonstrates internalisation of Bt-BEVs in hCMEC/D3 cells and trafficking to lysosomal compartments in both single-cell and co-culture systems (Fig. 3.7 and Fig. 3.10C, respectively). These results are in line with previous studies (Bielaszewska, Rüter et al. 2013, Jones, Booth et al. 2020), with Jones et al., also demonstrating co-localisation to lysosomal-compartments following 72 hr incubation, indicating that some Bt-BEVs may be able to resist lysosomal degradation (Jones, Booth et al. 2020).

Similarly, BEVs from an enterohemorrhagic *Escherichia coli* (E. coli) strain was used to show that following dynamin dependent-endocytosis in Caco-2 and human brain microvascular endothelial cells (HBMECs), *E. coli* BEVs are trafficked to lysosomes where it's associated toxin, hemolysin, is separated and translocated to mitochondria resulting in membrane permeabilization and induction of apoptosis. It was further demonstrated that following hemolysin dissociation from BEV-associated lysosomes, that a transient loss of lysosomal function occured. This study provides insight into the mechanistic details involved in the delivery of membrane-associated toxins (Bielaszewska, Rüter et al. 2013). Determining the fate of acquired Bt-BEVs that are not trafficked to lysosomal compartments is needed to fully understand the role of Bt-BEVs and their biological relevance in host-cell physiology.

3.4.3. Translocation of BEVs across epithelial and endothelial cell barriers

The concept that BEVs can cross epithelial barriers arises from evidence of vesicles of bacterial origin in regions outside the GIT. For example, vesicles predominantly from *Pseudomonas* spp. have been isolated from urine samples (Yoo, Rho et al. 2016). Furthermore, *E. coli* derived BEVs injected intraperitoneally have been shown to spread across the murine body, accumulating in the liver, lung, spleen and kidney, within 3 hrs of administration, eliciting a systemic inflammatory response indicated by increased TNF-α and IL-6 in the serum and bronchoalveolar lavage-fluid, showing that BEVs are effective mediators of long-distance communication *in vivo* (Jang, Kim et al. 2015). Similarly, Bt-BEVs administered via oral gavage have been shown to cross gut epithelial barriers, accumulating in the liver and lungs (Jones, Booth et al. 2020). There is also potential of detection of extracellular vesicles of bacterial origin

as clinical biomarker for disease diagnosis. For example, in the gut-brain axis disorder, autism spectrum disorder (ASD), evaluation of BEVs in urine revealed some consistency between members of bacterial species of which isolated BEVs where derived to reported alterations in gut microbiota composition in ASD patients (Lee, Park et al. 2017). Moreover, in a murine model of AD, the suitability of BEVs in blood for metagenome analysis was assessed, revealing significant alterations in blood BEVs profiles in AD-mice compared to healthy controls (Park, Choi et al. 2017), indicating a potential role of BEVs in gut-brain disorders. Recent work has attempted to elucidate whether BEVs are able to cross the BBB. Han et al., used *A. actinomycetemcomitans* derived BEVs to show their transport across the BBB following intracardiac injection in mice, using lectin injection to identify brain vessels (Han, Choi et al. 2019). Although the evidence provided of *A. actinomycetemcomitans* in the brain cortex wasn't compelling, it was later demonstrated in another study that *A. actinomycetemcomitans* BEVs administered via intravenous injection were delivered to microglia cells in a monocyte-specific GFP-knockin mouse model (Ha, Choi et al. 2020).

The transwell insert system, introduced in 1953 (Grobstein 1953), is a well-established, widely used system in cell biology to assess a variety of measures including transport, absorption, secretions and interactions between co-cultures. Caco-2 cells have been used in a variety of co-cultures models including those with macrophages (Tanoue, Nishitani et al. 2008), human microvascular endothelial cells (HMEC-1), human umbilical cord endothelial cells (HUVECs) (Maaser, Schoeppner et al. 2001) and monocytes (Smith, Gheux et al. 2018). In this present study, two-cell and three-cell ThinCert™ culture systems were utilised to assess *in vitro* uptake and transport of Bt-BEVs across epithelial and endothelial barriers within the gut-brain axis.

Firstly, transport across the gut-epithelial and brain-endothelial cells barriers was modelled using Caco-2 and hCMEC/D3 cells respectively (Fig 3.3B). In line with previous work by Jones et al., (Jones, Booth et al. 2020) addition of Bt-BEVs was shown to initially reduce TEER measurements, a marker for Caco-2 monolayer integrity, which improved steadily through the 24 hr period (Fig. 3.10A). Bt-BEVs

were then shown to cross the epithelial cell barrier (Fig. 3.10B) and be acquired by brain endothelial cells (hCMEC/D3) cultured in the basal compartment (Fig. 3.10C) were they co-localised to intracellular lysosomes (Fig. 3.10D), demonstrating the ability of Bt-BEVs to cross gut-epithelial barriers to access endothelial cells within the BBB. It is possible however, that initial increased permeability following Bt-BEV addition led to subsequent paracellular migration of Bt-BEVs across the ThinCert™ membrane. Determining expression levels of tight junction proteins, such as ZO-1 and occludens, could help determine whether the paracellular permeability of Caco-2 cell monolayer was compromised following Bt-BEV exposure.

Commensal gut microbes under homeostatic conditions have protective roles in gut immunity, but in conditions that result in microbial dysbiosis and barrier disruption, they can elicit potentially harmful effects, indicating that they're function is dependent on the environment (Kang, Ban et al. 2013). Bt has been well studied for its anti-inflammatory effects. In a murine model of dextran sulphate sodium (DSS) induced colitis, Bt was shown to alleviate inflammation (Charlet, Bortolus et al. 2020, Li, Hao et al. 2021) through mechanisms associated with modulation of tryptophan metabolism and $CD4^+$ T cell differentiation (Li, Hao et al. 2021). Bt also alleviates Salmonella-induced inflammation in Caco-2 cells via mechanisms involving reduced TNF, IL-8 and CXCL2 and increased TGF-β mRNA expression (Kelly, Campbell et al. 2004). Another dominant commensal member of the human intestine, the mucin-degrading *Akkermansia muciniphila* (*A. muciniphila*) has important roles in gut homeostasis (Derrien, Vaughan et al. 2004, Derrien, Van Baarlen et al. 2011). In inflammatory diseases such as IBS, the abundances of *A. muciniphila* is greatly reduced (Png, Lindén et al. 2010). Characterisation of BEVs in faeces from DSS-induced colitis murine models also released increases in BEV derived protein yield and BEV size distributions, indicating significant alterations in faecal BEVs in *in vivo* model of colitis. Metagenomic analysis also showed drastic differences in faecal BEVs from these mice, with reduced abundances of BEVs derived from the phylum Bacteroidetes and Verrucomicrobia compared to controls (Kang, Ban et al. 2013). Furthermore, RAW 263.7 macrophages and gut epithelial CT26 cell line treated with *A. muciniphila* BEVs, induced IL-6 production in a dose-dependent manner, but substantially lower than the proinflammatory effects of *E. coli* BEVs, reflecting a weaker potency of *A. muciniphila* BEVs (Kang, Ban et al. 2013). However, *A. muciniphila* BEVs were

shown to elicit anti-inflammatory protective effects in Ct26 cells pre-treated with *E.coli* BEVs, reducing IL-6 production, and was also shown to alleviate disease severity *in vivo* DSS-induced colitis murine model (Kang, Ban et al. 2013).

Recently, Moosavi *et al*., showed that *Faecalibacterium prausnitzii* BEVs significantly increased intestinal epithelial barrier permeability via upregulation of ZO-1 and occludin gene expression in Caco-2 cells (Moosavi, Akhavan Sepahi et al. 2020). Co-culture experiments with Caco-2 cells cultured on the apical side of a transwell membrane and RAW 264.7 macrophages in the basolateral compartment have also been used to assess epithelial permeability in response to inflammation. In this study, RAW 264.7 macrophages were stimulated with LPS to mimic gut inflammation, which resulted in increased TNF-α production from the macrophages, and a reduction in Caco-2 TEER measurements (Tanoue, Nishitani et al. 2008).

By administering Bt-BEVs in Caco-2 cells pre-treated with LPS could help determine the potential protective effects of Bt-BEVs and provide comparisons to help determine whether the effects of Bt-BEVs on Caco-2 permeability are weaker or stronger than a well-used and established inflammatory inducer, LPS. Measuring additional parameters of inflammatory responses such as cytokine production can help verify these effects.

In addition to gut epithelial barrier, the BBB provides an additional highly regulated barrier controlling the exchanges between the blood and brain compartments. Different approaches and cell types have been used to mimic the BBB *in vitro*. As with many *in vitro* models, primary cell cultures provide the most representative option to model in-situ systems, but are associated with drawbacks including limited yield, availability and cost (Bowman, Ennis et al. 1983, Rubin, Hall et al. 1991). Immortalised cell lines provide a reliable, accessible and cost effective alternate to primary cultures (Eigenmann, Xue et al. 2013). hCMEC/D3 cells form a contact-inhibited monolayer that express endothelial markers such as CD34, CD31, and VE-cadherin, maintaining their characteristics up to at least 35^{th} passages (Weksler, Romero et al. 2013). Unlike in *in vivo*, where BBB TEER is reported to reach well above 1000 Ωcm2 (Butt, Jones et al. 1990), hCMEC/D3 monolayers achieve substantially lower TEER values as reported in this present study (Fig. 3.11) in addition to various other reports (Hatherell,

Couraud et al. 2011). Some studies have reported higher TEER values upon culture with hydrocorticosterone (Förster, Burek et al. 2008), co-culture with other cell types in the NVU (Hatherell, Couraud et al. 2011) and exposure to flow-based sheer stress (Poller, Gutmann et al. 2008). In this present study,

The "normal" gut microbiota is essential in maintaining BBB integrity, as evidenced by GF mice that display increased BBB permeability and reduced expression of the tight junction proteins occludin and claudin-5 (Braniste, Al-Asmakh et al. 2014). Permeability across the BBB is not static, with colonisation of GF mice with microbiota from SPF mice, resulting in improved BBB integrity and upregulated expression of tight junction proteins (Braniste, Al-Asmakh et al. 2014). There is accumulating experimental and clinical evidence indicating associations between impaired BBB function and CNS diseases. Given microbial alterations associated with many CNS-related disorders, the ability of microbiota and in particular, BEVs, to serve as long-distance mediators in gut-brain communication is receiving growing interest.

H. influenzae derived BEVs have been shown to increase BBB permeability, in a dose- and time-dependent manner in an *in vivo* rat model, primarily mediated via LPS, implicating BEVs as delivery molecules of LPS *in vivo* (Wispelwey, Hansen et al. 1989). As mentioned earlier, recent evidence of BEVs to cross *in vivo* BBB and accumulate to microglia cells has emerged (Ha, Choi et al. 2020). To assess Bt-BEV transport across the BBB and immunomodulatory effects in the CNS in this present study, an *in vitro* two-cell culture system with hCMEC/D3 cells and BV-2 microglia cells was utilised (Fig. 3.3B). Following incubation with Bt-BEVs, BV-2 cells in the basal compartment were fixed and co-stained with microglia markers, Iba-1, TREM119 and CD45. Fluorescent microscopy revealed presence of DiO-labelled BEVs in BV-2 cells (Fig. 3.12) indicating Bt-BEV transport across the BBB endothelial cell barrier and uptake by microglia cells. Furthermore, ZetaView particle analysis (Fig. 3.13B) showed that fewer Bt-BEVs were transported across hCMEC/D3 monolayer cultured on Matrigel®-coated ThinCert™ insert indicating reduced barrier permeability, in line with TEER measurements (Fig. 3.11). With this in account, further analysis of the immunomodulatory effects of Bt-BEVs reveal that under high brain endothelial barrier permeability, substantially higher levels of extracellular TNF-α are secreted by BV-2 cells (Fig. 3.13C). These results suggest that low levels of Bt-

BEVs crossing the BBB may have not cause any adverse immunomodulatory effects, but in conditions where BBB integrity is impaired, higher concentrations of Bt-BEVs migrating across the barrier could elicit a greater inflammatory response.

3.4.4. Immunomodulatory effects of BEVs in CNS microglia

In this Chapter, Bt-BEVs were shown to be acquired by BV-2 cells, eliciting activation (Fig. 3.8) and immunomodulatory responses (Fig. 3.9). Several other studies using BEVs derived from other commensal and probiotic bacterial species have also demonstrated their interaction with macrophages and monocytes. For example, the probiotic *E. coli* Nissle 1917 is widely used for the treatment of intestinal inflammatory diseases and infections (Jacobi and Malfertheiner 2011) and it's BEVs have recently been shown to stimulate proliferation, immune-related enzymatic and phagocytic activities and IL-10 induced anti-inflammatory responses in RAW 264.7 macrophages (Hu, Lin et al. 2020), indicating possible mechanisms underlying the anti-inflammatory properties of *E. coli* Nissle 1917. Colonisation with another predominant commensal *Bacteroides* genus member, *B. fragilis*, (Fletcher, Coyne et al. 2009) has also been shown to prevent disease development through the upregulation of $CD11c^+$ $CD103^+$ dendritic cell (DC) and promotion of T regulatory (Treg) cell differentiation in an animal model of multiple sclerosis (MS), an inflammatory disease involving the CNS (Ochoa-Repáraz, Mielcarz et al. 2010). These immunomodulatory effects of *B. fragilis* are thought to be mediated via polysaccharide A (PSA) and it has been further demonstrated that *B. fragilis* selectively packages PSA into its BEVs and are internalised by dendritic cells in culture to induce the expression of IL-10 producing Treg cells via TLR-2 activation (Shen, Giardino Torchia et al. 2012). Furthermore, Bt-BEVs have been shown to induce regulatory colonic mucosa and blood derived DCs responses in healthy humans, but not in IBS patients (Durant, Stentz et al. 2020). Together these studies demonstrate the ability BEVs to interact with host peripheral immune cells, but there is a lack of studies exploring interactions of bacterial BEVs in CNS immune cells, particularly those derived from known commensals. Therefore, BV-2 cells used for the experiments in this chapter provide an insight into BEV interaction with CNS microglia cells.

Microglia activation is commonly determined by their morphology and cell surface marker expression. Iba-1 is widely used as a marker for microglia activation, with many studies relying on immunostaining methods to characterise their morphology, distribution and enumeration (Ito, Imai et al. 1998, Kanazawa, Ohsawa et al. 2002). Although a small but significant increase immuno-positive Iba-1 cells were quantified following incubation with Bt-BEVs (Fig. 3.8E), it did not reflect in its cellular expression (Fig. 3.8F). To further determine the activation response of BV-2 cells following Bt-BEV translocation across CNS blood vessel endothelial cell monolayers, additional microglia markers were used, and their immunoreactivity quantified (Fig. 3.12A). CD45 was used to confirm microglia activation identifying all microglia independent of their activation status has proved challenging. The identification of TREM119 as a highly enriched and specific marker for microglia in both mice and humans, however, provides a promising alternate (Park, Chen et al. 2016). Comparisons between Iba-1^{+}/CD45^{+}/TREM119^{+} Bt-BEV^{+} BV-2 cells, demonstrate inherent differences in Iba-1 and CD45^{+} cells that are not reflected by TREM119^{+} cell counts (Fig. 3.13A).

It may be useful to utilise complementary flow cytometric techniques, mRNA expression and protein analysis in parallel to immunohistochemical methods used here, to allow for more accurate enumeration of microglia cells. Another important consideration to take into account is that microglia activation is a dynamic process that had been classically characterised into two different phenotypes, the M1 classically activated (proinflammatory) and M2 alternative activated (anti-inflammatory) phenotypes that have a complex overlap of cell surface marker expression (Colton and Wilcock 2010, Ransohoff 2016). Incorporating phenotypic markers specific for the different activation states, including MHC-II, CD86 and CD32 for M1 activated microglia and CD206 for M2 activated microglia, would provide further insight into whether the Bt-BEV stimulated immune response in BV-2 cells is pro- or anti-inflammatory. Single cell RNA sequencing has also suggested that the simplified classification system for microglia status is inappropriate as it doesn't take into account the diversity of microglia reported (Boche, Perry et al. 2013), including bipolar/rod-shaped (Tam and Ma 2014) and multinucleated (Hornik, Neniskyte et al. 2014) microglia and "subpopulations" identified that reflect developmental stage, biological

events and pathological context (Holtman, Raj et al. 2015, Szulzewsky, Pelz et al. 2015, Keren-Shaul, Spinrad et al. 2017, Müller, Kohanbash et al. 2017).

Microglia activation can further be assessed indirectly through cytokine secretion and/or expression. In comparison to the significant increase in TNF-α secretion induced by Bt-BEVs (Fig. 3.9A), other studies have shown LPS to induce a substantially larger immunostimulatory response in BV-2 cells. For example, 100 ng/ml LPS has been shown to induce almost 4-fold higher TNF-α secretion (2 ng/ml) (Henn, Lund et al. 2009) compared to Bt-BEVs (Fig. 3.9A; ~530 pg/ml). Similarly, whereas Bt-BEVs induced a statistically significant increase in nitrite production (Fig. 3.9B), LPS has been shown to induce a much larger increase in nitrite production in BV-2 cells in a dose-dependent manner (~25 μM, ~45 μM and ~55 μM from 1, 10 and 100 μg/ml LPS, respectively) (Scheiblich, Roloff et al. 2014).

Although LPS is associated with the outer membrane of gram-negative bacteria, LPS structure and potency differs amongst species (Raetz and Whitfield 2002). LPS from *Bacteroides* spp. have a different lipid A structure than that of *E. coli* LPS, and whereas *E. coli* LPS is the most potent enterotoxin known, the toxicity of *Bacteroides* LPS remains an area of controversy (Magnuson, Weintraub et al. 1989, Mancuso, Midiri et al. 2005). Stimulation of HUVECs with Bt-LPS and *B. fragilis* LPS, show that they both possess immunostimulatory effects, albeit at a much lower level than *E. coli* LPS (Berezow, Ernst et al. 2009). Heterogeneity in the lipid A region within individual species also presents a confounding issue and it remains to be determined what effects lipid A heterogeneity has on the innate immune response (Bainbridge, Karimi-Naser et al. 2008, Berezow, Ernst et al. 2009). Despite the lack of clarity, it is estimated that the healthy human gut contains ~300 mg of *Bacteroides* LPS (Jacobson, Choudhury et al. 2018) and analysis of endotoxin concentrations in Bt-BEVs of 1.68 EU/ml indicate that the endotoxins concentrations are within safe levels (Ahmadi Badi, Moshiri et al. 2020). It is also possible that the immunostimulatory response observed (Fig. 3.9) in Bt-BEV treated BV-2 cells were a result of contaminants enhancing its stimulatory ability. Ultimately, these results demonstrate that *E. coli* LPS elicits a more reliable and robust inflammatory response, which is substantially higher than that induced by Bt-BEVs, indicating that although the results were statistically significant, they may not be of any biological significance. Assessment of the

inflammatory response to higher concentrations of Bt-BEVs, in addition to potential protective effects against LPS, is required to robustly assess the modulatory effects of Bt-BEVs on BV-2 cells. Further research is needed to help uncover the complexity underlying the structure and function of membrane-associated endotoxins.

3.4.5. Proliferative effects of BEVs on non-differentiated neurons in the CNS

A three-cell culture system was used to assess Bt-BEV transport across epithelial and endothelial cell barriers (Caco-2 cells and hCMEC/D3 cells, respectively) and subsequent uptake and interactions with neurons (modelled by non-differentiated SH-SY5Y cells) (Fig. 3.3C). Evidence of Bt-BEV translocation across epithelial and endothelial cell monolayers and uptake by CNS neurons was obtained (Fig. 3.15).

Non-differentiated SH-SY5Y cells are continually proliferative cells, resembling immature catecholaminergic neurons (Lopes, Schröder et al. 2010, Xie, Hu et al. 2010) and express markers indicative of proliferation (e.g. PCNA) and differentiation inhibiting basic-helix-loop-helix transcription (bHLH) factors (e.g. ID1, ID2 and D3) (López-Carballo, Moreno et al. 2002). Despite no alterations observed in SH-SY5Y $PCNA^+$ cells following Bt-BEV treatment in the three-cell culture system (Fig. 3.15F), Bt-BEVs elicited some effect on the proliferative activity of neurons indicated by a small but significant reduction in mean fluorescent intensity of $PCNA^+$ staining (Fig. 3.15G). In comparison and as expected, LPS induces major changes in proliferative activity, demonstrated by reduced number and mean fluorescent intensity of $PCNA^+$ cells a dose dependent manner (Fig. 3.15F, G). In line with PCNA cell counts, Bt-BEVs do not alter ND1 expression, demonstrated by no significant differences in mean fluorescent intensity of ND1 staining (Fig. 3.15), whereas following LPS treatment, a significant increase in mean fluorescent intensity suggests priming of SH-SY5Y for differentiation. These results indicate that although Bt-BEVs may induce immunomodulatory effects on microglia cells, they do not elicit adverse effects on neurones. In addition, to verify proliferation rates, it may also be prudent to assess the effects on cell apoptosis by using methods such as TUNEL assay.

What factors trigger the reactivation of the cell cycle in neurons remains unclear, but it is likely that activation of cell cycle elements required interactions between microglia, astrocytes and neurons. For example, media from non-stimulated BV-2 cells significantly improves the viability of LPS-treated (1 μg/ml) SH-SY5Y cells indicating that microglia release neurotrophic factors (Fernández-Calle, Galán-Llario et al. 2020). Furthermore, a positive correlation is observed between nitrite production in LPS-stimulated BV-2 cells and SH-SY5Y cell viability upon incubation with BV-2 conditioned media (Fernández-Calle, Galán-Llario et al. 2020), demonstrating that microglia are activated upon detection of stimuli and contribute to neuronal cell death (Herrup and Yang 2007). It may therefore be the case that Bt-BEVs induce a more potent anti-proliferative or potential apoptotic effects on SH-SY5Y cells via the activation of BV-2 microglia cells and secretion of proinflammatory mediators. Mixed cultured of BV-2 and SH-SY5Y cells could also provide a more realistic model to in situ environments and allow direct interactions between different cells.

In this study, non-differentiated SH-SY5Y cells were used, but SH-SY5Y cells can be driven towards a number of mature phenotypes including cholinergic, adrenergic or dopaminergic depending on methods of differentiation and culture conditions (Xie, Hu et al. 2010). Differentiation of SH-SY5Y cells results in reduced proliferation rate and increased expression of neuron specific enolase (NSE) (Påhlman, Ruusala et al. 1984, Encinas, Iglesias et al. 2000) and ND1 (López-Carballo, Moreno et al. 2002).

3.4.6. Constraints in current BEV research

3.4.6.1. BEV production and isolation

Experiments conducted in this Chapter used Bt-BEVs ranging from 50-200 nm in size (Fig. 3.4) were isolated from cultures grown in a chemically defined medium collected at a single time point. They were used in their crude heterogeneous populations and not separated according to their size; therefore, compositional heterogeneity cannot be eliminated from the data collected. It is becoming more apparent that a single bacterial species can produce a huge diversity of membrane vesicular structures (Kaplan, Chreifi et al. 2021), with varying metabolite and protein profile of Bt-BEVs observed when culturing in different media (Bryant, Stentz et al. 2017) indicating that a more

robust and extensive characterisation methods are needed. Together with standardisation of protocols, this can help enable reproducibility and reduce discrepancies between studies, by producing BEV isolates with a higher yield and purity.

One of the main factors that is known to strongly influence BEV vesiculation is bacterial growth phase (Zavan, Bitto et al. 2019) and stress conditions, such as heat shock (Katsui, Tsuchido et al. 1982, Thompson, Naidu et al. 1985, Willén, Carlén et al. 2000, Olofsson, Vallström et al. 2010). General isolation methods for BEVs first remove bacterial cells by filtration, followed by ultracentrifugation and purification steps (Kim, Choi et al. 2014, Klimentová and Stulík 2015). More recent BEV studies are tending towards better characterisation of BEVs prior to assessing their interaction with host cells (Chew, Chung et al. 2021, Melo, Pinto et al. 2021). Melo *et al*., showed that *H. pylori* grew well in chemically defined Ham-F12 media and that many proteins expressed on BEVs were differentially expressed depending on the time point at which BEVs were isolated, and contained proteins from membrane, periplasmic and cytoplasmic compartments of the parent bacterium (Melo, Pinto et al. 2021). Furthermore, *H. pylori* BEV production was shown to significantly increase during the growth phase, with continual accumulation through to the death phase. *H. pylori* BEVs from the early growth phase contained smaller amounts of low molecular weight proteins and associated virulence proteins, which were shown to increase considerably after 36 hrs culture. They also demonstrated that BEVs isolated from clinical *H. pylori* strains had similar, but not identical characteristics to the lab-based strains (Chew, Chung et al. 2021).

Although overlooked, the biogenesis of BEVs is an important factor to consider. Currently there are several known factors that influence BEV biogenesis, including the number and distribution of lipoprotein-peptidoglycan cross-links, stress-response induced by accumulation of mis-folded proteins, envelope proteins and LPS and peptidoglycan fragments causing bulging of the outer-membrane or alterations in dynamics of the lipid membranes (Schwechheimer, Sullivan et al. 2013, Schwechheimer, Kulp et al. 2014, Schwechheimer and Kuehn 2015, Schwechheimer, Rodriguez et al. 2015). These factors modulate size and number of BEVs and cargo content. The mechanisms behind selective packaging of cargo, including proteins and

RNA, remains unclear (Chiou, Kageyama et al. 2018, Fitzgerald, Freeman et al. 2018) although may vary between species/strain and may require the development of analysis tools to individually assess BEVs, instead of NTA systems which assesses whole populations (Margolis and Sadovsky 2019).

3.4.6.2. BEV quantification

Accurate quantification of BEVs forms an integral step that influences downstream experimental assays to explore their composition and biological functions (Klimentová and Stulík 2015). In this chapter, Bt-BEVs were characterised using a combination of protein assays and NTA. Bt-BEVs were used at a concentration of $1x10^{11}$/ml determined using NTA, with protein content determined prior to labelling with the Alexa Fluor® protein dye. Quantification of protein content present the most common method for quantifying BEVs, with a variety of different protein assays available (Bradford, Lowry, bicinchonic acid (BCA) and Qubit assays). However, protein content can vary considerably depending on bacterial growth stage, bacterial strain and species, culture conditions and BEV size (Klimentová and Stulík 2015, Bitto, Zavan et al. 2021) and using this method to solely quantify BEVs has its limitations. Recent studies have therefore used NTA to quantify BEV production and size distribution (Gerritzen, Martens et al. 2017, Turner, Bitto et al. 2018, Jones, Booth et al. 2020, Wang, Eagen et al. 2020, Bitto, Zavan et al. 2021). NTA enables the quantification of BEVs, using very small amounts, irrespective of their protein content and provides details on the size distribution of particles, useful when comparing cultures grown at different conditions and that contain different cargo (Gerritzen, Martens et al. 2017), as well as enabling quantification of fluorescently labelled particles (Fig. 3.4). Recently, Bitto *at al.*, undertook a detailed comparison of between strain, species and growth-stage dependent differences in BEV yield, composition and immunostimulatory properties from *H. plyori* and *P. aeruginosa* cultures. They demonstrated that the traditional method for quantifying BEVs using protein assays, wasn't sufficient to differentiate between the differences between the parameters that affect BEV size and composition determined using NTA. Furthermore, significant differences in protein content of BEVs were reported using different proteins assays available (mentioned above). Although detailed characterisation of BEVs in studies assessing biological and experimental outcomes is lacking, it is apparent that a

standardised and integrated approach to accurately quantify BEVs is needed, especially in order to draw comparisons across BEVs isolated from different bacterial strains and growth condition.

Currently, analysis of the biological implications arising from vesicle diversity are unclear. BEV preparations can contain a number of different type of vesicles. In gram-negative bacteria, like Bt used for the experiments in this Thesis, BEV isolations will include outer-membrane vesicles, but also other vesicles such as outer-inner membrane vesicles (Pérez-Cruz, Delgado et al. 2015) and vesicles produced by cell lysis rather than the natural blebbing of the outer membrane (Turnbull, Toyofuku et al. 2016). Therefore, effective definitions of vesicle homogeneity and heterogeneity is required to fully understand their role in bacteria and host cell functions (Margolis and Sadovsky 2019). Challenges still remain in efficiently separating BEV based on size to yield a homologous populations. Density gradient chromatography (DGC) provides one approach for size-based sorting of BEVs, but is associated with difficulties in scaling up the methods. Size exclusion chromatography (SEC) has also been used to separate heterogeneous *A. actinomycetemcomitans* derived BEV populations into subpopulations of large (~350 nm) and small (<150 nm) sized populations (Collins, Nice et al. 2021)

3.4.6.3. BEV labelling

Quantifying BEV uptake into host cells remains to be optimised. In this chapter, Bt-BEVs were labelled with either a lipophilic dye or Alexa Fluor® fluorescent protein tag, with free-dye removed by ultracentrifugation and size exclusion purification (SEP) column, respectively, to visualise uptake and intracellular fate (Fig. 3.4). Membrane integration of lipophilic fluorescent dye is a widely used method for labelling vesicles. However, there are numerous limitations associated with lipophilic dyes in BEV research, including risk of BEV aggregation (Gangadaran, Hong et al. 2018), formation of micelles in the solution due to the highly lipophilic nature of the dyes (Morales-Kastresana, Telford et al. 2017) and leaching of fluorescent membrane dye to other intercellular membranes (Mulcahy, Pink et al. 2014). This presented a challenge when quantifying Bt-BEV uptake into cells as it was difficult to distinguish between lipophilic-labelled vesicles or contaminated artifacts (Fig. 3.5), also reported

by Takov *et al*., (Takov, Yellon et al. 2017) and therefore it is possible that the actual uptake of DiD/DiO-labelled vesicles was masked by the presence of contaminants, further highlighting the needed to increased BEV purity. Due to these limitations surrounding the use of lipophilic membrane dyes, the Alexa Fluor® fluorescent protein label was used in conjunction in the experiments undertaken in this chapter. Comparisons in Bt-BEV^{+} cells between the lipophilic and protein labelled Bt-BEVs, reveal significant differences in their uptake, possibly due to limitations in confidently identifying and quantifying uptake. It may be prudent to include negative controls that consist of the lipophilic dye in the same buffer solution as Bt-BEVs and to compare vesicle size/quaintly before and after labelling. Another important aspect to consider is the method in which free dye is removed from vesicles. Whilst ultracentrifugation is the most common method used, SEC may present an alternate to achieving higher purity BEV isolates.

BEVs can also be labelled via a bioengineering method such as fluorescent protein fusion with vesicular proteins. In *E. coli* for example, heterologous green fluorescent protein (GFP) fusion to the membrane associated toxin, ClyA, was used to generate engineered BEVs displaying GFP-fused to ClyA. The resulting labelled-BEVs were shown to be highly fluorescent and easily tracked upon interaction with host epithelial cells (Kim, Doody et al. 2008). Protein fusion requires the identification of a suitable carrier protein displayed on the bacterial cell surface provides potential for redirecting the generated BEVs to target specific cell types to elicit desired therapeutic effects. Although Kim et al., (Kim, Doody et al. 2008) recorded no adverse effects on bacterial growth rate following GFP-protein fusion to CylA care must be taken when identifying a suitable membrane protein and potential effects on bacterial function. The fluorescent protein tags, Alexa Fluor® 488/647 used in this present study, label BEV membrane associated proteins in BEV preparations and do not require bioengineering, which can be time consuming and challenging, but it may be worthwhile exploring this method for future studies as it potentially reduces downstream handling of BEVs during the labelling process.

BEV uptake into host cells remains to be optimised. As demonstrated by microphotographs and Z-stacks generated, Bt-BEVs are observed in various focal

planes surrounding cells (Fig. 3.6). Due to their nano-sized, it can often be difficult to distinguish intracellular single vesicles and those that have formed clusters without high resolution imaging.

3.4.6.4. *In vitro* models of the BBB

Although a full understanding of the BBB *in vivo* is lacking, recent transcriptomic and proteomic analysis have helped provide a more detailed set of characteristics for which *in vitro* models can mimic (Baker, Martin et al. 2002, Tamashiro, Dalgard et al. 2012). Obtaining primary cultures of blood vessel endothelial cells is limited by difficulties in obtaining fresh tissue regularly, with high batch-batch variation (Blasi, Barluzzi et al. 1990, Henn, Lund et al. 2009). In addition, primary cells of brain endothelial cells undergo rapid differentiation and senescence, limiting their use (Bernas, Cardoso et al. 2010). Therefore generation of immortalised brain endothelial cells provide a favourable, stable, high yield and homogenous alternate. Indeed, hCMEC/D3 cells form monolayers of tightly packed endothelial cells, that has been shown to express many BBB-specific markers, including tight-junction proteins, adhesion molecules and chemokine receptors. They have been shown to retain most of the morphological and functional characteristic of brain endothelial cells, thereby providing a reliable and well characterised model of the BBB (Hinze and Boucrot 2018, Jones, Booth et al. 2020). A limitation to hCMEC/D3 cells is they're reduced claudin-5 expression compared to intact brain microvessels (Mahe, Fisson et al. 2001, Guillemin and Brew 2004, Hanisch and Kettenmann 2007, Henn, Lund et al. 2009) resulting in low TEER values (Fig. 3.11). As high junctional tightness is an important feature of the *in vivo* BBB, there have been several methods identified that have shown to improve *in vitro* TEER values of hCMEC/D3 cells, including activation of Wnt/β-catenin canonical pathway (Paolinelli, Corada et al. 2013), Wnt/planar cell polarity pathway (Horvath, Nutile-McMenemy et al. 2008, Henn, Lund et al. 2009, Scheiblich, Roloff et al. 2014, Gao, Shen et al. 2019), exposure to sheer stress (Griep, Wolbers et al. 2013) and co-culture with other cells in the NVU (Horvath, Nutile-McMenemy et al. 2008). Although there are many advantages to use of *in vitro* cell culture based models, including the control of environmental factors and ability to obtain detailed analysis, the development of an *in vitro* BBB model that better recapitulates the *in vivo*

physiological parameters is required, especially in moving forward to translational studies.

Culturing brain endothelial cells as a monoculture on a solid support (glass coverslips or plastic cell culture dish) enables easy locating, identification and imaging of cells, but are often too simple to assess transport across the BBB and the static conditions don't reflect the in situ physiology (Sivandzade and Cucullo 2018). The ThinCert™ transwell system used in this Chapter allows for the culture of cells on the microporous semipermeable membrane, providing separation between the luminal and apical compartments. Using this system also enables to co-culture cells that mimic the luminal, vascular and parenchymal compartments (Fig. 3.3B and C). Limitations to the ThinCert™ inserts include lack of exposure to sheer stress that could limit the maintenance of BBB properties (Cucullo, Hossain et al. 2011). Development of the microfluidic platform provides an alternate to static transwell cultures, enabling the mechanical and biochemical modulation of BBB function. In this system, the semi-permeable porous membrane is enclosed between two layers of plastic support. Cells cultured on the membrane can be subjected to mechanical sheer force by regulating the flow of media through the top and bottom of the channels, as well as introducing components (such as cytokines, nutrients etc) that can flow across the cells (Griep, Wolbers et al. 2013). There is flexibility with this design, allowing the connection of multiple cell "chips" to mimic different organ systems. Other models utilise intricate structures involving artificial capillary like structures, where endothelial cells can be cultured in the lumen of hollow semi-permeable fibres inside a sealed chamber. Cells cultured in this system have been shown to produce high TEER values, but due to the technical skills required and limitations surround visualisation of intraluminal compartments, there is a lack of consistency between studies (Cucullo, Couraud et al. 2008, Sivandzade and Cucullo 2018). Emulating the physical *in vivo* physiology can also be extended to CNS cells embedded within the parenchyma, for example, culturing microglia, astrocytes, and other CNS cells in a 3-dimensional (3D) matrix (Haw, Tong et al. 2014).

BV-2 cells derived from raf/myc-immortalised murine neonatal have been extensively studied as a suitable alternate to primary microglia. (Blasi, Barluzzi et al. 1990, Henn, Lund et al. 2009). BV-2 cells display similar morphological properties, expression of

microglia markers, cytokine secretion and functional properties to primary microglia (Blasi, Barluzzi et al. 1990, Henn, Lund et al. 2009). Cells within the host reside in heterogenous states containing quiescent, senescent, apoptotic, terminal differentiated cells that present different preferred endocytic routes (Hinze and Boucrot 2018). Primary cell cultures contain heterogenous cell populations that better represent the heterogeneity seen *in vivo* (Hinze and Boucrot 2018, Jones, Booth et al. 2020), but this can often make it challenging to investigate specific signal transduction pathways (Mahe, Fisson et al. 2001, Guillemin and Brew 2004, Hanisch and Kettenmann 2007, Henn, Lund et al. 2009). Although similar activations responses are seen between the two, primary microglia appear far more sensitive to LPS (Scheiblich, Roloff et al. 2014). Comparative analysis of primary microglia and BV-2 cells, showed that upon stimulation with 1 μg/ml LPS a larger increase in nitrite production (7.5 μM versus 2 μM), a 3-fold increase in Iba-1 expression and increased TNF-α secretion (3000 pg/ml verses 2000 pg/ml) was reported in primary cells compared to BV-2 (Horvath, Nutile-McMenemy et al. 2008). Another consideration when working with primary microglia is the region from which microglia are isolated. Microglia from different anatomical regions of the brain display different reactions to responses due to their specific regional adaptations (Mahe, Fisson et al. 2001, Hanisch and Kettenmann 2007). BV-2 cells respond more uniformly in response to stimuli (Blasi, Barluzzi et al. 1990, Henn, Lund et al. 2009), providing consistency when undertaking research. These considerations should therefore be considered when extrapolating data obtained from immortalised microglia cells.

3.5. Concluding remarks

BEVs are increasingly being viewed as mediators in long-distance microbiota-gut-brain communication pathways. Due to their ability to cross host barriers, be acquired by a range of host cells and deliver cargo, they provide an intriguing possibility of being utilised therapeutically to deliver drugs for the treatment of various diseases, including non-gastrointestinal disorders. The data presented in this Chapter provides insights into the possibility of BEVs from dominant commensal members of the human gut microbiota, Bt, to cross epithelial and endothelial cell barriers and induce immunomodulatory effects in CNS resident macrophaes. Further investigation into the

possible neuroprotective effects of Bt-BEVs in the context of the gut-brain axis is needed.

To help facilitate this a more robust, replicable and quantifiable approach to isolate and determine BEV and cargo delivery into host cells is needed to help elucidate the underlying mechanisms of the processes involved and bring consistency and comparability in the field of BEV research.

3.6. Methods

3.6.1. Preparation of bacterial cultures and BEV isolation

Bt VPI-5482 was grown under anaerobic conditions at 37°C in 500 ml BDMr media containing 2.61 g KH_2PO_4 (Sigma, P5655), 7.03 g K_2HPO_4*$3H_20$ (Sigma, C7902), 15 mM NaCl (Sigma, 31434), 8.5 mM $(NH_4)_2SO4$ (Sigma, 31119), 30 mM glucose (Sigma, G7021), 0.1 mM $MgCl_2$*$6H_2O$ (Sigma, M2393), 50 μM $CaCl_2$*$2H_2O$ (Sigma, C7902), 0.2 mM L-Histidine (Sigma, H8125), 2 μM Hemin (Sigma, 51280), 100 nM vitamin B12 (Sigma, V2876), 6 μM vitamin K3 Menadione (Sigma, M5625), 4.1 mM L-cysteine hydrochloride (Sigma, W778567) and 1.4 μM $FeSO_4$*$7H_2O$ in deionised H_2O. Bacterial cultures at OD_{600} 1.5-2.5 were collected, centrifuged at 6037 x g for 30 min at 4°C and supernatants filtered through 0.22 μM pore-size polyethersulfone membrane (Satorius, 180C5) to remove cell and debris. The supernatants were then concentrated by crossflow ultrafiltration (100 kDa MWCO, Vivaflow 50R, Satorius) and the retentate rinsed once with 500 ml PBS (pH 7.4). The BEV suspensions were concentrated to 1 ml in sterile PBS, filtered through 0.22 μM pore-size syringe-filters (Hydrostat) and stored at 4°C.

3.6.2. Bt-BEV labelling

The lipophilic dyes 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiD) and 3,3-dioctadecyloxacarbocyanine perchlorate (DiO) and Alexa Fluor® Protein Labelling kit was used to label isolated Bt-BEVs. For lipophilic labelling, Bt-BEVs ($1x10^{12}$/ml) were labelled with 5% (v/v) DiO/DiD Vybrant cell

labelling solution (Molecular Probes, V-22886, V22887) by incubating at 37°C for 30 min. Unbound dye was removed by washing 3 x PBS using centrifugal filters (100 kDa, Merck UFC510024). For protein labelling, the protein content of Bt-BEVs were first determined using BCA protein assay kit for low protein concentrations (Abcam, ab207002), and if greater than 2 mg/ml, Bt-BEVs were diluted accordingly in 0.1 M sodium bicarbonate and transferred to a vial of Invitrogen™ AlexaFluor™488/647 reactive dye (ThermoFisher, A20173, A10235). The reaction mixture was gently stirred for 1 hr at room temperature and resulting conjugates purified through a purification column containing purification resin (Bi0-Rab BioGel P-30 fine size exclusion purification resin which separates free-dye from protein MW>40000). The labelled protein fractions were then collected, and and Bt-BEV size/distribution analysed using Nanoparticle tracking analysis.

3.6.3. Cell cultures

The human cerebromicrovascular endothelial cell line, hCMEC/D3, was cultured at 37°C, 5% CO_2 in endothelial cell growth basal medium-2 (EBM-2, Lonza, CC3156) supplemented with EBM-2 endothelial SingleQuots kit (Lonza, CC4176) and 1% Penicillin/Streptomycin (Sigma, P4333). Cells were cultured on either 1:20 collagen 1 (Thermo Fisher, A1048301) or 1:20 Corning® Matrigel® Growth Factor Reduced (GFR) Basement Membrane Matrix (Life Sciences, 354230). For experiments, cells were cultured without vascular endothelial growth factor (VEGF) to enhance expression of cell junction proteins (Cristante, McArthur et al. 2013). The human colonic epithelial cell line, Caco-2 (ECACC-86010202), was cultured at 37°C, 5% CO_2 in MEM (Sigma, M5650) supplemented with 10% fetal bovine serum (FBS) (Sigma, F0804), 1% L-glutamine (Sigma, G7513), 1% Penicillin/Streptomycin (Sigma, P4333). For murine microglia, BV-2 cells (AMSBIO, AMS.EP-CL-0493) cells were cultured at 37°C, 5% CO_2 in MEM (Sigma, M5650) supplemented with 10% FBS (Sigma, F0804) and 1% Penicillin/Streptomycin (Sigma, P4333). For non-differentiated neuronal cultures, SH-SY5Y cells were cultured in EMEM supplenmented with 1% L-glutamine (Sigma, G7513), 1% Penicillin/Streptomycin (Sigma, P4333) and 15% FBS (Sigma, F0804).

3.6.3.1. One-cell culture experiments

hCMEC/D3 cells were cultured on 1:20 collagen coated 12-well chamber slides (IBIDI, 81201) and BV-2 cells on uncoated glass-coverslips in 24-well culture plates (Fig. 3.3A).

3.6.3.2. Two-cell culture experiments

For the *in vitro* gut-brain model, Caco-2 cells were cultured on the PET membrane of ThinCert™ inserts, 0.4 μM pore diameter (Greiner Bio-one, 622641) and hCMEC/D3 cells cultured on collagen-coated glass coverslips in the basal compartment of 24-well culture plates (Fig. 3.3B). For the *in vitro* BBB model, hCMEC/D3 cells were cultured on the underside of ThinCert™ PET membrane pre-coated with either collagen, Matrigel® or uncoated. BV-2 cells were cultured on glass coverslips in the basal compartment of 24-well culture plates (Fig. 3.3B).

3.6.3.3. Three-cell culture experiments

Caco-2 cells were cultured on the PET membrane of ThinCert™ inserts, 0.4 μM pore diameter (Greiner Bio-one, 622641) and hCMEC/D3 cells cultured on the underside on either collagen, Matrigel® or non-coated PET membrane. SH-SY5Y cells were cultured on glass coverslips in the basal compartment of the 24-well culture plate (Fig. 3.3C).

3.6.4. Fluorescence microscopy

Labelled Bt-BEVs ($1x10^{11}$/ml) or PBS were added to cell cultures for 24 hrs at 37°C, 5% CO_2. Cells were fixed using 4% paraformaldehyde, permeabilised with 0.25% Triton-X100 in PBS (Sigma, X100) and blocked with 10% goat serum (Sigma, G9023). Alexa 488-phalloidin (1:1000, Thermo Fisher, A12379) was used to visualise intracellular membranes, anti-LAMP1 (1:100,) for lysosomes, recombinant rabbit monoclonal anti-Iba-1 (1:500, Abcam, ab178846) for microglia activation, recombinant rabbit monoclonal anti-TREM119 antibody (1:250, Abcam, ab209064) and recombinant rabbit monoclonal anti-CD45 antibody (1:250, Abcam, ab40763) for microglia markers, and goat anti-rabbit IgG (1:250, Abcam, ab205718). All secondary antibodies used were Invitrogen™ Alexa Fluor®594 goat anti-rabbit Ig (1:1000, Invitrogen, 27117) and Alexa Fluor®488 goat anti-rabbit IgG (1:1000, Abcam,

ab150077). Hoechst 33342 nuclear stain (1:2000, Thermo Fisher, H1399) was used for nuclear visualisation and cells mounted using Invitrogen™ ProLong™ Diamond Antifade mountant (Thermo Fisher, P36961).

Cells were imaged using a Zeiss Axio Imager M2 microscope equipped with 40x/air objective and Zen blue software (Zeiss). In addition, Zeiss LSM880 confocal microscope equipped with 63x/1.4 oil DIC objective and Zen black software (Zeiss) was used to obtain higher resolution images. Fluorescence was recorded at 405 nm (blue), 488 nm (green), 594 nm (red) and 647 nm (far-red). Fluorescent intensity of Iba-1 staining in BV-2 cells was quantified using sum fluorescent pixel intensity of the field of view (FOV) using a macro written in Image J/FIJI v1.52p. Quantification of cell numbers were performed manually on ImageJ/FIJI v1.52p software using raw, unprocessed images.

3.6.5. Transepithelial electrical resistance

Transepithelial electrical resistance measurements were undertaken using Transwell inserts, 0.4 μM pore diameter (Greiner Bio-one, 622641) in 24-well culture plates. Caco-2 monolayers were seeded onto the apical compartment of the Transwell PET membrane until fully confluent. Bt-BEVs ($1x10^{11}$/ml) or PBS were then added to the apical compartment and TEER measurements recorded using an EVOM2 epithelial voltmeter with chopstick electrode (World Precision Instruments Inc.).

For hCMEC/D3 monolayer optimisation, hCMEC/D3 cells were seeded on either collagen -coated, Matrigel-coated or uncounted membranes of ThinCert™ inserts and TEER measurements recorded daily for 9-days.

3.6.6. Nitrite concentrations

Nitrite (NO_2^-) concentrations in cell culture medium following exposure to Bt-BEVs were determined using the Griess Reagent system (Promega, G2930). A nitrite standard reference curve was generated for each assay undertaken. Briefly, media from samples collected was centrifuged to remove cell debris, aliquoted and stored at -20°C until use. In a 96-well flat-bottom enzymatic assay plate, 50 μl of samples were added to wells in duplicate. To this 50 μl of Sulphanilamide solution was added and incubated

in the dark at room temperature for 5-10 min. Following this 50 µl of NED solution was added and further incubated in the dark at room temperature for 5-10 min. Absorbance between 520-550 nm was recorded within 30 mins using a microplate spectrophotometer (BioRad Benchmark Plus™).

3.6.7. TNF-α concentrations

TNF-α secretion from BV-2 cells was measured using the Invitrogen^{TM} TNF-α mouse uncoated ELISA (Thermo Fisher, 88-7324-88) according to manufacturer's protocols.

3.6.8. Nanoparticle Tracking Analyser

ZetaView ® Nanoparticle tracking analyser (ParticleMetrix) was used to detect bacterial BEVs (488 nm) at 100-400 nm. Sample dilutions were prepared in 1 ml particle-free H_2O. With the fluorescent filter mounted, 1 ml of diluted sample was injected. The following measurements were used: run video acquisition>multiple acquisitions, low bleaching, dose sub volume, number of experiments=11, number of cycles=1. Analysis of 488 nm particles was then recorded.

3.6.9. Statistical analysis

All statistical analysis was conducted on GraphPad Prism 9.2.0.

4. Discussion

Bt is one of the most abundant symbionts in the human GIT. This gram-negative anaerobe has a 4779-member proteome devoting ~18% of its genes to an extensive repertoire of hydrolyses facilitating the degradation of complex and other wise indigestible dietary polysaccharides (Salyers 1984, Xu, Bjursell et al. 2003, Eckburg, Bik et al. 2005, Martens, Chiang et al. 2008, Porter, Luis et al. 2018). The ability of Bt to stably colonised and adapt to the fluctuating environment of the human GIT is further contributed to by its dynamic cell surface architecture that promotes interactions with and evasion of the mucosal immune system (Lee, Donaldson et al. 2013, Porter, Luis et al. 2018). Emerging evidence supporting its anti-inflammatory, mucosal barrier promoting properties (Wrzosek, Miquel et al. 2013, Brown, Ke et al. 2019, Delday, Mulder et al. 2019, Li, Hao et al. 2021) have made it a become a versatile model organism of the gut microbiota and a promising candidate for microbial therapeutic applications.

4.1. Bt and its major fermentation outputs regulate EEC populations

The results displayed in Chapter 2 provide evidence to show that Bt can singularly normalise EEC populations and networks in the intestinal epithelium of GF mice. Furthermore, the major fermentation metabolites of Bt reproduce the effects of the bacterium, suggesting that Bt mediates its effects, on EEC populations at least through its fermentation products. Whether all three fermentation products, acetate, propionate or succinate, contributed to this effect is yet unknown. Bt is able to produce multiple organic acids due to its large repertoire of genes involved in metabolic pathways (Porter and Larsbrink 2022). The proportions of organic acids produced by Bt vary considerably amongst different strains and depending on nutrition available in their environment. The production ratio also likely differ significantly according to whether they are in their native gut habitat or outside in artificial culture, and may affect the genetic repertoire or enzyme function involved in polysaccharide utilisation (Macfarlane and Macfarlane 2003, Adamberg, Tomson et al. 2014, Lu, Sethu et al. 2018, Porter and Larsbrink 2022).

The role of FFAR signalling in EECs beyond regulating hormone expression and secretion is limited. FFAR are amongst many nutrient GPCR expressed on EECs, each with its own specificity to individual metabolites and macromolecules (Nohr, Pedersen et al. 2013, Husted, Trauelsen et al. 2017). Moreover, GPCR expression on EECs are not static and can be influenced by availably of nutrients (Widmayer, Küper et al. 2012, Remely, Aumueller et al. 2014, Peiris, Aktar et al. 2018) suggesting that there is plasticity within the expression profile of nutrient receptors on EECs. Whether this is reflected in some of the reported health benefits of increased SCFA concentrations is unclear (Chambers, Viardot et al. 2015, Chambers, Byrne et al. 2018).

It was recently demonstrated that SCFA exposure at physiologically relevant concentrations alters the expression of specific nutrient receptors (taste receptors) and peptide hormone transcripts in STC-1 cells. The most significant change was observed with TAS1R1, where a 6.7-fold increase in expression following incubation with SCFAs (acetate, propionate and butyrate in a 3:1:1 ratio with final concentration of 10 mM) was detected. These effects were then confirmed to be mediated by propionate and butyrate as treatment with acetate alone did not alter TASR1 expression. Intriguingly, SCFAs did not alter the expression levels of FFAR2 or FFAR3 in STC-1 cells during the 5 hr incubation (Shackley, Ma et al. 2020). Moreover propionate, but not butyrate significantly reduced expression of CCK mRNA (Shackley, Ma et al. 2020). This contradicts previous reports of increased CCK mRNA levels following propionate incubation in GLUTag and the human NCI-H716 EEC line, although a longer period of stimulation (24 hr) was used (Zhang, Grosfeld et al. 2019), suggesting that the effects of propionate may be dependent on acute or chronic exposure. The reduced CCK mRNA expression reported here could also be a result of reduced gene expression or increased translation of CCK mRNA to protein (Shackley, Ma et al. 2020). Indeed, the reported opposing effects on CCK mRNA and protein levels following exposure to butyrate has been processed to entirely be dependent on continuing protein synthesis (Roche, Cordier-Bussat et al. 1996). These could be mediated via mechanisms involving FFAR signalling or HDAC inhibition. Given that the associated reduced CCK mRNA expression in this study were predominately by propionate but not butyrate, which is the primary HDAC inhibitor, it can be plausible that increased CCK synthesis contributed to its reduced expression at the transcript

level (Shackley, Ma et al. 2020). However, owing to inherent limitations of using immortalised EEC lines, the results may not be representative of the in-situ behaviour of EECs in the intestine. Whether Bt and/or APS induces the expansion of EEC populations or increases peptide hormone content in EECs remains unclear and requires further methods (mRNA, secretion and histone acetylation activity analysis) to accurately determine hormone gene expression and protein levels and to identify possible nutrient receptors involved.

It has recently been proposed that microbial fermentation products mainly activate nutrient receptors after being absorbed across the epithelium, in contrast to previous belief that nutrient GPCRs were restricted solely to the apical membrane (Reimann, Tolhurst et al. 2012). There is evidence of FFAR2 being expressed on the basolateral membrane and neuropod basal extensions of EECs (Karaki, Mitsui et al. 2006, Engelstoft, Egerod et al. 2008). It could also be plausible that apical sensing of metabolites via FFARs is not compatible as luminal metabolite concentrations are too high (~50-100 mM) (Cummings, Pomare et al. 1987, Tan, Mahadeva et al. 2014) meaning that apically located FFARs would be maximumly activated, independent of the subtle fluctuations in metabolites concentration. It is also likely this could lead to receptor desensitisation, therefore it is likely that following absorption across the epithelium, metabolites stimulate FFARs expressed on the basolateral membranes, immune cells and afferent nerve fibres (Nohr, Pedersen et al. 2013, Husted, Trauelsen et al. 2017). Indeed, Bt has been shown to promote T cell differentiation in a DSS-induced murine colitis model. Bt regulates these effects by increasing levels of acyl hydrogen receptor (AHR) ligands, thereby enhancing activation of the AHR in specific T cell subtypes (Li, Hao et al. 2021). At a molecular level, AHR activation by Bt treatment was associated with altered methylation of the transcription factor Foxp3 promotor (Li, Hao et al. 2021).

Molecular mechanisms that underpin the symbiotic host-bacterial relationship. Despite this the ability of a single bacterial species to regulate EEC populations and distribution along the GIT, is a promising insight into the role of the gut microbiome in health.

4.2. Bt-BEVs as mediators in gut-brain communication

The results display in Chapter 3 provide evidence supporting the role of BEVs in intercellular signalling mediators in the gut-brain axis. Using cell culture-based models, it was demonstrated that Bt-BEVs can cross intestinal epithelial and brain blood vessel endothelial cells and be taken up by CNS microglia and neurones. Upon internalisation most BEVs are trafficked to intracellular lysosomes, indicating that most are fated for degradation. Whether they can resist degradation or are able to target other intracellular organelles remain to be determined.

Recent investigations by Badi et al., into the morphology, diameter, protein content and endotoxin content of Bt-OMVs revealed that vesicles extracted ranged between 30-110 nm in diameter (Ahmadi Badi, Moshiri et al. 2020) like vesicles isolated for experiments conducted in chapter 3. However, it cannot be concluded whether these were pure OMV isolates as gram-negative bacteria are known to co-produce other types of vesicles, including OIMVs. Transmission electron microscopy can help assess morphology of isolated vesicles but determining the proportions of single or double-membrane vesicles remain challenging.

4.3. Future directions

The use of GF models used in Chapter 2 holds some limitations as they display aberrant intestinal morphology and function. The presence of the gut microbiota is associated with a higher epithelial turnover (~53 hr), whereas in the absence of gut microbiota epithelial transit time from the crypt base to villus tip is around 115 hr (Savage, Siegel et al. 1981). Generating intestinal organoids that maintain self-renewal and cellular diversity that more closely resembles *in vivo* conditions would provide a valuable tool in the study of EEC interactions. Organoids also provide potential for expansion of EEC populations, generating larger pools of these otherwise rare cells. It may be the case that short-term 2D crypt derived cultured do not possess the potential to fully recapitulate *in vivo* EECs subtypes and hormone expression repertoire. Further work is needed to optimise this system, and to determine the extent to which they differ from 3D organoid models. Whether FFAR expression profiles on EEC are influenced

by external nutrient levels remains to be elucidated. Contrary to past belief, it is also possible that given the presence of basolateral nutrient receptors, EECs within 3D intestinal organoids can respond to nutrients added to the media they are maintained within, and do not require exposure to the luminal (apical) membrane.

For the experiments undertaken in chapter 3, replication with suitable positive and negative controls, are needed. It is particularly important to compare Bt-BEV responses to that of LPS, and whether either pre- or post-treatment with Bt-BEVs confer any protective or preventative effects against LPS-induced damage. Determining whether exposure and internalisation of Bt-BEVs induces transcriptional levels alterations will support immunohistochemical data.

It will be interesting to determine the significance of vesicle diversity especially as this field remains largely unexplored. A multifaceted approach for efficient and comprehensive BEV characterisation, will help broaden the understanding of BEVs in biological functions, improve experimental reproductivity and allow comparisons between studies. Therefore, future work should include to develop and optimise Bt culture and BEV isolation, to enable higher yield purity, using NTA and protein assays to provide detail on vesicle enumeration and size distribution. Generating mixed-culture models to better recapitulate *in vivo* BBB, where numerous interactions between cells within the NVU contribute to the function and integrity of the *BBB in vivo*. Generating models using primary cultures will also provide useful insight into whether these cells respond in similar ways to Bt-BEVs as immortalised cell lines. Translating this to *in vivo* animal models will then further validate the ability of bacterial BEVs as significant intracellular and intercellular mediators in microbial-host communication.

Overall, this the results presented in this Thesis can be used towards developing novel approaches where microbial metabolites and products can be used as biomarkers and therapeutic treatment. For many diseases, particularly neurological and neurodegenerative diseases, obtaining early disease diagnosis is often challenging and invasive. BEVs and metabolites can be utilised to develop novel biomarkers for disease. Indeed, the metabolic profile of BEVs is reported to be different amongst

health and patients with neurodegenerative diseases (Wei, Wei et al. 2019) and could be used as a non-invasive, relatively straight-forward approach for diagnosis.

In addition to development of biomarkers, the current use of BEV in vaccine delivery provides promising potential for drug delivery. Microbial metabolites too have also attracted therapeutic interest and are currently being tested in clinical trials to assess neurological, metabolic, and GI functions. However, the functions of these metabolites are often highly context dependent and can have differential effects on the molecular aetiology of disease. In cases where metabolites cause or contribute to disease pathophysiology, inhibition of receptor activation or downstream signalling may be required for the pharmacological intervention.

Appendix A

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Regulation of Enteroendocrine Cell Networks by the Major Human Gut Symbiont *Bacteroides thetaiotaomicron*

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Gut microbes have critical roles in maintaining host physiology, but their effects on epithelial chemosensory enteroendocrine cells (EEC) remain unclear. We investigated the role that the ubiquitous commensal gut bacterium *Bacteriodes thetaiotaomicron* (Bt) and its major fermentation products, acetate, propionate, and succinate (APS) have in shaping EEC networks in the murine gastrointestinal tract (GIT). The distribution and numbers of EEC populations were assessed in tissues along the GIT by fluorescent immunohistochemistry in specific pathogen free (SPF), germfree (GF) mice, GF mice conventionalized by Bt or *Lactobacillus reuteri* (Lr), and GF mice administered APS. In parallel, we also assessed the suitability of using intestinal crypt-derived epithelial monolayer cultures for these studies. GF mice up-regulated their EEC network, in terms of a general EEC marker chromogranin A (ChrA) expression, numbers of serotonin-producing enterochromaffin cells, and both hormone-producing K- and L-cells, with a corresponding increase in serum glucagon-like peptide-1 (GLP-1) levels. Bt conventionalization restored EEC numbers to levels in SPF mice with regional specificity; the effects on ChrA and L-cells were mainly in the small intestine, the effects on K-cells and EC cells were most apparent in the colon. By contrast, Lr did not restore EEC networks in conventionalized GF mice. Analysis of secretory epithelial cell monolayer cultures from whole small intestine showed that intestinal monolayers are variable and with the possible exclusion of GIP expressing cells, did not accurately reflect the EEC cell makeup seen *in vivo*. Regarding the mechanism of action of Bt on EECs, colonization of GF mice with Bt led to the production and accumulation of acetate, propionate and succinate (APS) in the caecum and colon, which when administered at physiological concentrations to GF mice via their drinking water for 10 days mimicked to a large extent the effects of Bt in GF mice. After withdrawal of APS, the changes in some

EEC were maintained and, in some cases, were greater than during APS treatment. This data provides evidence of microbiota influences on regulating EEC networks in different regions of the GIT, with a single microbe, Bt, recapitulating its role in a process that may be dependent upon its fermentation products.

Keywords: intestinal microbiota, *Bacteroides thetaiotaomicron*, germfree mice, enteroendocrine cells, short chain fatty acids

INTRODUCTION

A mutualistic relationship exists between the intestinal microbiota and the host in which commensal microbes provide the host with essential protective and metabolic functions, including fermentation of complex plant-based carbohydrates to produce metabolites such as short chain fatty acids (SCFAs) that are an important energy source for host cells (McNeil, 1984; Bergman, 1990). In turn, the host provides the microbiota with nutrients essential for their colonization and survival (Savage, 1977; Tremaroli and Backhed, 2012). Chronic metabolic diseases including metabolic syndrome, obesity and diabetes have been associated with structural and/or functional changes in the intestinal microbiota and principally, the prokaryome (Carding et al., 2015). The causal nature of these associations remains to be determined, although alterations in energy extraction from food (Backhed et al., 2004; Turnbaugh et al., 2006), increased nutrient harvesting (Tremaroli and Backhed, 2012) and appetite signaling (Plovier and Cani, 2017; Covasa et al., 2019) are potential mechanisms. Animal models and in particular germfree (GF) rodents have been instrumental in advancing our understanding of the complexity of the intestinal microbiota and providing mechanistic insights of microbial-host interactions at the epithelial interface (Tremaroli and Backhed, 2012).

Enteroendocrine cells (EECs) are scattered throughout the entirety of the epithelium of the gastrointestinal tract (GIT) and are key sensors of microbial metabolites in the intestinal lumen. They sense changes within the luminal environment and relay signals via the production and secretion of peptide hormones, which act on local nerve endings of the enteric nervous system or other cells within the intestinal mucosa that converge on hypothalamic feeding circuits to regulate and coordinate metabolism and food intake (Beutler et al., 2017). Via the circulatory system and vagal nerves their influence can extend beyond the GIT, affecting the function of organs such as the brain, liver and adipose tissues (Gribble and Reimann, 2016). EECs are divided into subgroups depending on their secreted hormones and location along the GIT. Prominent subsets include L, K and enterochromaffin cells (EC). L cells secrete mainly glucagon-like peptide-1 (GLP-1) or peptide YY and are found throughout the GIT but are more densely populated in the colon. K cells secrete glucose-dependent insulinotropic polypeptides (gastric inhibitory peptide, GIP) and are mainly found in the upper small intestine. Enterochromaffin cells, found throughout the GIT, make up the single largest population of endocrine cells in the intestinal epithelium and produce mainly serotonin or 5-hydroxytryptamine (5-HT) (Sjolund et al., 1983).

GLP-1 and GIP are the primary incretin hormones that cause the release of insulin from pancreatic beta-cells following meal ingestion (Baggio and Drucker, 2007). The administration of probiotic bacteria to obese and diabetic mice increases glucose tolerance, L cell number, intestinal proglucagon mRNA and plasma GLP-1 levels, suggesting that intestinal microbes can play a role in altering glucose homeostasis and EEC activity (Cani et al., 2008). However, our understanding of how the intestinal microbiota initiates signaling in EEC is incomplete. Key insights have been obtained from studies using wildtype or transgenic strains of germfree animals and by examining the impact of substrates and metabolites of microbiota metabolism. Evidence for the ability of the intestinal microbiota to influence L cells directly has come from germfree mice expressing a proglucagon reporter gene in which conventionalization with an unfractionated microbiota has been shown to modulate the L cell transcriptome in the ileum (Arora et al., 2018). Amongst microbial metabolites, SCFAs have been the most intensively studied with those produced from the fermentation of dietary fiber increasing GLP-1 and peptide YY (PYY) levels in tissues and plasma (Keenan et al., 2006; Zhou et al., 2006, 2008). SCFAs signal through G-protein coupled receptors (GPCRs) that co-localize with EECs (Karaki et al., 2006), such as free fatty acid receptor (FFAR) 1 and 3 (also known as GPR41 and 43, respectively, expressed on L cells) (Tazoe et al., 2008, 2009; Tolhurst et al., 2012; Nohr et al., 2013). GPR43-deficient mice display lower GLP-1 plasma levels and reduced glucose tolerance highlighting the importance of these receptors in microbial signaling in EECs (Tolhurst et al., 2012). In addition, oligofructose supplementation increases GIP plasma levels as well as affecting microbial composition (Girard, 2008; Tolhurst et al., 2012). Furthermore, GIP is involved in fat metabolism (Yip and Wolfe, 2000) and therefore is a potential target for microbiota modulation in obesity. Using two bacteria to conventionalize germfree mice a role for the GPR41 receptor in regulating host energy balance has been identified in a process involving bacterial modulation of PYY expression (Samuel et al., 2008). Aside from metabolic processes, the intestinal microbiota can affect neuronal signaling processes by altering 5-HT production. For example, the numbers of EECs are reduced in number in GF rats (Uribe et al., 1994), while the presence of indigenous spore-forming bacteria, mainly from the *Clostridial* spp., promotes 5-HT biosynthesis through increasing Tph1 expression, a rate-limiting enzyme involved in the biosynthesis of 5-HT tryptophan

Abbreviations: APS, acetate, propionate, succinate; Bt, *Bacteroides thetaiotaomicron*; ChrA, chromogranin A; EC, enterochromaffin cells; EEC, enteroendocrine cells; GIP, gastric inhibitory peptide; GF, germfree; GIT, gastrointestinal tract; GLP-1, glucagon-like peptide-1; 5-HT, -hydroxytryptamine; Lr, *Lactobacillus reuteri*; SCFA, short chain fatty acids; SPF, specific pathogen free.

(Yano et al., 2015; Zelkas et al., 2015). Conversely, 5-HT stimulates the growth in culture of bacterial species including *E. coli* and *Rhodospirillum* (Oleskin et al., 1998), suggesting a bi-directional relationship exists between EEC signaling and the gut microbiota.

Collectively, these studies suggest the intestinal microbiota has profound effects on EECs including regulation of production and secretion of their peptide hormones, which may occur via products of microbial metabolism acting directly on EECs. Here, we have undertaken a study to obtain more evidence of these putative mechanisms using conventional and GF mice to investigate the role that the ubiquitous and prominent commensal gut bacterium *Bacteroides thetaiotaomicron* (Bt) and its major metabolic output (acetate, propionate and succinate) (Hooper et al., 2002; Wrzosek et al., 2013; Curtis et al., 2014) have on EEC networks in the murine GIT.

MATERIALS AND METHODS

Bacterial Strains and Culturing

Bt (VPI 5482, ATCC) was grown anaerobically at 37°C in brain heart infusion medium (Oxoid) supplemented with 15 μM hemin. *Lactobacillus reuteri* (Lr; 100-23, DSMZ) was grown anaerobically at 37°C in MRS medium (Difco Laboratories).

Animal Handling

C57BL/6 mice of 8–12 weeks of age were housed in a specific pathogen free (SPF) Disease Modeling Unit (DMU) at the University of East Anglia (UEA), Norwich, United Kingdom and were maintained on standard chow at all times throughout the study. All experiments were conducted in accordance with the Home Office Animals (Scientific Procedures) Act 1986 under the license number PPL80/2545 at the UEA. C57BL/6 germfree (GF) mice were maintained in sterile isolators in the Quadram Institute Germ Free Facility within the DMU with the GF status being continuously monitored by microscopy, aerobic and anaerobic culturing, and PCR for bacterial contamination. GF mice were conventionalized by administrating 0.1 ml (1.4×10^9 cells/ml) of Bt or Lr in sterile PBS by oral gavage. Conventionalized GF mice were maintained in individual ventilated cages for up to 10 days. To assess extent of colonization, contents of the GIT were cultured under anaerobic conditions and colony counts determined. Additional aerobic and anaerobic cultures were performed to exclude contamination. In some experiments GF mice were administered via their drinking water a cocktail of APS at levels comparable to those in the gut lumen consisting of sodium acetate (95 μM Sigma-Aldrich), sodium propionate (29 μM, Sigma-Aldrich) and sodium succinate (5.6 μM, Sigma-Aldrich) (Mineo et al., 2006) for 10 days after which the APS-containing drinking water was replaced with regular drinking water (wash out) for a further 10 days.

Blood and Tissue Sampling

Sampling was carried out at the same time of day for all experiments. Blood samples were taken by cardiac puncture following euthanasia with 0.1 ml of Dipeptidyl peptidase IV (DPP-IV) inhibitor (BIO-TECHNE LTD.) per ml of blood, centrifuged at 1,000–2,000 × *g* for 10 min and the serum removed, aliquoted and stored at −20°C prior to analysis. The entire intestinal tract was excised, the contents removed by flushing with sterile Dulbecco's Phosphate Buffered Saline (DPBS), prior to dividing into anatomically distinct segments (duodenum, jejunum, ileum, proximal colon and distal colon) that were fixed in 10% neutral buffered formalin (Sigma-Aldrich) for 24 h at 20–22°C followed by 24 h in 70% ethanol at 4°C. Tissues were then processed through a xylene/alcohol dehydration and clearing series followed by wax infiltration. Segments were embedded in paraffin wax prior to sectioning (5 μm) and mounting on SuperFrost®Plus glass slides (VWR). ELISA (Millipore, MMHMAG-44K) was used to quantitate GLP-1 levels in serum.

Intestinal cfu

Content was obtained from all regions of the intestine (duodenum, jejunum, ileum, cecum, proximal colon and distal colon) from GF mice 5 days post- conventionalization with Bt, and weighed, prior to addition of 400 μl PBS to each sample. Samples were briefly vortexed, centrifuged at 1000 rpm for 10 min at 20–22°C and serial two-fold dilutions carried out and plated on BHI agar plates. Plates were incubated in an anaerobic cabinet (37°C, 5% CO_2) for 48 h. Colonies were counted and used to calculate the CFU/g of contents at 5-days post-colonization with Bt in germ-free mice.

Immunohistochemistry

Tissue sections were rehydrated through histoclear and a graded ethanol series. Following washing in dH_20, slides were heated in citric acid buffer (10 mM, pH 6) (Sigma-Aldrich) for antigen retrieval, washed further in Tris-buffered saline with Tween-20 (TBS-T) and incubated for 16 h at 4°C with either a rabbit polyclonal anti-GLP-1 (ab22625, Abcam), mouse monoclonal anti-GLP-1 antibody (ab23468, Abcam), rabbit monoclonal anti-GIP antibody (ab209792, Abcam), mouse monoclonal anti-GIP (021-04, Santa Cruz), rabbit polyclonal anti-Chromogranin A (ChrA) antibody (sc-13090, Santa Cruz Biotechnology), goat polyclonal anti-5-HT antibody (ab66047, Abcam) and Hoechst nuclear stain (Thermo Fisher). Unless specified, control antibodies were obtained from Abcam; rabbit IgG (ab37415) and monoclonal IgG (ab172730), mouse IgG2a (ab18415), mouse IgG_1 (IS5-21F5, Miltenyi Biotech), and goat IgG (ab37373). Tissues were washed in TBS-T and incubated with Alexa Fluor594 goat anti-rabbit Ig (27117, Invitrogen), Alexa Flour488 anti-mouse IgG (Thermo Fisher A11001), or Alexa Fluor594 donkey anti-goat Ig (A11057, Invitrogen) for 30 min at 20–22°C. Tissues were mounted using ProLong™ Diamond Antifade mountant (Thermo Fisher). The hemi-villous crypt region in each section of the GIT was used to enumerate EECs (**Supplementary Figure 1**) with the total number of epithelial cells in the same hemi-villous crypts also determined. A minimum of 20 hemi-villus crypts were counted for each section with at least 10 sections from each tissue sample and experimental group using a minimum of 3 mice per group to obtain EEC cell counts.

Metabolite Analysis by Nuclear Magnetic Resonance (NMR)

Acetate, butyrate, propionate and succinate were quantified in the contents of the duodenum, distal colon (SPF, GF, and GF-Bt), and cecum (SPF, GF, GF Bt, and GF Lr) (n = 5 ea.) using ^{1}H NMR spectroscopy. Samples were prepared by mixing ∼50 mg of the sample with 12 times the volume of phosphate buffer-D2O (0.1 M K_2HPO_4, 0.1 M NaH_2PO_4, 145.1 μM TSP-d4 mixed 1:1 with deuterium oxide [D2O]). The ^{1}H NMR spectra were recorded on a 600 MHz Bruker Advance spectrometer (Bruker BioSpin GmbH, Germany). Each ^{1}H NMR spectrum was acquired with 64 scans, a spectral width of 12,500 Hz, and an acquisition time of 2.62 s. The "noesygppr1d" pre-saturation sequence was used to suppress the residual water signal with low power selective irradiation at the water frequency during the recycle delay (D1 = 2 s) and mixing time (D8 = 0.15 s). A 90_ pulse length of 8.8 μs was set for all samples. Spectra were transformed with a 0.1 Hz line broadening and manually phased in TopSpin 3.9.1, and the chemical shift scale referenced to TSP. The spectra were then baseline corrected, removing the broad envelope between 0.7 and 4.5 ppm using AMIX 3.9.15 (underground removal tool, filter width = 20 Hz). Acetate, butyrate, propionate, and succinate were quantified using the Chenomx NMR Suite 8.12.

Intestinal Crypt Isolation and Culture

The intact small intestine was flushed with ice-cold DPBS, opened longitudinally and villi removed by gentle scraping using a glass coverslip. Tissues were then cut into 5–8 mm pieces, vigorously washed 5 times in ice-cold DPBS and transferred to 50 ml tubes containing 15 ml Gentle Cell Dissociation Reagent (Stem Cell Technologies) and incubated at 20–22°C for 15 min on a rolling platform. Tissues were then washed in ice-cold DPBS to release the crypts and filtered to remove excess debris using a 70 μm cell strainer (Corning). The crypt suspensions were then centrifuged at 300 × g for 3 min at 20–22°C. Supernatant was removed, and crypt pellets were resuspended in IntestiCult Organoid Growth Medium Mouse (Stem Cell Technologies) supplemented with Penicillin/Streptomycin, and Y-27632 ROCK inhibitor (Stem Cell Technologies) to prevent anoikis. The crypts were then plated onto glass coverslips (Agar Scientific Ltd.) in 24 well cell culture plates (Greiner Bio-One Ltd.) coated with 1:20 dilution (in DPBS) of Matrigel Basement Membrane Matrix (Scientific Laboratory Supplies) for 20–24 h to form semi-confluent monolayers. Monolayers were fixed in 10% neutral buffered formalin, washed with DPBS and permeabilized with 0.25% Triton X100 in DPBS, then incubated in blocking buffer (DPBS containing 10% goat serum (Sigma-Aldrich). Cultures were then incubated with rabbit polyclonal anti-GLP-1 antibody (ab22625, Abcam), rabbit monoclonal anti-GIP antibody (ab209792, Abcam), or rabbit polyclonal anti-ChrA antibody (sc-13090, Santa Cruz Biotechnology) and mouse anti-E-cadherin antibody (610181 BD Transduction Laboratories) for 2 h at 20–22°C. Following further washes in DPBS, monolayers were incubated for 30 min with the secondary antibodies, Alexa Fluor594 goat anti-rabbit (37117, Invitrogen) and Alexa Fluor488 goat anti-mouse (A11001, Invitrogen), followed by Hoescht (Thermo Fisher). The monolayers were washed with H_20, coverslips carefully removed and mounted on glass slides using ProLong Diamond Antifade mountant (Thermo Fisher).

Statistical Analysis

For box and whisker plots, the box extends from the 25th to 75th percentiles with the horizontal line within the box representing the median and the whiskers representing the minimum and maximum values. Other graphed date sets are expressed as mean ± SEM. For immunohistochemistry, data was analyzed by fitting a mixed model (GraphPad Prism 8.0). Statistical analysis was performed using a two-way ANOVA with $p < 0.05$ defined as significant with Geisser-Greenhouse correction not used.

RESULTS

To determine the impact of the intestinal microbiota on EEC networks, the distribution and number of several key EEC subsets (i.e., K, L, and EC cells) were compared in SPF and GF mice, and in GF mice after mono-conventionalization with the commensal gut bacteria Bt or Lr, or after administering physiological concentrations of the major fermentation products (acetate, propionate, and succinate; APS) of Bt. EEC numbers were determined by counting antibody stained cells within the hemi-villous crypt region (**Supplementary Figure 1**) counting a minimum of 20 hemi-villus crypts for each section and at least 10 sections from each tissue sample and experimental group using a minimum of 3 mice per group.

Distribution of EECs in the GIT of SPF and GF Mice

The small intestine of adult SPF and GF mice was divided into the anatomically distinct duodenum, jejunum and ileum. Although EECs can express more than one hormone (Egerod et al., 2012; Habib et al., 2012; Sykaras et al., 2014), they can be globally identified by ChrA expression which is stored and secreted by the majority of EECs (Massironi et al., 2016). Immunohistochemical staining of formalin fixed sections showed that cells reactive with anti-ChrA antibodies had the funnel shape morphology typical of EECs and were evenly distributed along the length of the intestine of SPF mice (**Figure 1A** and **Supplementary Figure 2**). Non-specific, background staining with isotype matched control antibodies was very low or absent (**Supplementary Figure 2**). In GF mice all regions of the small intestine contained significantly higher numbers ($p < 0.05$ to 0.01) of ChrA-expressing EECs compared to SPF mice (**Figure 1A**). In the colon, the distribution and number of ChrA-expressing cells were similar in SPF and GF mice with no regional differences being apparent after comparing the proximal and distal colon (**Figure 1A**). To assess if the differences and similarities noted in ChrA$^+$ EECs in SPF and GF mice reflected those of distinct populations of EECs, the distribution and number of GLP-1, GIP and 5-HT expressing cells were examined.

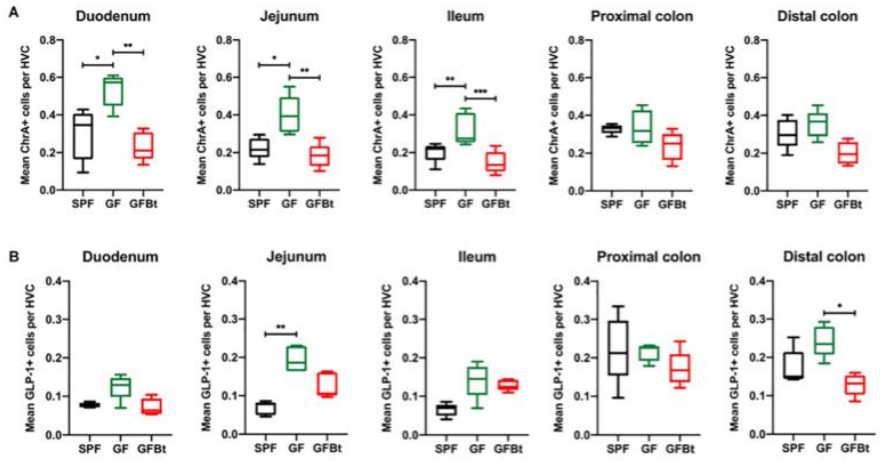

FIGURE 1 | Impact of colonization of GF mice with Bt on the distribution of ChrA$^+$ and GLP-1$^+$ EECs. ChrA$^+$ **(A)** and GLP-1$^+$ **(B)** cells were counted in the hemi-villus crypt (HVC) of sections of tissue obtained from different regions of the GIT of SPF ($n = 10$), GF ($n = 11$) and GF mice mono-colonized by Bt (GFBt, $n = 10$) mice. The box of the box whisker plots extends from the 25th to 75th percentiles with the horizontal line within the box representing the median and the whiskers representing the minimum and maximum values. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Regional Variation in Individual EEC Populations in the GIT of SPF and GF Mice

Analysis of GLP-1$^+$ cells in GF and SPF mice using two different (mouse and rabbit-derived) anti-GLP-1 antibodies revealed that GLP-1$^+$ cells were equivalent along the entire length of the small intestine in both groups of animals with the exception of the jejunum where they were present in significantly higher numbers ($p < 0.01$) in GF mice (**Figure 1B** and **Supplementary Figures 2** and **4**). Analysis of plasma levels of GLP-1 in fed mice showed that GF mice had higher levels than SPF mice, although the differences were not statistically significant (**Figure 2**). The distribution of GIP-expressing EECs in the small intestine of SPF and GF animals was the same as that of GLP-1 expressing EECs with significant differences only evident in the jejunum (**Figure 3A** and **Supplementary Figure 3**). In the colon, and in contrast to GLP-1 expressing EECs that were similar in SPF and GF animals, an approximately two-fold increase in GIP-1 expressing cells were seen in the distal colon of GF mice (0.188 ± 0.02 versus 0.084 ± 0.014 cells/hemi-villus crypt of GF and SPF mice, respectively; **Figure 3A**). In light of previous inconsistent findings regarding the presence of GIP-expressing EECs in the colon (Jorsal et al., 2017; Billing et al., 2019; Roberts et al., 2019), we used two different anti-GIP (mouse and rabbit-derived) antibodies to identify GIP expressing cells in the colon of both SPF and germfree mice (**Supplementary Figure 5**). After Bt-conventionalization the levels of GIP expressing cells along the GIT were reduced to the levels seen in SPF mice (**Figure 3A**). 5-HT expressing EEC also displayed region-specific difference in their distribution in the GIT of GF versus SPF mice (**Figure 3B** and **Supplementary Figure 3**) with significantly higher numbers of positive cells ($p < 0.01$) in the jejunum and throughout the colon of GF mice (**Figure 3B**). It was not possible to detect any discrete staining with isotype matched control antibodies (**Supplementary Figure 3**). The regional variations in EEC subset numbers is unlikely to be due to any bias in the sectioning or presentation of the tissues as the number of epithelial cells within the hemi-villous crypts of sections of the same regions of the GIT from different mouse strains were equivalent (**Supplementary Figure 1**).

EEC in Cultured Intestinal Crypt-Derived Epithelial Monolayers

The ability to establish cultures of the intestinal epithelium that reflect the architecture and distribution of differentiated cell types seen *in vivo* (Sato et al., 2009; Sato and Clevers, 2013) provides a valuable and tractable *in vitro* system to interrogate microbe-host cell interactions at the molecular and cellular level. To determine if such culture systems can faithfully replicate the different profiles of EECs seen in sections of preserved tissues of SPF and GF mice, we examined EEC in monolayer cultures established from small intestinal crypts of SPF, GF, Bt conventionalized GF mice, and GF treated with APS.

Representatives of the differentiated epithelial cell lineages including EEC and mucus-producing goblet cells were

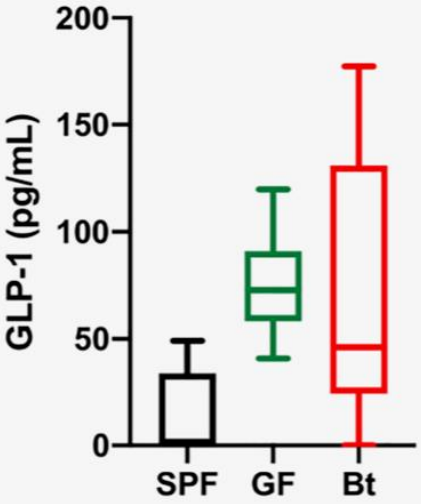

FIGURE 2 | Serum GLP-1 levels. GLP-1 levels in the serum of SPF ($n = 5$), GF (GF, $n = 11$) and Bt mono-colonized GF mice (GFBt, $n = 15$) were determined by ELISA. The box of the box whisker plots extends from the 25th to 75th percentiles with the horizontal line within the box representing the median and the whiskers representing the minimum and maximum values.

readily detected in cultured two-dimensional epithelial cell monolayers (**Supplementary Figure 6**). However, analysis of EEC populations in the cultured monolayers revealed inconsistencies in the distribution of EEC subsets compared to tissues preserved and processed directly *ex vivo*. In particular, the epithelial monolayer cultures established from GF mice showed no differences in the number of GLP-1$^+$ or ChrA$^+$ cells compared to those from SPF mice. By contrast, a significant increase in GIP$^+$ cells ($p = 0.005$) was seen in monolayer cultures established from GF mice compared to SPF mice small intestine (**Supplementary Figure 6b**), similar to that seen in intact tissue sections (**Figure 3A**). Generally, therefore, our analysis of secretory cell cultures from whole small intestine showed that intestinal monolayers are variable and with the possible exclusion of GIP expressing cells, do not accurately reflect the EEC cell makeup seen *in vivo*. We therefore relied on immunohistochemistry of intact tissues for the analysis and more accurate enumeration of EECs in subsequent experiments.

Bt Mono-Conventionalization of GF Mice Alters EEC Populations *in vivo*

The ability of commensal gut microbes to directly influence the makeup of the EECs network was assessed by conventionalizing GF mice with Bt, a universal and prominent member of the mammalian intestinal microbiota (Salyers, 1984) and comparing the distribution and numbers of EECs populations in the small and large intestine pre- and 10 days post-conventionalization. Bt was found throughout the length of the GIT of conventionalized mice which individually showed considerable variation in colonizing density based on cfu determinations of the luminal contents of different regions of the GIT (**Supplementary Table 1**). However, a consistent finding in all conventionalized animals was that the cecum and colon contained the highest levels of Bt as previously noted (Wrzosek et al., 2013; Curtis et al., 2014), and Bt colonization resulted in significant changes in EEC populations to the extent that they more closely resembled the profile and number of EEC seen in SPF mice. This was exemplified by the analysis of ChrA$^+$ EECs pre- and post-conventionalization which showed a significant reduction in ChrA$^+$ EECs throughout the small intestine of Bt conventionalized mice to levels comparable to that of SPF mice ($p < 0.01$to 0.001, **Figure 1A** and **Supplementary Table 2**). In the colon, Bt colonization also reduced the number of ChrA-expressing cells compared to both SPF and GF mice although the differences were not statistically significant (**Figure 1A** and **Supplementary Table 2**).

Analysis of individual EEC populations revealed subtle differences in the impact of Bt colonization on their regional distribution and/or numbers. For GLP-1 expressing EECs the effect of Bt was most apparent in the distal colon where it significantly reduced ($p < 0.05$) the number of positive cells (**Figure 1B** and **Supplementary Table 2**). By contrast, the impact of Bt conventionalization on GIP$^+$ EECs was more profound with significant reductions ($p < 0.05$ to 0.01) in cell numbers seen in both the small (duodenum and jejunum) and large (proximal and distal) intestine post-Bt conventionalization making them comparable to that of SPF mice (**Figure 3A** and **Supplementary Table 2**). A similar effect was noted for 5-HT-expressing EECs with significant reductions ($p < 0.05$) post-Bt conventionalization seen in the jejunum and in particular, throughout the colon ($p < 0.001$) (**Figure 3B** and **Supplementary Table 2**).

The Effects of Bt on EEC Networks Are Not Seen With an Unrelated Gut Commensal Bacterium

To determine if the effects of Bt on EEC networks after colonizing GF mice were specific to this bacterium, GF mice were conventionalized with a strain of Lr (100-23) isolated from the rat GIT that is able to stably colonize GF mice (Wesney and Tannock, 1979). The data shown in **Figures 4**, **5** (and **Supplementary Table 3**) show striking differences in the effects of Bt and Lr on EEC networks in the GIT post-conventionalization. Whereas Bt generally reduces the number of EECs in GF mice, Lr either had no significant effect (GLP-1$^+$ cells, **Figure 4B**) or the opposite effect and significantly increased numbers of EECs as seen in the jejunum and proximal colon for ChrA$^+$ cells (**Figure 4A**), in the duodenum for GIP$^+$ cells (**Figure 5A**), and throughout the colon for 5-HT$^+$ cells (**Figure 5B**).

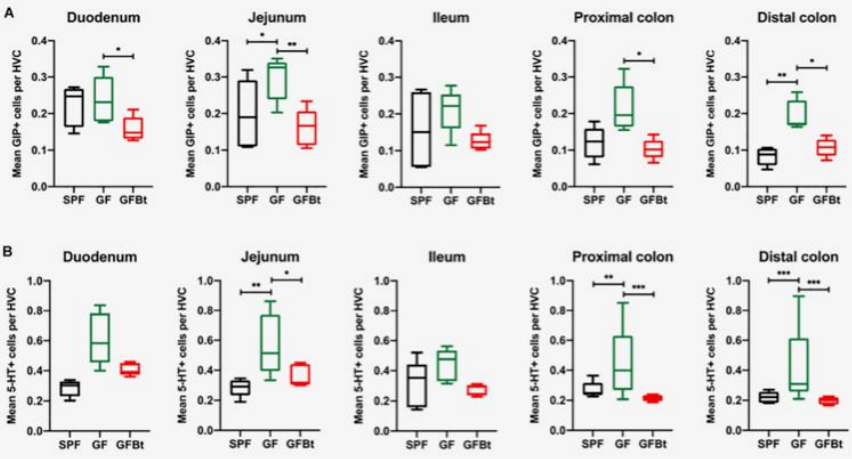

FIGURE 3 | Impact of colonization of GF mice with Bt on the distribution of GIP$^+$ and 5-HT$^+$ EECs. GIP$^+$ **(A)** and 5-HT$^+$ **(B)** cells were counted in the hemi-villus crypt (HVC) in sections of tissue obtained from different regions of the GIT of SPF ($n = 10$), GF ($n = 11$) and GF mice mono-colonized by Bt (GFBt, $n = 10$) mice. The box of the box whisker plots extends from the 25th to 75th percentiles with the horizontal line within the box representing the median and the whiskers representing the minimum and maximum values. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

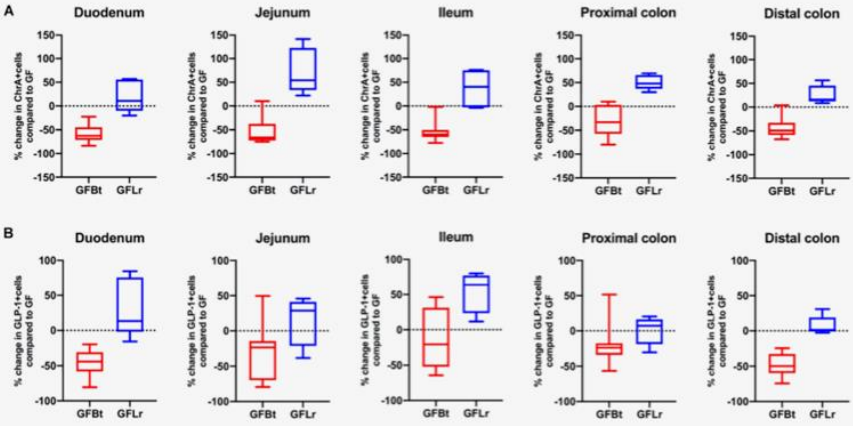

FIGURE 4 | The effect of Bt on ChrA and GLP-1 EECs is not replicated with another gut commensal bacterium. **(A)** Percentage change of ChrA$^+$ **(A)** or GLP-1$^+$ **(B)** EECs throughout the GIT of GF mice after mono-colonization with Bt (GFBt, $n = 10$) or Lactobacillus reuteri (GFLr, $n = 5$) compared to the number of GIP$^+$ cells in GF ($n = 11$) mice, which is shown as a dashed horizontal line and is set at zero. The box of the box whisker plots extends from the 25th to 75th percentiles with the horizontal line within the box representing the median and the whiskers representing the minimum and maximum values.

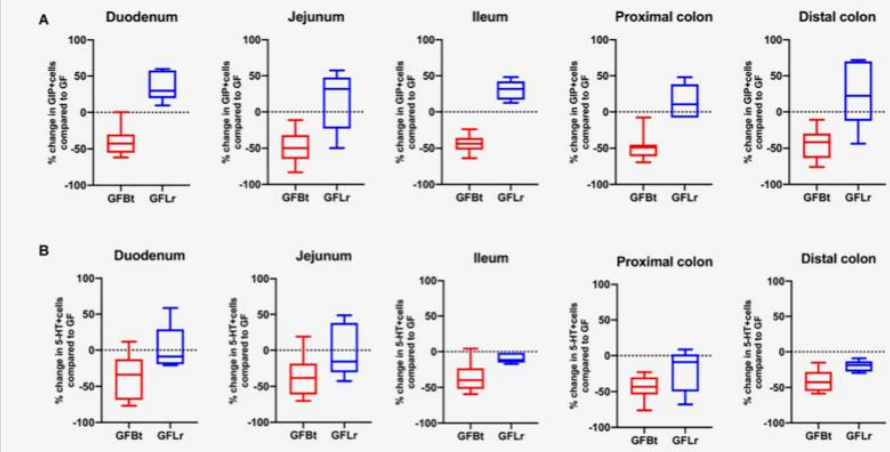

FIGURE 5 | The effect of Bt on GIP and 5-HT EECs is not replicated with another gut commensal bacterium. **(A)** Percentage change of GIP$^+$ **(A)** or 5-HT$^+$ **(B)** EECs throughout the GIT of germfree mice after mono-colonization with Bt (GFBt, $n = 10$) or Lactobacillus reuteri (GFLr, $n = 5$) compared to the number of GIP$^+$ cells in GF ($n = 11$) mice, which is shown as a dashed horizontal line and is set at zero. The box of the box whisker plots extends from the 25th to 75th percentiles with the horizontal line within the box representing the median and the whiskers representing the minimum and maximum values.

APS Reproduces the Effect of Bt Conventionalization on EECs Cells *in vivo*

Considering that EECs are highly enriched in free fatty acid receptors that contribute to physiological responses to microbially produced metabolites and SCFA (Lu et al., 2018), we investigated if these products, and in particular the fermentation products of Bt, mediated the effects of Bt on EEC networks.

First, we set out to confirm that the principle fermentation products of *Bacteroides* polysaccharide metabolism (acetate, propionate and succinate) (Hooper et al., 2002; Wrzosek et al., 2013; Curtis et al., 2014) were produced in Bt-conventionalized mice. NMR-based analysis was used to quantify these metabolites in the luminal contents of the duodenum, cecum and distal colon, 10 days post-conventionalization. As shown in **Supplementary Table 4**, all of the metabolites were present at very low levels (0.01–0.09 mM) in the GIT of GF mice. Post Bt colonization, the levels of acetate, succinate and propionate increased in the cecum and distal colon (0.26–2.35 mM). Strikingly, the levels of succinate in the cecum increased by >200-fold compared to those in both GF and SPF mice, as seen previously in C3H/HEJ mice post Bt colonization (Curtis et al., 2014). As expected, Bt conventionalization had no impact on butyrate levels (0.01 to 0.02 mM and 0.01 to 0.02 mM pre- and post-conventionalization, respectively) consistent with Bt not being a butyrate producer. To determine if the Bt fermentation products acetate, propionate and succinate (APS) could reproduce and provide an explanation for the effects of the bacterium itself on EEC populations seen *in vivo*. GF mice were administered via their drinking water APS in amounts corresponding to those present in the cecum of SPF rodents maintained on regular chow (95 μM acetate, 29 μM propionate and 5.6 μM succinate) (Mineo et al., 2006). Ten days later intestinal tissues were removed and examined for EECs.

The impact of Bt-APS on ChrA$^+$ cells was comparable to that seen after Bt conventionalization of GF mice with a reduction in the number of positive cells throughout the GIT and in particular in the small intestine which showed an approximate 50% reduction (**Figure 6A**), as seen with Bt conventionalization (**Figure 1**). The impact of APS on GLP-1$^+$ cells was more variable with the most apparent reductions in positive cells seen in the duodenum, jejunum and proximal colon (**Figure 6B**). Bt-APS administration had a similar effect on GIP+ (**Figure 7A**) and 5-HT$^+$ cells (**Figure 7B**) as that of ChrA$^+$ cells with reductions in positive cells seen throughout the small intestine and the colon.

To determine if the effects of administering Bt-APS on EEC were dependent upon constant exposure to APS, GF mice were treated with Bt-APS for 10 days followed by a 10 day wash out period prior to EEC analysis. For ChrA-expressing cells the removal of Bt-APS led to a rebound effect and increase in the number of positive cells in the small intestine and in particular, in the jejunum and ileum where the levels significantly exceeded that of non-treated GF mice (**Figure 6A** and **Supplementary Table 5**). Amongst individual EEC subsets some interesting and contrasting effects were noted. For GLP-1-expressing cells the removal of Bt-APS resulted in further and significant reductions in the proportion of positive cells throughout the small intestine

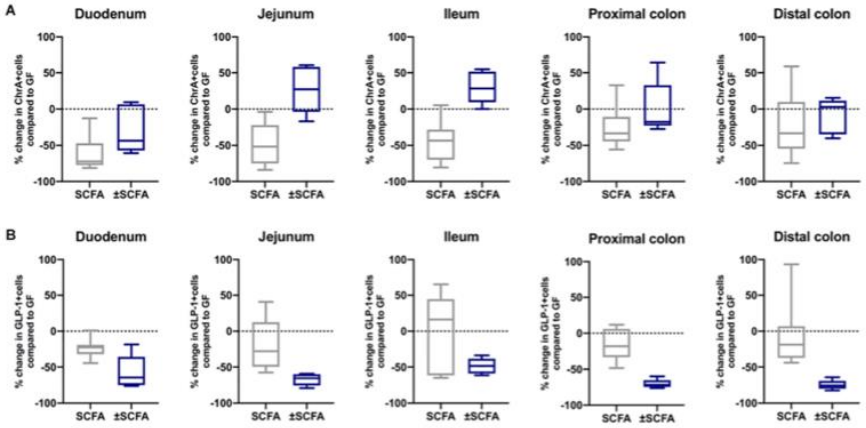

FIGURE 6 | Effects of APS on ChrA$^+$ and GLP1$^+$ EECs in GF mice. ChrA$^+$ **(A)** and GLP-1$^+$ **(B)** EECs were enumerated in tissue sections obtained from different regions of the GIT of GF either 10 days after administering APS (95 μM acetate, 29 μM propionate and 5.6 μM succinate) via drinking water, or after an additional 10 day washout period (± APS) and were compared to the number of ChrA$^+$ and GLP-1$^+$ cells in GF mice ($n = 11$), which is shown as a dashed horizontal line and is set at zero. The box of the box whisker plots extends from the 25th to 75th percentiles with the horizontal line within the box representng the median and the whiskers representing the minimum and maximum values. The horizontal dashed line represents values of GF mice which was set at zero.

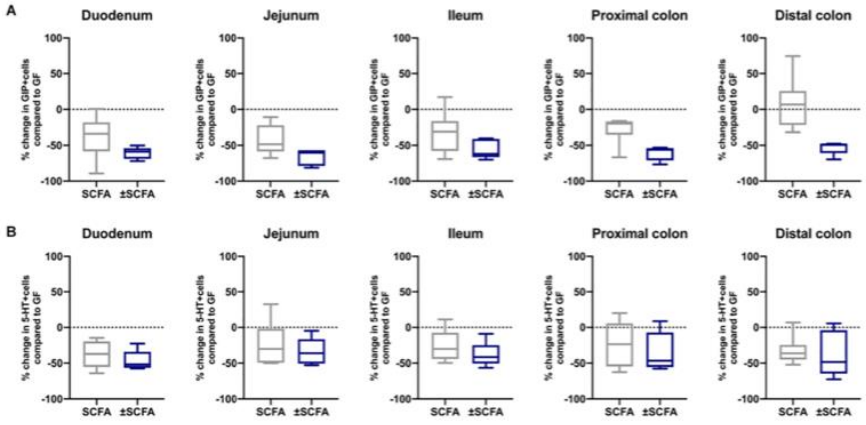

FIGURE 7 | Effects of APS on GIP$^+$ and 5-HT$^+$ EECs in GF mice. GIP$^+$ **(A)** and 5-HT$^+$ **(B)** EECs were enumerated in tissue sections obtained from different regions of the GIT of GF either after 10 days after administering APS (95 μM acetate, 29 μM propionate and 5.6 μM succinate) via drinking water for 10 day, or after an additional 10 day washout period (±APS) and were compared to the number of GIP$^+$ and 5-HT$^+$ cells in GF mice ($n = 11$), which is shown as a dashed horizontal line and is set at zero. The box of the box whisker plots extends from the 25th to 75th percentiles with the horizontal line within the box representing the median and the whiskers representing the minimum and maximum values.

and colon (**Figure 6B** and **Supplementary Table 5**) with the number of GLP-1 expressing cells in the small intestine now being comparable to that of SPF mice. A similar albeit more regionalized effect was seen for GIP-expressing cells with the withdrawal of Bt-APS resulting in further significant reductions in positive cells in the jejunum and in the proximal and distal colon (**Figure 7A** and **Supplementary Table 5**). In contrast, the withdrawal of Bt-APS had no significant impact on 5-HT expressing cells (**Figure 7B** and **Supplementary Table 5**).

DISCUSSION

A major challenge to developing a more detailed understanding of the nature of microbiota-EEC interactions that underpins the development of new evidence-based treatments for disorders affecting the GIT and other connected organ systems, is identifying which microbes are important and how they contribute to this crosstalk. Our recent study describing the ability of Bt to promote neurogenesis within the enteric nervous system of Bt conventionalized GF mice with accompanying effects on L-cells and EC cells (Aktar et al., 2020) prompted us to investigate in greater depth the mechanism and selectivity of the effect of Bt on EEC in the current study. The results presented here show for the first time that Bt is directly involved in shaping EEC networks throughout the mouse GIT in a process that is related to, and may be dependent on, their metabolism and production of succinate and the SCFAs acetate and propionate.

There are several cases reported in the literature of individual gut microbes, when administered exogenously, affecting host physiology. These include normalization of an autism phenotype seen in offspring of immunologically challenged dams by *B. fragilis* (Hsiao et al., 2013). Similarly, microbe-depleted mice showed altered behavior (Hoban et al., 2016) and probiotic treatment of normal mice with *L. rhamnosus* reduces anxiety and depression-related behaviors (Bravo et al., 2011). The SCFA receptor Gpr41 expressed on EEC, was shown, using GF mice, to act as a regulator of host energy balance through effects that are dependent upon the gut microbiota, although this was considered to be via cholecystokinin-containing cells of the upper intestine rather than L-cells. Such studies exemplify the ability of individual species to have potent influences at sites remote from the GIT. Their fundamental mechanisms of action, however, are not addressed. We reinstated one microbial species (Bt, a major constituent of the mammalian intestinal microbiota) in mice that were otherwise GF from birth. Thus, the role of Bt in postnatal development of EEC could be determined without quorum- or network-mediated effects that could be responsible in antibiotic-depleted or exogenously supplemented normal animals. In a similar study to ours, GF mice were mono-colonized with either *E. coli* or Bt for 4 weeks (Wichmann et al., 2013). This study reported that GLP-1 positive cells in the proximal colon are increased by colonization with Bt but not by *E. coli*, although there were regional differences to our results. This study also found serum GLP-1 increased in GF mice as we did. Other studies conventionalizing GF mice with specific microbiota agree with our findings. For example, Turnbaugh and colleagues (Turnbaugh et al., 2006) showed that conventionalization of GF mice with an obesity-associated mouse gut microbiome induces an increased capacity for energy harvest (Turnbaugh et al., 2006). Reigstad and co-workers (Reigstad et al., 2015), through the use of GF and humanized mice, showed that gut microbiota are important determinants of enteric 5-HT production and homeostasis, as we observed in the proximal and distal intestine of Bt conventionalized GF mice. We also demonstrated the selectivity of the effect of Bt on EEC networks as seen by the inability of another unrelated rodent gut commensal bacterium, Lr, to replicate the effects of Bt on EECs in mono-conventionalized GF mice.

The ability of Bt to influence EEC throughout the GIT is perhaps not surprising considering *Bacteroides* species are found in close association with the mucus that coats intestinal epithelial cells (Bry et al., 1996) and are therefore juxtaposed with EEC. It is important to note, however, that the impact of Bt on EEC is not uniform throughout the GIT with some but not all EEC subsets being modulated to the same degree, suggestive of a both a regionalized and subset specific effect of Bt on EEC. Unlike Billing et al. and Roberts et al. (Billing et al., 2019; Roberts et al., 2019) who using "omics based approaches were unable to detect *Gip* expression in the mouse colon, we detected the presence of GIP-expressing cells in the proximal and distal colon of both germfree and SPF mice." Whilst we cannot entirely exclude the possibility that this is the result of non-specific antibody reactivity, similar findings to ours have been reported in human studies using immunohistochemistry and mRNA analyses to detect GIP expression in the distal colon (Jorsal et al., 2017). Discrepancies in detecting GIP expressing cells in the mouse colon may, in addition to experimental design and methodological differences, be related to variations in environmental conditions within different animal facilities. Each facility has their own unique combination of various and numerous attributes of animal husbandry that impact on the bacterial communities within each facility and on the microbiome of their occupants that can influence host physiology and phenotype (Rausch et al., 2016).

It is particularly noteworthy that Bt exerts effects on EEC in the small intestine, which is at odds with the conventional view of Bt being a resident of the anoxic cecum and colon. However, we have shown the ability of Bt to colonize both the small and large intestine of GF mice, comparable with its presence in regions of the small intestine of healthy humans (Mallory et al., 1973). This could therefore provide a possible route for its global effect on EEC networks. Alternatively, Bt might act via non-cognate interactions and through the production of metabolites or other mediators that are absorbed from the intestinal lumen and then disseminated throughout the body via the circulatory or nervous systems.

Among the various pathways and products that could be responsible for the effects of Bt on EEC we investigated their major products of polysaccharide fermentation, acetate, propionate and succinate (Wrzosek et al., 2013; Curtis et al., 2014). We confirmed that all three metabolites are produced and accumulate in the cecum and colon of Bt-conventionalized GF mice. High levels of succinate are particularly noteworthy

and replicate prior studies of Bt colonized, antibiotic-pre-treated, C3H/HeJ mice demonstrating a 200-fold increase in cecal succinate levels post-Bt colonization (Curtis et al., 2014). Amongst SCFA, acetate, propionate, and butyrate are the most abundant (≥95%) (Cook and Sellin, 1998) and are present in an approximate molar ratio of 60:20:20 in the colon and stool (Cummings et al., 1987; Hijova and Chmelarova, 2007; Binder, 2010). The prominence within the human colon of *Bacteroides* which make up ∼25% of the total anaerobes (Salyers, 1984), and are adept glycan metabolisers (Salyers et al., 1977) and producers of high levels of acetate (Wrzosek et al., 2013; Curtis et al., 2014), helps explain the prominence of acetate amongst SCFA in the colonic lumen. The importance of acetate and other SCFAs to the host is exemplified by the fact that they provide ∼10% of our daily caloric requirements (McNeil, 1984; Bergman, 1990). In addition, propionate stimulates intestinal gluconeogenesis (De Vadder et al., 2014) and contributes to protecting the integrity of the blood-brain barrier (Hoyles et al., 2018), whereas succinate is a key intermediary in several metabolic pathways, playing an important role in the elimination of reactive oxygen species (Tretter et al., 2016). What emerged from our study is that a mixture of acetate, propionate and succinate administered in physiologically appropriate concentrations and molar ratios (Mineo et al., 2006) was able to recapitulate the effect of Bt, with certain exceptions. For example, the regulation of EEC in the colon was weakly affected by APS compared to Bt, which may indicate there is reduced access of oral APS to the colon compared with the small intestine. Alternatively, there may be additional factors and metabolites to APS that convey the efficacy of Bt in the colon. The inability of Lr, which produces a similar profile of SCFA (including acetate and propionate but not succinate) to Bt (Kahouli et al., 2015) to replicate the effects of Bt on EEC supports this proposal. Surprisingly, in several cases, the effects of APS were greater in the colon after a 10-day washout period. This may be indicative of the effects of APS being gradual in onset, and/or their initial effect persisting and being amplified. A long-lasting effect could also arise as a consequence of influencing epithelial stem cells and driving production of EEC lineage cells as recently demonstrated in a SCFA (acetate, propionate, butyrate)-murine and human intestinal enteroid co-culture model system (Pearce et al., 2020). In this *in vitro* culture system, butyrate was shown to be the most effective SCFA in increasing ChrA expression (Pearce et al., 2020). This may explain the loss of $ChrA^+$ cells we observed *in vivo* after washout of the butyrate-deficient APS cocktail. The absence of a long lasting effect of APS on ChrA cells might also reflect a separate population of EEC not otherwise labeled in our study that accounted for its transient effect, such as PYY containing L-cells (Aktar et al., 2020) or tuft cells (Sutherland et al., 2007).

Our finding that epithelial cell monolayers generated from small intestinal crypts of GF mice do not accurately reflect the EEC makeup or response to Bt (with the exception of GIP^+ cells) and APS seen *in vivo* is at odds with their increasing use as a physiologic model of intestinal response to stimuli including microbes and nutrients (Leushacke and Barker, 2014; Pearce et al., 2018; Yin et al., 2019). Our contradictory findings may relate to our use of GF mice, as SPF mice are the usual source of intestinal crypts (Petersen et al., 2014; Roberts et al., 2019). However, the finding that the transcriptome and proteome of small intestinal stem cell-derived organoids from SPF and GF mice co-cluster (Hausmann et al., 2020), would argue against this possibility although in this study no account was made for any possible differences in EEC distribution or number. Indeed, whereas our analysis relied on cellular comparisons of EEC in epithelial cell monolayer cultures versus in intact tissue, other studies have used single or multi-omics-based approaches in comparative studies (Lindeboom et al., 2018; Beumer et al., 2020; Hausmann et al., 2020; Ohki et al., 2020). Other possible confounding factors include comparing EECs in two-dimensional epithelial cell monolayers versus stem cell-derived three-dimensional organoids, other methodological differences including the age of the mice used, where in the small intestine crypts are obtained from Fuller et al. (2012), the duration of culture, and the type and concentrations of growth and differentiation factors used. Additional multidisciplinary studies incorporating both molecular and cellular methodologies are required in order to address these discrepancies, and to determine what aspects of EEC physiology can, and can't, be faithfully represented by crypt-derived epithelial cell monolayers from conventional versus GF mice.

CONCLUSION

We have used conventional and GF mice to demonstrate that the intestinal microbiota is required for regulation of EEC networks, and that a single microbe, Bt, can recapitulate its role in a process that may be dependent on their metabolism and production of APS. Since Bt is a major human symbiont, these findings have implications for novel interventions for the maintenance of human health via the microbiome.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

All experiments were conducted in accordance with the Home Office Animals (Scientific Procedures) Act 1986 under the license number PPL80/2545 at the UEA.

AUTHOR CONTRIBUTIONS

SC, AP, and LA conceived and designed the experiments. AM, LA, and SC wrote the manuscript and SC supervised the research. SC, AM, RS, EJ, AG, and AP executed the experimental work. MD undertook the chemometric and metabolite analysis. SC, AM, AP, and LA carried

out the data interpretation. AM carried out the statistical analysis. All authors revised, read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.575595/full#supplementary-material

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