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The Microbiota and Colon Cancer Risk: Cause or Consequence?



*Submitted to the National University of Ireland, Cork
in fulfilment of the
requirements for the degree of Doctorate of Philosophy*

Thesis presented by

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Declaration

I hereby declare that this thesis is the result of my own work and has not been submitted in whole or in part elsewhere for any award. Any assistance and contribution by others in this work is duly acknowledged within the text.

Jonathan Keane

List of Publications

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Abstract

Background: Colorectal cancer (CRC) has a complex aetiology involving both genetic and environmental factors. Recently, attention has turned to the influence of the gut microbiota upon the development of CRC. The human gut microbiota is altered in CRC and certain microbial patterns are associated with increased cancer risk, but definitive identification of mechanisms and specific bacteria involved has proven elusive as species associated with human CRC are not consistent across studies. Moreover, research into the role of the microbiota in CRC has primarily focussed on changes that exist in and around tumour sites at a single point in time. This has made it difficult to differentiate between changes that promote cancer initiation in healthy tissue from changes that are a result of tumour development. We therefore tracked changes in the host and microbiota throughout the course of colon cancer development in mouse models of sporadic and familial CRC to elucidate their contribution to disease. Our aim was to identify changes in each of these criteria and establish their temporal relationships in order to generate a timeline of alterations and define causative and consequential events. One possible mechanism by which the gut microbiota may affect host processes leading to the development of CRC is the production of bacterial metabolites. We furthermore investigated the role of one of these metabolic pathways; in particular changes in free bile acids formed by the enzyme *bile salt hydrolase (bsh)*, which is widespread in the microbiota.

Results: We observed pro-tumorigenic changes in the microbial composition of our model of sporadic CRC prior to detection of host neoplastic transformation. The microbiota present in the gut of our familial model also differs prior to the onset of disease with the potential to contribute to disease initiation. Our longitudinal studies also identified a distinct set of microbial alterations associated with disease progression. This included a reduction of taxa associated with suppression of inflammation prior to the onset of pro-inflammatory

signalling in our sporadic model, followed by enrichment for cancer-associated taxa later. In our intervention study, mice administered a research strain of *E. coli* overexpressing *bsh* displayed significantly fewer intestinal polyps compared to *E. coli* or PBS controls, possibly mediated by an enrichment of hydrophilic bile acid tauro-ursodeoxycholic acid and bile acid signalling.

Conclusion: These results suggest that the microbiota is dynamic over time and may act as an instigating factor in CRC development. Moreover, it highlights prophylactic treatment of the gut microbiota as a potential avenue to reduce cancer risk. This could be explored by administration of probiotics, for example *bsh*-expressing species, to human subjects in a prospective clinical trial of CRC risk.

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Abbreviations

5-FU	5-fluorouracil
7-keto-LCA	7-keto-lithocholic acid
ABC	Avidin-biotin complex
Acetyl-CoA	Acetyl coenzyme A
ACF	Aberrant crypt foci
AKT	RAC-alpha serine/threonine-protein kinase
ALDH	Aldehyde dehydrogenase
ANOVA	Analysis of variance
AOM	Azoxymethane
AP-1	Activator protein 1
APC	Adenomatous polyposis coli <i>or</i> Antigen presenting cell
APC ^{MIN}	Adenomatous polyposis coli, multiple intestinal neoplasia
APC ^{mNLS}	Adenomatous polyposis coli, mutant nuclear localisation signal
ASBT	Apical sodium-dependant bile acid transporter
ATP	Adenosine triphosphate
BA	Bile acid
Bai	Bile acid inducible
BAR	Bile acid receptor (also known as FXR)
BAT	Bile acid-CoA:amino acid N-acyltransferase
BAX	B-cell lymphoma 2-associated X
Bcl-2	B-cell lymphoma 2
Bcl-xL	B-cell lymphoma-extra large
Beta (β)-Trcp1	E3 ubiquitin ligase transducin repeat containing protein 1

BLAST	Basic local alignment search tool
BRCA	Breast cancer gene
B _{REG}	Regulatory B cell
BSEP	Bile salt export pump
BSH	Bile salt hydrolase
CA	Cholic acid
CA-d4	Deuterated cholic acid
CAC	Colitis-associated cancer
CAF	Cancer-associated fibroblast
CAG	Co-abundance group
CAR	Constitutive androstane receptor
CCND1	Cyclin D1
CD	Crohn's disease
CD#	Cluster of differentiation #
CDCA	Chenodeoxycholic acid
CDCA-d4	Deuterated chenodeoxycholic acid
Cdk4	Cyclin-dependent kinase 4
cDNA	Complementary deoxyribonucleic acid
CFU	Colony forming unit
CIMP	CpG island methylator phenotype
CIN	Chromosomal instability
CKK	Cholecystokinin
c-myc	Cellular myelocytomatosis
COG	Clusters of orthologous groups
COX	Cyclooxygenase

CpG	5'-cysteine-phosphate-guanine-3'
CRC	Colorectal cancer
CTL	Cytotoxic T lymphocyte
CTLA	Cytotoxic T-lymphocyte-associated protein
Cxcl	C-X-C chemokine ligand
CYP	Cytochrome P450
DAB	3,3'-diaminobenzidine
DC	Dendritic cell
DCA	Deoxycholic acid
DCC	Deleted in colorectal carcinoma
DEPC	Diethyl pyrocarbonate
DHCA	Dehydrocholic acid
DMH	1,2-dimethylhydrazine
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DSS	Dextran sodium sulphate
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
Elk-1	E26 transformation-specific-like-1
ELR	Glutamic acid-leucine-arginine
EMT	Epithelial–mesenchymal transition
EpCAM	Epithelial cell adhesion molecule
ERK	Extracellular signal-regulated kinases

FAP	Familial adenomatous polyposis
FDR	False discovery rate
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
FLASH	Fast length adjustment of short reads
FMT	Faecal microbial transfer
FOB	Faecal occult blood
FXR	Farnesoid X Receptor (also known as BAR)
g	Gravity
G-	Glycine-conjugated bile acid
G1-to-S	Gap 1 to synthesis phase
G2/M	Gap 2 to mitotic phase
GADD	Growth arrest and DNA damage-inducible
GB	Gnotobiotic
GF	Germ-free
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GPBAR1	G protein-coupled bile acid receptor (also known as TGR5)
GSK3 β	Glycogen synthase kinase 3 β
GST	Glutathione-S-transferase
GTPase	Guanosine triphosphatase
HAT	Histone acetyltransferase
HCA	Hyochoic acid
HCC	Hepatocellular carcinoma
HDAC	Histone deacetylase
HDCA	Hyodeoxycholic acid

HNPPC	Hereditary non-polyposis colorectal cancer
HSD	Honest significant difference
HSDH	Hydroxysteroid dehydrogenase
HZ	Heterozygote
i.p.	Intraperitoneal
I-BABP	Ileal bile acid binding protein
IBD	Irritable bowel disease
IDO	Indoleamine 2,3-dioxygenase
IFN	Interferon
IHC	Immunohistochemistry
IL	Interleukin
iNOS	Inducible nitric oxide synthase
JAK	Janus kinase
KO	Knockout (superscript) <i>or</i> Kegg orthologue
KW	Kruskal-Wallis
LAB	Lactic acid bacteria
LAG-3	Lymphocyte-activation gene 3
LCA	Lithocholic acid
LEF	Lymphoid enhancer-binding factor
Lgr5	Leucine-rich repeat-containing G-protein coupled receptor 5
LOH	Loss of heterozygosity
LOX	Lipoxygenase
LPS	Lipopolysaccharide
LRH-1	Liver receptor homolog-1
LXR	Liver X receptor

MAM	Methylazoxymethanol
MAPK	Mitogen-activated protein kinase
MCA	Muricholic acid
MCP	Macrophage chemo-attractant protein
MDM2	Mouse double minute 2 homolog
MDSC	Myeloid-derived suppressor cell
MGMT	O ₆ -methylguanine-DNA-methyltransferase
MHC	Major histocompatibility complex
Min	Multiple intestinal neoplasia
miRNA	Micro ribonucleic acid
MLH1	MutL homolog 1
M-MDSC	Monocytic myeloid-derived suppressor cell
MMP	Matrix metalloproteinase
MNNG	Methylnitronitrosoguanidine
MNU	N-nitroso-N-methylurea
MoCA	Murocholic acid
mRNA	Messenger ribonucleic acid
MSI	Microsatellite instability
MWU-test	Mann-Whitney U-test
NADP	Nicotinamide adenine dinucleotide phosphate
NCRI	National cancer registry Ireland
NF-κB	Nuclear factor kappa-B
NK	Natural killer
NO	Nitric oxide
NOS	Nitric oxide synthase

NSAID	Non-steroidal anti-inflammatory drug
NTCP	Na ⁺ -taurocholate co-transporting polypeptide
N-terminus	Amino terminus
OATP	Organic anion-transporting peptides
Olfm4	Olfactomedin 4
Ost	Organic solute transporter
OTU	Operational taxonomic unit
P16INK4a	Inhibitor of CDK4
PBS	Phosphate-buffered saline
PCoA	Principle coordinate analysis
PD	Phylogenetic diversity
PD-1	Programmed death-1
PERMANOVA	Permutational multivariate analysis of variance
PGE ₂	Prostaglandin E ₂
PICRUSt	Phylogenetic investigation of communities by reconstruction of unobserved states
PIK3CA	Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha
PKC	Protein kinase C
PMN-MDSC	Polymorphonuclear myeloid-derived suppressor cell
PXR	Pregnane X receptor (also known as SXR)
PyNASt	Python nearest alignment space termination
QIIME	Quantitative insights into microbial ecology
qPCR	Quantitative real time polymerase chain reaction
Raf	Rapidly accelerated fibrosarcoma
RAR	Retinoic acid receptor

RAS	Rat sarcoma
Rb	Retinoblastoma protein
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
rRNA	Ribosomal ribonucleic acid
RTK	Receptor tyrosine kinase
RXR	Retinoid X receptor
SCFA	Short chain fatty acid
SHP	Small heterodimer protein
SMAD	Small mothers against decapentaplegic
Sox9	SRY-Box 9
SPSS	Statistical package for social sciences
STAT	Signal transducer and activator of transcription protein
SXR	Sensing X receptor (also known as PXR)
T-	Taurine-conjugated bile acid
TAM	Tumour associated macrophage
Tap1	Transporter associated with antigen processing 1
TCF	Transcription factor
TCR	T cell receptor
TGF	Transforming growth factor
TGR5	Takeda G Protein-Coupled Receptor 5 (also known as GPBAR1)
T _h	Helper T cell
TIIDM	Type II diabetes mellitus
TIM-3	T-cell immunoglobulin and mucin-domain containing-3
TIMP	Tissue inhibitor of metalloproteinase

TME	Tumour microenvironment
TNF	Tumour necrosis factor
TNM	Tumour-node-metastasis
T _{REG}	Regulatory T cell
TS	Thymidylate synthase
UC	Ulcerative colitis
UCA	Ursocholic acid
UCG	Uncultured group
UDCA	Ursodeoxycholic acid
UPL	Universal probe library
UPLC-MS	Ultra performance liquid chromatography – tandem mass spectrometry
URB	Uncultured rumen bacterium
VDR	Vitamin D receptor
VEGF	Vascular endothelial growth factor
Wnt	Wingless mouse mammary tumour virus integration site family
WT	Wild-type
WT1	Wilm's tumour 1

1.0.1 Introduction

The following research project will investigate the role of the microbiota in colorectal cancer (CRC) using two mouse models. This literature review will describe CRC and those models. It will then introduce the microbiota and delineate our understanding of its role in health and disease, as well as highlight the gaps in our knowledge relating to CRC. One mechanism by which the microbiota can contribute to disease is by the production of microbial metabolites. Our research focusses on one particular set of such processes, the microbial metabolism of endogenous bile acids, which we will define in the following section, before presenting our aims and objectives.

1.1 Cancer

Cancer is a complex family of diseases characterised by abnormal cell growth with the potential to spread to other parts of the body. It is a multi-step process that develops through epigenetic changes and mutations in multiple genes, including loss of function of tumour suppressor genes and gain of function of oncogenes. In 2000, Hanahan and Weinberg described a series of six “hallmarks” acquired by most types of cancers during their development. These hallmarks are sustained proliferative signalling, replicative immortality, activated invasion and metastasis, induced angiogenesis, cell death resistance and evasion of growth suppressors. In 2011, they added a further two hallmarks, deregulation of cellular energetics and avoidance of immune destruction, as well as two enabling characteristics, tumour-promoting inflammation and genome instability/mutation (Figure 1.1) (1, 2).

Cancer represents the second leading cause of death globally, responsible for approximately 9.6 million deaths per year (3). Cancer overtook circulatory diseases to become the number

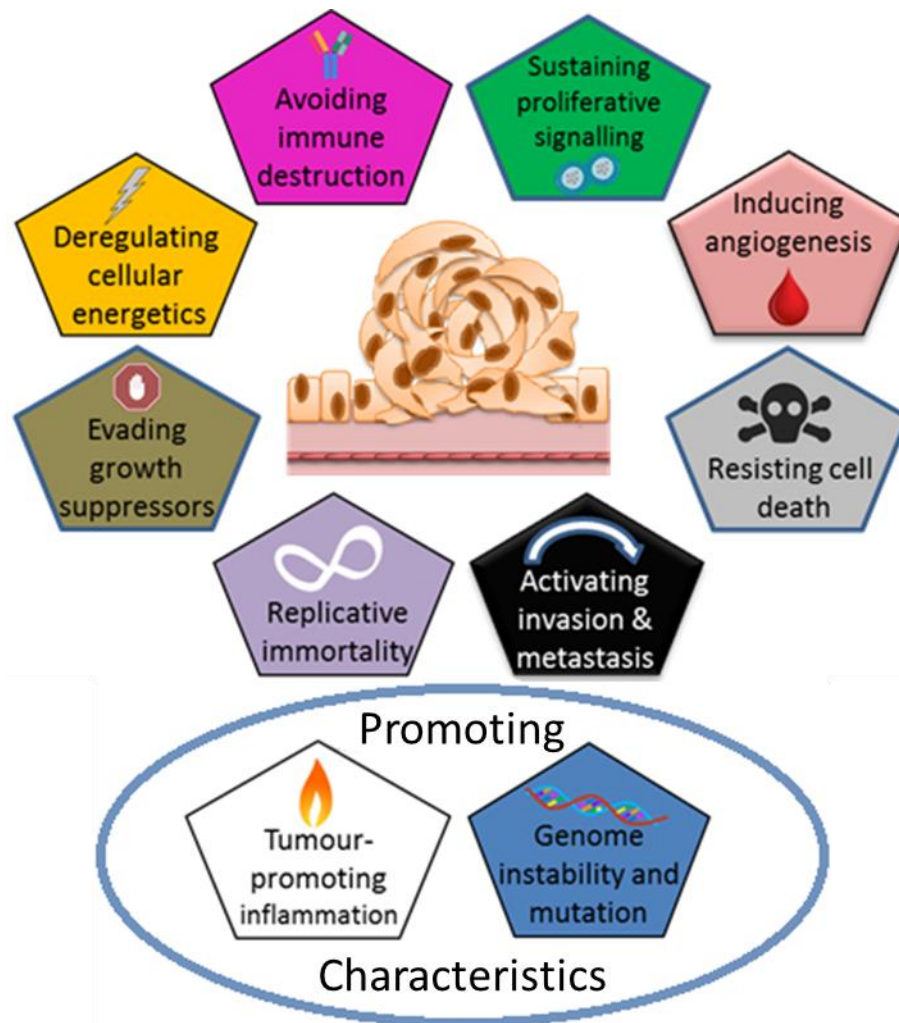


Figure 1.1: Hallmarks of cancer. Hanahan and Weinberg described eight “hallmarks” of cancer, as well as two enabling characteristics, genome instability/mutation and tumour-promoting inflammation, which are present in virtually all cancers (1, 2). These factors combine to create malignant disease.

one cause of death in Ireland in 2016, accounting for 30.7% of total fatalities at a rate of 2.0 per 1,000 population (4). Approximately 40,000 new cancer cases are diagnosed in Ireland every year with a total cost to the economy of €1.4bn in 2009, and this rate is expected to double by 2040 according to the National Cancer Registry Ireland (NCRI) (5-7).

1.1.1 Colon Cancer

Colorectal cancer (CRC) is a major cause of cancer-related death worldwide, with the NCRI predicting that the incidence of CRC will increase by ~130% by 2040 (7). Of the €1.4bn cost burden of cancer to Ireland, €158m was associated with CRC (6). CRC is the 3rd most common cancer both in Ireland (excluding non-melanoma skin cancer) and globally. It is also the 3rd highest cause of cancer-related death in Ireland and 2nd globally (3, 8).

1.1.1.1 Risk factors in CRC

Lifestyle choices play a large role in colon cancer risk, with up to 90% of cases believed to involve environmental factors (9). These factors include diet, obesity, smoking, alcohol consumption and a sedentary lifestyle (10, 11). Diet itself is believed to account for around 30% of the total cancers in the western world, second only to tobacco as a preventable cause of cancer, and for 80% of the increased CRC rates between developed and developing countries (12, 13). Indeed, the “Western diet” which is high in fat, protein and sugar and low in fibre, fruit and vegetables has been the focus of recent research investigating the high rates of CRC.

1.1.1.2 Types of Colon Cancer

CRC has a complex pathogenesis but cases fall into three broad categories, sporadic, inflammation-associated and genetic. The majority of cases (approximately 75%) are sporadic with no direct inherited or comorbid cause (14). Sporadic cancer generally occurs later in life and displays a progressive accumulation of mutations in genes such as *APC*, *RAS*, *SMAD4* and *TP53* and subsequent genomic instability (14).

Approximately 20% of CRC cases display a familial inheritance pattern (15). Hereditary Non-Polyposis colorectal cancer (HNPCC) accounts for ~5% of these cases, while Familial Adenomatous Polyposis (FAP) accounts for ~1%. HNPCC, also known as Lynch syndrome, is an autosomal-dominant condition caused by mutations in DNA mismatch repair proteins, leading to a 50-70% lifetime risk of CRC (15). FAP is also an autosomal-dominant condition caused by mutations in the *adenomatous polyposis coli (APC)* gene which causes hundreds to thousands of adenomatous polyps to develop in the large intestine, and has a CRC risk approaching 100% in middle age (16).

Thirdly, inflammatory bowel disease (IBD)-related CRC is responsible for approximately 2% of the annual mortality from CRC overall, but 10–15% of the annual deaths in IBD patients (17). Here, chronic inflammation and epithelial disruption in the large intestine provide the genotoxic background that promotes cancer initiation and progression. IBD-CRC patients, specifically those suffering from ulcerative colitis (UC) and Crohn's disease (CD), are also affected at a younger age than sporadic CRC patients and have a 5-year survival rate of 50%. Indeed, patients with IBD have a CRC relative risk of 0.8 – 23 and undergo close monitoring, treatment with immune-suppressor drugs and frequent colectomy to address both the IBD and CRC risk (18). Relative risk is approximately twice as high in UC patients compared to CD, possibly due to the involvement of a greater area of the colonic epithelium (19).

1.1.1.3 Staging of Colon Cancer

Colon cancers are divided into five stages, 0 to IV. These summarise the tumour-node-metastasis (TNM) characteristics of the disease. “Tumour” describes how deeply the tumour has penetrated the intestine epithelium and bowel lining. “Node” relates to whether or not tumour cells are detected in regional lymph nodes, while “metastasis” refers to whether the cancer has spread to other body parts. Each stage is described in Table 1.1.

1.1.1.4 Pathogenesis of CRC

Colon cancer development normally follows a gradient process, in which various genetic mutations gradually accumulate over time. Adenocarcinomas make up 95% of colon cancer cases. They are believed to arise from aberrant crypt foci (ACF); abnormal lesions in the colonic mucosa identifiable by their raised and thickened epithelium and enlarged luminal openings (20). Some ACF can eventually progress to benign adenomatous polyps, which then develop into an advanced adenoma with high grade dysplasia. This is followed by progression to carcinoma and ultimately to metastasis (Figure 1.2).

The progression from normal epithelium to ACF, adenoma, adenocarcinoma and metastasis is a stepwise process as initial mutations disable cellular maintenance systems, allowing the accumulation of further cytogenetic damage and assumption by the cell of additional tumorigenic characteristics. The approximate rate in which key mutations appear in the sequence is presented in Table 1.2.

Another tumorigenic pathway which bypasses the exophytic adenomatous stage and is believed to account for around one third of CRC cases is termed the *de novo* or flat adenomatous stage. These tumours also arise from ACF that are not elevated above the

Table 1.1: Stages of CRC (modified from Hardy *et al.* (21))

Stage	Characteristics	Survival (%)
Stage 0	Carcinoma in situ	90-100
Stage I	Tumour invades the submucosa or muscularis propria	90-100
Stage II	Tumour invades the subserosa or surface of the visceral peritoneum	75-85
Stage III	Tumour cells have spread to the regional lymph nodes	30-40
Stage IV	Cancer has distant metastases	<5

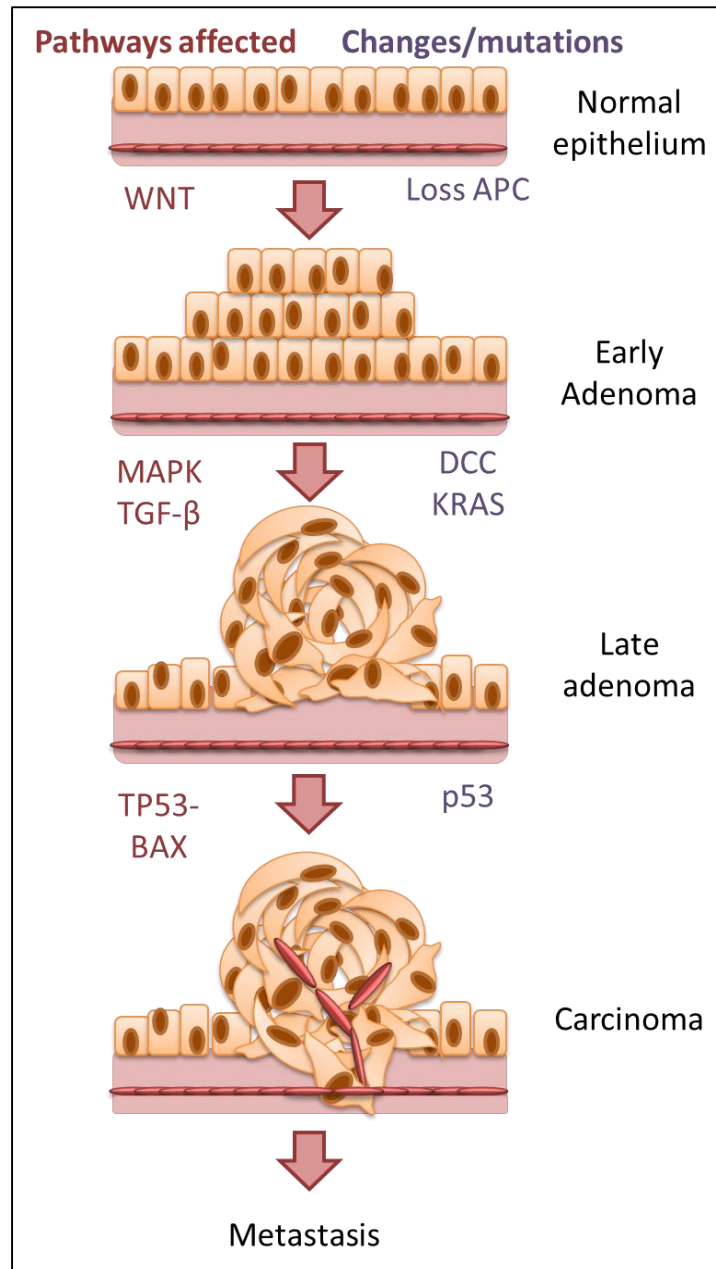


Figure 1.2: The adenoma-carcinoma sequence and associated pathways. Loss of APC is an initiating mutation in many colorectal cancers that leads to loss of cell cycle control and abnormal proliferation. Exposure to additional genetic insults such as chronic inflammation or repeated exposure to dietary carcinogens, e.g. heterocyclic amines and heme iron, can cause an accumulation of mutations in the colonic epithelium and the subsequent loss of proto-oncogenes such as KRAS, Deleted in Colorectal Cancer (DCC) and TP53. This can cause the sequential progression of normal epithelium through the early and late adenoma stages into invasive carcinoma and metastasis.

Table 1.2: Mutations in CRC progression (modified from Alrawi *et al.* (20))

Lesion	Mutation Frequency (%)				
	APC	B-Catenin	KRAS	DCC	TP53
ACF	<5	0	10-95	0	0
Adenoma	40-65	0	0-40	0	0
Adenocarcinoma	40-80	15	40-60	40-70	50-80

colonic mucosa making it harder to identify and hence are less well understood (22, 23). These ACF differ from classical ACF by early dysregulation of β -catenin, fast growing crypts and more consistent progression to tumours in mouse models (24).

1.1.1.5 Key Genes and Pathways in CRC

Mutations in the Wnt signalling pathway are regarded as the instigating factor in colorectal cancer development and mutations in *APC* are the most common mutation in these cancers, being found in 80% of colorectal adenomas and carcinomas (25, 26). *APC* forms part of the β -catenin destruction complex and loss of *APC* leads nuclear accumulation of β -catenin and expression of the TCF/LEF pathway. These transcription factors are involved in the activation of *cyclin D1*, *Cdk4* and *c-myc* which control G1-to-S phase cell cycle transition, and their constitutive activation leads to aberrant cell proliferation, differentiation and migration (27-29). Germ-line mutations of *APC* in humans lead to FAP and a similar phenotype occurs due to β -catenin stabilising mutations even in the presence of wild-type *APC* (30, 31).

Humans have three *RAS* genes; *KRAS*, *NRAS* and *HRAS*; with *KRAS* mutated in ~45% of colon cancers. It is part of the mitogen activated protein kinase (MAPK) signalling pathway and encodes a guanosine triphosphatase (GTPase) which regulates cell proliferation and survival. It is activated in response to a ligand binding a receptor tyrosine kinase (RTK) such as epidermal growth factor receptor (EGFR) (32). Aberrant activation of *KRAS* without concurrent *APC* mutation usually leads to self-limiting hyperplastic lesions but *KRAS* mutation occurring downstream of initiating mutations in *APC* can result in tumour progression (33). There is also evidence that activated *KRAS* is required for tumour maintenance, as withdrawal of inducible *Ras* from mouse models of CRC leads to tumour

regression (34-36). This could involve the role of KRAS in the Warburg effect, which is the utilisation of aerobic glycolysis by malignant tumour cells (37).

P53 is a tumour suppressor transcription factor described as the “guardian of the genome” (38). In normal conditions, p53 is subject to several layers of post-translational regulation, primarily binding and ubiquitylation by mouse double minute 2 homolog (MDM2) and degradation by the proteasome (39). It is activated by alteration of normal cell homeostasis such as DNA damage or oxidative stress, upon which it can halt cell cycle progression and trigger pathways involved in DNA repair or apoptosis to prevent damaged cells from proliferating (40). The mechanisms for deciding between these two pathways have not been determined. P53 can halt cell cycle progression at G1/S phase by up-regulating *CDKN1A* (p21) or at G2/M via GADD45 to allow DNA repair enzymes to act (41). If the damage is beyond repair, it can instigate programmed cell death which is believed to be its primary tumour-suppressor function (42). It can achieve this by up-regulating pro-apoptotic genes, down-regulating anti-apoptotic genes and via transcription-independent mechanisms (38). P53 is inactivated on over half of human cancers and this leads to loss of this tumour suppressive effect, tumour progression, metastasis, and worse prognosis (43).

Deleted in Colorectal Carcinoma (DCC) is another gene down-regulated in more than half of colorectal cancers. DCC is a transmembrane receptor which suppresses apoptosis when bound to its ligand netrin-1 but promotes apoptosis when netrin-1 is absent via a novel caspase-dependent pathway (44, 45). DCC can act as a tumour suppressor in the absence of netrin-1 via this pro-apoptotic function, but also as an oncogene in the presence of its ligand or activating mutation by suppressing apoptosis (44). Similar to *KRAS*, mutation in *DCC* is not an activating mutation in CRC but contributes to CRC progression downstream of *APC*.

1.1.1.6 Genetic Instability in CRC

CRCs can arise from one or a combination of three different mechanisms, namely chromosomal instability (CIN), CpG island methylator phenotype (CIMP), and microsatellite instability (MSI). Aneuploidy and loss of heterozygosity (LOH) are major factors in CIN tumours which constitute most sporadic tumours (~85%) and are most likely caused by dysfunctional chromosome duplication or segregation during mitosis (46). Other than *APC*, mutations in the CIN pathway include the activation of proto-oncogenes such as *KRAS*, *BRAF* and deactivation of tumour suppressors *p53*, *p15*, *p16*, *BRCA1*, retinoblastoma (*RB*), *SMAD4*, *DCC* and loss of heterozygosity in 17p and 18q chromosome arms (47-50).

Microsatellite instability (MSI) involves inactivating mutations in the DNA mismatch repair genes that are responsible for correcting DNA replication errors, due to genetic alterations in short repeated sequences called microsatellites. Microsatellites are repetitive sequences of DNA generally featuring 1-6 base pairs repeated 5-50 times. Errors in repair of this microsatellite DNA can cause frameshift mutations and MSI as seen in HNPCC and 12-15% of sporadic CRCs (51-54). There is an overlap of genes involved in the CIN and MSI pathways which contain microsatellites including genes for *TGFβ*, *CCND1* and *SMAD* (55). MSI abnormalities are observed in HNPCC ACF in 100% of cases, highlighting its importance as an early event in this form of familial CRC (20)

The CpG island methylator phenotype (CIMP) involves hypermethylation of CpG dinucleotides in the promotor regions of key genes leading to their epigenetic silencing (56). This promotor methylation is observed in ~25% of CRC. CRC-associated genes affected by CIMP silencing include *BRAF*, *p16INK4a*, *MGMT* and *PIK3CA* (55). Moreover, the DNA mismatch repair protein MLH1 is silenced in this manner in 80% of sporadic MSI colorectal

cancers, with the DNA methyltransferase inhibitors representing a new class of drugs targeting this aspect of carcinogenesis (57, 58)

Finally, microRNAs (miRNAs) are small, non-coding RNA molecules containing ~22 nucleotides involved in gene silencing and post-transcriptional regulation of gene expression by base-pairing with mRNA (55, 59). Recent research has highlighted the role of miRNA dysregulation in cancer. miRNAs are involved in aberrant proliferation and differentiation, evading apoptosis and growth suppression, promoting invasion, metastasis and angiogenesis. Attention is now turning to using miRNAs as biomarkers for tumorigenesis and as targets for cancer treatment (60-62).

1.1.1.7 The Tumour Microenvironment

Tumours are composed of both tumour cells and non-tumour cells, and the surrounding environment becomes significantly altered once neoplastic transformation has begun. This tumour microenvironment (TME) consists of blood vessels, immune cells, fibroblasts and the extracellular matrix (ECM) surrounding the tumour and plays a central role in tumour maintenance and progression.

1.1.1.7.1 Stroma: The stromal tissue of a tumour is the collection of supportive and connective tissue comprising different cell types, ECM and the vasculature. Immune cells in the stroma initially provide an anti-tumour response but this can be co-opted by the tumour to contribute to pathogenesis.

Remodelling of the ECM is a feature of the TME. This is largely achieved by the production of matrix metalloproteinases (MMPs) by cancer-associated fibroblasts (CAFs). Degradation of the ECM by MMPs allows infiltration by endothelial cells leading to angiogenesis (63). Growth factors are also released from the ECM, such as FGFR1 and TGF β , which can promote angiogenesis, as well as some potential inhibitors, highlighting the complex role of MMPs in the TME (64-68).

Degradation of the ECM can lead to tumour cell growth as well as an endothelial cell migration, facilitating invasion and metastasis. Indeed, inhibitors of MMPs have been shown to reduce cancer cell invasiveness *in vitro* and *in vivo* (69-71).

1.1.1.7.2 Angiogenesis: Angiogenesis plays a critical role in tumorigenesis. Tumours may exist *in situ* for months or years limited to 2-3mm diameter until alterations in the TME lead to the growth of vasculature allowing further growth, either by the production of pro-angiogenic factors or down-regulation of angiogenic inhibitors (72, 73). These mediators of angiogenesis in cancer, especially vascular endothelial growth factor (VEGF), are a target for anti-cancer drugs but concerns exist about the ability of anti-angiogenic therapy to select for aggressive cancer cells and possibly drive invasion and metastasis (74-76), while clinical trials showed only modest survival benefits (77-79).

VEGF is up-regulated in around half of CRC cases (80). It acts on endothelial cells to stimulate proliferation and invasion by the vasculature (81-83). It also stimulates the expression of anti-apoptotic proteins and promotes the survival of these cells (84-86). Hypoxia and necrosis induce the expression of VEGF which is exploited by tumours proliferating beyond their available blood supply, as evidenced by the specific induction of VEGF in tumour cells immediately proximal to necrotic foci (87, 88). VEGF expression is also correlated with

activation of oncogenes during carcinogenesis and can be promoted by inflammatory mediators common in the tumour microenvironment such as interleukin (IL)1 β and IL6, and can be induced by tumour necrosis factor (TNF) α in an nuclear factor (NF)- κ B-dependent manner (87, 89-92).

1.1.1.7.3: Cytokines and Chemokines in the Tumour Microenvironment: A dysregulated immune response plays a key role in tumour initiation and promotion. This can be via pro-inflammatory signals which induce DNA damage, inhibit apoptosis and stimulate cell proliferation and angiogenesis, and anti-inflammatory mediators which can allow tumour cells to evade immune surveillance. The normal immune response to tissue injury is self-limiting while the response in cancer is chronic and uncontrolled, so tumours have been described as “wounds that do not heal”.

A dysregulated immune response and chronic inflammation is a significant risk factor for CRC, for example by the production of reactive oxygen species (ROS) by NADPH oxidase or reactive nitrogen species (RNS) by nitric oxide synthase, whose production can be promoted by cytokines such as TNF α , IL6 and TGF β . ROS and RNS cause oxidative stress to the cell which can lead to tumorigenesis (93-96). As such, some cytokines and chemokines that are important in the immune response to cancer can also promote tumorigenesis in some contexts.

IL1 β , IL6 and TNF α are pro-inflammatory cytokines which can act as tumour initiators by producing ROS and RNS, and as tumour promoters by mechanisms such as suppressing apoptosis and driving proliferation and angiogenesis (97-101). They also form part of the anti-tumour response, such as the activation and trafficking of T-cells of the adaptive immune system (100). These complex effects can depend on factors such as the location or

degree of expression (100, 101). For example, TNF α expression in macrophages and mast cells is associated with higher survival, while a poorer prognosis is associated with TNF α in the tumour stroma (93, 102, 103). In contrast, IL10 is also associated with anti-inflammatory responses. IL10 can reduce pro-tumorigenic inflammation by suppressing NF- κ B signalling but can also allow tumours to evade immune surveillance and facilitate apoptosis resistance, with elevated levels of IL10 associated with poor prognosis in B cell lymphoma (93, 104-108).

Chemokines are a class of chemotactic cytokines (109). Generally, *glutamic acid-leucine-arginine* (ELR)-positive chemokines such as CXCL1, CXCL2 and CXCL5 are pro-angiogenic while ELR-negative chemokines are angiostatic (110). CXCL1 is the most abundant chemokine expressed by TAMs and has been implicated in cancer growth and metastasis via NF- κ B activation (111). CXCL2 is also involved in tumorigenesis, with knockdown of CXCL2 in a metastatic human colon cancer cell line (LoVo cells) leading to a decrease in the expression of cancer stem cell markers (112). CXCL5 is also associated with cancer proliferation and metastasis and is over-expressed in late-stage human cancers, where it correlates with poor prognosis (113-116).

1.1.1.7.4 Immune Cells in the Tumour Microenvironment: The pro- and anti-tumorigenic roles of key immune cells are presented in Figure 1.3. Cancer-associated fibroblasts (CAFs) are the predominant cell in the stroma and can promote angiogenesis as well as primary tumour growth, invasion and metastasis (117-123). CAFs induce an inflammatory environment by overexpressing cyclooxygenase (COX)-2 as well as chemoattractants such as macrophage chemoattractant protein (MCP)-1, granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL8 which promote infiltration of neutrophils and other pro-inflammatory leukocytes (124-128).

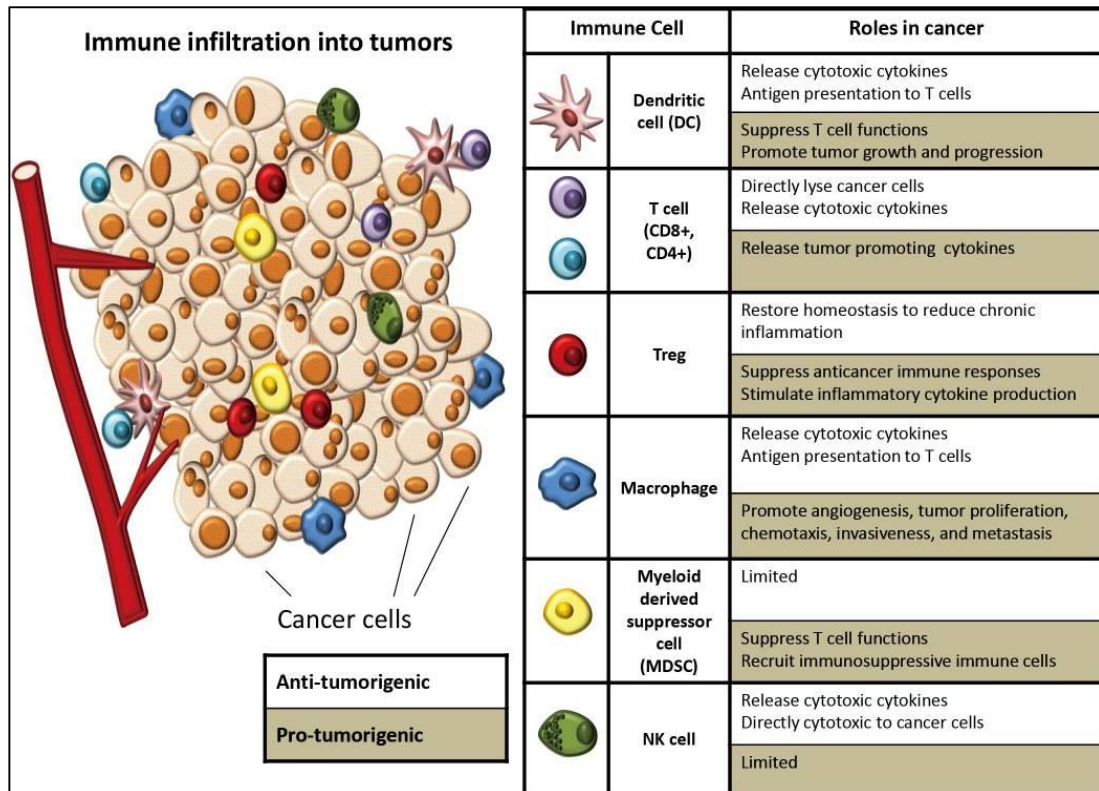


Figure 1.3: Key cells in the tumour microenvironment. A range of immune cells can be found in different parts of the tumour microenvironment at different stages of cancer development. These can produce pro- and anti-inflammatory cytokines and chemokines that can have pro or anti-tumorigenic effects depending on a number of factors, including the level and duration of their expression, their location and the receptors expressed on the surrounding stromal, immune and tumour cells. Figure from Markman and Shiao, 2015 (129).

Myeloid-derived suppressor cells (MDSCs) are a family of immune cells similar to immature monocytes and neutrophils with potent immune-suppressive activity (130). They are divided into two classes; monocytic MDSCs (M-MDSC) and polymorphonuclear (also called granulocytic) MDSCs (PMN-MDSC), with PMN-MDSCs predominant in tumour-bearing mice (131). MDSCs suppress immune function by a number of mechanisms including the production of ROS and RNS which suppress CD8⁺ T-cells (132-136), by depleting L-arginine and L-cysteine which leads to the down-regulation of the T cell receptor (TCR) complex, and by expansion of regulatory T (T_{REG}) cells (137-139). MDSCs also interfere with T-cell trafficking by reducing L-selectin in naïve T-cells, and nitration of chemokines such as CCL2 which inhibits T-cell infiltration (140, 141). As well as inhibiting the anti-tumour T-cell response, MDSCs suppress natural killer (NK) cells and dendritic cells (DCs), and cross-talk between MDSCs and macrophages increases production of immune-suppressive IL10 by MDSCs and reduces expression of anti-tumorigenic IL12 by macrophages (142-144). This suppression facilitates immune evasion by the tumour and contributes to cancer progression, with MDSCs associated with advanced disease and poor prognosis in human cancers, whilst elimination of MDSCs increases the CD4- and CD8-mediated anti-tumour response (145).

Natural killer (NK) cells are potent anti-cancer effectors of the innate immune system. They can generate a cytotoxic response to cancer cells which do not express Major Histocompatibility Complex (MHC)-I fragments, leading to necrosis or apoptosis of the targeted cell (146). NKs also mediate the downstream inflammatory response by production of cytokines and chemokines (147). These cells are often reduced in cancer and low NK activity is associated with poor prognosis (148-150). As a result, methods for improving the anti-tumorigenic activity of NK cells have been investigated including adoptive transfer of NK cells, enhancing NK cell activation and proliferation, and breaking NK cell anergy (146, 151).

Dendritic cells (DCs) are antigen-presenting cells (APCs) of the immune system with both pro- and anti-tumorigenic roles in cancer development (152). In their role as APCs, they present antigens to T-cells to activate the adaptive immune response. Despite the presence of DCs in the TME, immune surveillance fails which suggests impaired DC function. This may be due to hypoxia, increased levels of lactate and adenosine, decreased pH and expression of IL10 in the TME (152). As well as failing to prime an anti-tumour response, DCs can promote tumour progression by overexpressing pro-tumorigenic IL6 and galectin-1, and can induce the differentiation of naive CD4⁺ T-cells into IL10-producing T_{REG} cells leading to immune suppression (153, 154). Reactivating or normalising DCs' anti-tumour response could be an effective cancer treatment, and vaccination using DCs activated and loaded with tumour antigen-associated peptides *ex vivo* induced a favourable immune response (155).

Macrophages are a class of immune cells with important functions in antigen-presentation and phagocytosis (156-158). They also have pro- and anti-inflammatory and pro- and anti-tumorigenic roles. In cancer, M1-type macrophages generate a chronic inflammatory environment via production of cytokines such as IL6 and TNF α which induce mutagenesis and apoptosis-resistance (159). Later, tumour-associated macrophages (TAMs) promote angiogenesis, migration, invasion and metastasis in the primary tumour via production or stimulation of factors such as VEGF, EGF and MMPs. At this stage, the predominant macrophage phenotype in the TME changes to the M2 immune-suppressive type, facilitating immune evasion by the tumour (160, 161). A meta-analysis of human studies shows strong evidence that macrophage density in the TME correlates with poor prognosis (162).

T-cells are classed according to their expression of T-Cell Receptor (TCR) as either $\gamma\delta$ or $\alpha\beta$. The $\alpha\beta$ T-cells are further subdivided into CD8⁺ cytotoxic T-cells (CTLs) and CD4⁺ helper T-cells (T_h) cells. CTLs can have potent cytotoxic effects on cancer cells by releasing granules containing perforin and granzymes, and can also induce FasL-mediated apoptosis (163). Their

effectiveness is reduced in tumours by deficiency of APCs such as DCs and macrophages, and the maintenance of an immuno-suppressive environment by CAFs, TAMs and T_{REG} cells. A subset of pro-tumorigenic CTLs which are deficient in perforin but express pro-inflammatory cytokines have also been identified (164).

CD4⁺ T_h cells include T_h1, T_h2, T_h17 and T_{REG} cells. T_h1 cells suppress tumorigenesis by producing IFN γ , TNF α , IL2 and IL12 by activating and regulating CTLs, and by inducing the secretion of IL1 β and IL6 by macrophages (165, 166). This demonstrates that even pro-inflammatory cytokines associated with cancer risk can be effective against tumour cells when accurately managed.

T_h2 cells have been associated with increased cancer risk by inducing proliferation, angiogenesis, invasion and metastasis via a number of mechanisms including inducing VEGF expression, activation of EGF-producing TAMs, and tumour-promoting inflammation (167-171). However this is an oversimplification of their role and other facets of T_h2 immune response, such as production of IL4 and infiltration of eosinophils, can inhibit tumour growth (172). Moreover, T_h2 cells do not rely on presentation of Major Histocompatibility Complex (MHC)-I molecules as CTLs do, and so can respond to MHC-I-negative tumours (173). T_h2 cell transfection has also been shown to eliminate tumour cells in cancer models by inducing eosinophil chemotaxis, macrophage activation and tumour necrosis, in a T_h1- and CTL-dependent manner (173-175). A similar result was observed following T_h2 cell adoptive transfer into myeloma and B-cell lymphoma cancer models, which eradicated cancer cells by inducing inflammation and macrophage infiltration in a manner independent of B-cells, Natural Killer T cells, CTLs cells and IFN γ (176).

T_h17 cells are also associated with increased cancer risk by inducing inflammation, proliferation, angiogenesis and recruitment of pro-tumorigenic neutrophils, but are also involved in the anti-tumour response by activating CTLs (177-180).

In contrast, T_{REG} cells contribute to cancer progression by their immuno-suppressive properties. Tumours display preferential recruitment of T_{REG} cells which may constitute 20-30% of the CD4⁺ cells in the TME, as opposed to 5-10% of the normal pool (181). T_{REG} cells correlate with poor prognosis in a number of cancers and these cells have been shown to enhance pro-tumorigenic actions of CAFs, tumour cells, macrophages, MDSCs and regulatory B cells (B_{REG}), while inhibiting tumour-suppressive NK cells and CTLs (182, 183). However, T_{REG} cells can also reduce cancer risk by down-regulating excess inflammation and are associated with improved prognosis in some cancers, including CRC (181, 184-186).

1.1.1.8 Clinical Treatment of CRC

Treatment for CRC depends on stage of the cancer. Surgery alone is used in Stage 0 and I, and has a high rate of success. Chemotherapy is used to treat Stage III and IV and occasionally Stage II. Radiotherapy is not routinely used due to the sensitivity of the bowel to radiation (187). The major chemotherapy agents used to target CRC include 5-fluorouracil (5-FU), principally a thymidylate synthase (TS) inhibitor; capecitabine, which is metabolised to 5-FU; irinotecan, an inhibitor of topoisomerase I; oxaliplatin, a non-targeted cytotoxin believed to inhibit DNA synthesis by forming crosslinks between DNA strands; and trifluridine/tipiracil, a compound drug comprising cytotoxin trifluridine and the thymidine phosphorylase inhibitor tipiracil (188-192). Targeted therapies are also available such as bevacizumab, which inhibits VEGF with limited success; cetuximab, which is an EGFR inhibitor; and regorafenib, a multi-kinase inhibitor approved for use against metastatic CRC (193-195).

More recently, immunotherapy has been successful in improving the prognoses of many cancers. Unfortunately, progress in using immunotherapy to treat CRC has been slower. Programmed Death-1 (PD-1) inhibitors have been shown to effectively treat only MSI CRC deficient for mismatch repair (MMR-d) which comprise ~15% of sporadic CRCs (196-199).

Tumours up-regulate PD-L1 to suppress the immune response by binding to PD-1 receptor on effector T cells, a defence that can be targeted by PD-1 inhibitors. Anti-CTLA-4 immunotherapy drugs have also so far proved ineffective in CRC, although studies using anti-CTLA-4 in combination therapy are ongoing, as well as trials on immunotherapy targets LAG-3, TIM-3 and IDO (198). Finally, recent studies have shown that cancer treatment can be affected by the composition of the host microbiota. For example, studies have suggested a role for the microbiota in PD-1 immunotherapy for melanoma, non–small cell lung cancer, and renal cell carcinoma, as well as in response to chemotherapy (200-203). Clinical response to PD-L1 correlated with the relative abundances of *Ruminococcaceae* and *Akkermansia muciniphila*, and oral supplementation of *A. muciniphila* increased the recruitment of CCR9⁺CXCR3⁺CD4⁺ T-cells into mouse tumours (200, 201).

Microbial interventions have also been considered for the treatment for CRC and these are addressed in section 1.3.2.1.

1.2 Mouse Models of CRC

In this thesis, I have used two common mouse models of colorectal cancer; APC^{MIN} mice, a model of familial CRC; and AOM-treated mice, a model of sporadic CRC.

1.2.1 APC^{MIN} Mice – A Model of Familial CRC

Research into familial CRC makes use of mouse models characterised by defects in the *Apc* gene. Several models encompassing different *Apc* mutations have been established, with the *multiple intestinal neoplasia* (APC^{MIN}) model predominant in the literature. Familial Adenomatous Polyposis (FAP), the hereditary human cancer, is also caused by mutations in the *APC* gene.

1.2.1.1 Adenomatous Polyposis Coli, the β -catenin Destruction Complex and Wnt

APC is a tumour suppressor gene that forms part of the intracellular β -catenin destruction complex where it facilitates the phosphorylation of β -catenin by glycogen synthase kinase 3 β (GSK3 β), leading to β -catenin being marked for degradation by E3 ubiquitin ligase TrCP1 (β -Trcp). Once marked with ubiquitin, β -catenin is degraded by the proteasome by the normal pathway. Disruption of the β -catenin destruction complex thus leads to the activation of the Wnt signalling pathway, as was described in section 1.1.1.5 *Key Genes and Pathways in CRC*. Dysregulation of APC-mediated β -catenin degradation can therefore lead to loss of cell cycle control in a cell-autonomous manner (204).

1.2.1.2 APC^{MIN} Mice

Mice heterozygous for the *Apc* gene are well established as models for familial CRC. The first *Apc* mouse mutant generated, referred to as the *multiple intestinal neoplasia (Min)* model (205), developed around 30 tumours in C57BL/6J mice, mostly in the small intestine. The mutation was identified as fully penetrant and autosomal dominant, with mice demonstrating a shortened lifespan of ~119 days. This mutation was subsequently identified as a nonsense mutation at amino acid 850 of APC (206) on proximal mouse chromosome 18 (207).

Subsequent studies determined that the development of intestinal neoplasia was a result of Knudson's two-hit hypothesis, involving loss of the wild-type APC protein as a result of random mutation or mitotic recombination, thus causing a loss of heterozygosity (LOH) of *Apc* (208, 209).

APC localises to the nucleus as well as the cytoplasm and regulates the nuclear export and cytoplasmic degradation of β -catenin via two N-terminal nuclear export signals (210, 211). Zeineldin *et al.* investigated the nuclear role of APC by generating a mouse model with a compromised APC nuclear import signal (APC^{mNLS}) which displayed increased tumorigenesis when treated with genotoxic agent azoxymethane (AOM) and colitis-inducing dextran sulphate sodium (DSS) (212). The authors suggest that aberrant Wnt signalling is insufficient for tumorigenesis and that inflammation is also necessary, highlighting another study in which mice treated with a single dose of AOM fail to develop tumours without subsequent DSS-induced colitis (213). They propose *Apc*-mutant mice undergo tumorigenesis due to an additional anti-inflammatory role of APC and show how APC^{mNLS} mice display increased inflammation and a reduction in goblet cells and mucin production, possibly contributing to epithelial barrier permeability. Mutations in *K-ras* and *p53* have not been observed in APC^{MIN} tumours despite being common in human sporadic cancer, although their short lifespans

prevent the accumulation of mutations and transition from benign adenoma to invasive carcinoma (214-216).

1.2.2 Inducible Models of Sporadic CRC

As a model for sporadic cancer, mice can be treated by a number of genotoxic agents that cause widespread untargeted mutation in the murine colonic epithelium. This reflects the accumulation of genetic damage seen in the human intestine which can lead to CRC in later life. The first chemically-induced models of CRC involved mice fed dibenzanthracene and 20-methylcholanthrene (217). Later, hydrazine in cycad flour was identified as a possible colonic carcinogen in humans, which caused tumours in the liver, kidney, lung and intestine when fed chronically to rats (218). The carcinogen was subsequently identified as cycasin, which is hydrolysed to methylazoxymethanol (MAM) in the liver (219-221). Later studies used 1,2-dimethylhydrazine (DMH), which is metabolised to azomethane and azoxymethane (AOM). AOM is subsequently hydroxylated to form MAM in the liver (Figure 1.4) (220, 222-225). AOM has now become the preferred method as it is more stable in solution and requires fewer metabolic steps to become active, and hence can be used in lower doses (226, 227).

1.2.2.1 Azoxymethane-Treated Mice

Repeated administration of AOM to rodents results in the development of intestinal tumours, and is a well-studied model of sporadic CRC. The reactive metabolite(s) of AOM initiate cancer development by alkylating guanine residues in DNA, thereby facilitating base mispairing. The AOM-induced carcinogenic insult ultimately leads to tumours primarily in the distal large intestine which reflects the predominant localization of spontaneous CRC in humans. These tumours, however, rarely display invasion or metastasis (228).

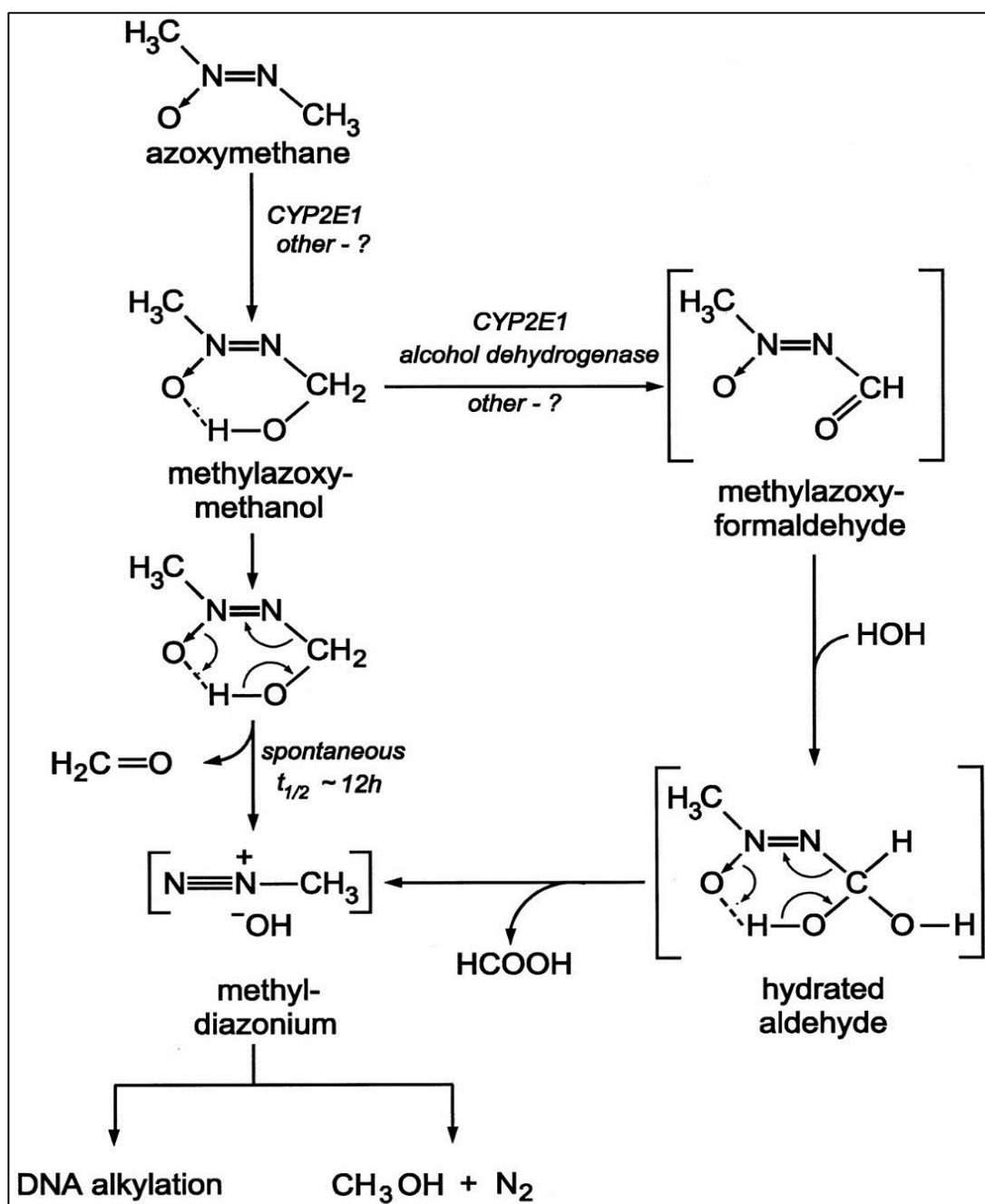


Figure 1.4: Two metabolic pathways of AOM and MAM activation. AOM is hydroxylated by P450 enzymes in the liver to form MAM, which is in turn oxidised to the methyldiazonium ion which has the capacity to alkylate DNA. Alternatively, MAM may decay spontaneously to the methyldiazonium ion. Figure from Sohn *et al.*, 2001 (229).

1.2.2.2 Strain Sensitivity to AOM

Different mouse strains have been shown to exhibit differential sensitivity to tumorigenesis following AOM administration (Table 1.3) (230-233). Sensitive strains such as A/J mice can develop over 40 colonic adenomas after AOM treatment while resistant strains such as AKR/J develop <1. This can reflect the genetic background of the mouse but must also account for the environmental conditions in which the mice are housed, including the diet and the gut bacteria that the mice acquire, as well as procedural differences such as route of administration and dosage (234). This is further complicated by interaction between confounding factors, such as between diet and strain, and an apparent complex and polygenic interaction between backgrounds observed in crossed strains (234). Tumours produced were of similar morphology regardless of AOM sensitivity suggesting similar pathways lead to tumorigenesis in each strain (235). A study on the relative levels of DNA alkylation in susceptible and resistance mouse strains suggested that the difference is not a result of the number of DNA methyl adducts produced, nor were CYP2A1 levels found to correlate with tumour susceptibility, suggesting that the foundation for differential strain susceptibility is downstream of DNA alkylation (226, 236). Back-cross experiments showed that tumour susceptibility is not inherited as a dominant trait and suggested a “resistance factor” exists in resistant AKR/J mice. AOM-susceptible A/J mice did show an increase in apoptosis, proliferation and expression of c-myc mRNA in the top third of colonic crypts compared to resistant AKR/J mice, suggesting a role for cell turnover (232).

1.2.2.3 Pathways to CRC in AOM-Treated Mice

While the mutations induced by AOM administration are untargeted, studies in mice and rats have revealed that AOM-induced tumours resemble human CRC in many respects at the molecular level. In particular, AOM-induced tumours display aberrant protein expression for

Table 1.3: Strain sensitivity to carcinogens (modified from Rosenberg *et al.* (233))

Strain	Carcinogen	Tumours per Mouse	References
A/J	DMH, AOM	9.2–41.6	(234, 237-240)
SWR/J	DMH, AOM	9.5–16	(237, 239-241)
FVB/N	AOM	3.6	(235)
C57Bl/6b	DMH, AOM, MAM	0–5.5	(235, 237, 241-244)
BALB/cJ	AOM	1–2	(245-247)
AKR/J	DMH, AOM, MAM	<1.0	(234, 235, 237, 239, 240, 246, 248)

APC and altered cellular localization of β -catenin (249). Moreover, consistent with findings in human CRC, AOM-induced tumours have mutations of *K-ras*. Levels of enzymes involved in prostaglandin and nitric oxide synthesis, such as COX-2 and inducible nitric oxide synthase, have also been reported to be elevated (249). In contrast, *Apc* mutations are less common in ACF but are observed in adenomas and adenocarcinomas, while mutations in *Tp53* are uncommon in chemically-induced models of carcinogenesis in rodents (250-253). Moreover, in a study of genomic instability on AOM-induced models, 75% of mice showed no evidence of CIN but 81% showed low level MSI with high level MSI observed in an A/J colon tumour-derived cell line (254). Finally, similar to humans whereby CRC incidence rates are generally higher among males than females, the incidence of CRC in DMH-treated outbred male Wistar rats was higher than in female rats, although no differences were observed in AOM-treated inbred mice (234, 255).

1.3 The Gut Microbiota

The human microbiota is the community of bacteria, archaea, protists, fungi and viruses that live in and on the human body (256). The cells of our microbiota are estimated to outnumber our nucleated human cells by a ratio of about 13:1, about 70% of which occupy our gastrointestinal tract (203, 257), although this ratio drops to approximately 1.3:1 when enucleated hematopoietic cells are included (258). These gut microbiota, most importantly gut bacteria, play vital roles in immune modulation, metabolism, inhibition of pathogens and structural development (259-262). They have also been implicated in intestinal disease, including CRC and IBD (263, 264). The gut “microbiome” is sometimes used synonymously with the gut “microbiota” but can also refer to the full collection of genes present in the microbiota of a community. The “gut microbiota” will henceforth be used to describe the gut bacteria only.

1.3.0.1 16S rRNA Gene Sequencing

16S ribosomal RNA (rRNA) is a component of the prokaryotic ribosome used for phylogenetic reconstruction. The DNA region coding bacterial 16S rRNA comprises nine hypervariable regions separated by conserved regions that can be targeted with universal primers. 16S rRNA gene sequencing provides high-throughput and affordable taxonomic analysis of communities such as the microbiota, but disadvantages include biases introduced by the universal primers, copy number variation or sequencing platform. Another limitation is the inability to consistently annotate to species level, with fewer than 20% of operational taxonomic units (OTUs) assigned to a species in an evaluation of 16S rRNA gene sequencing methodologies (265). OTUs are functional clusters, into which 16S rRNA sequences sharing an arbitrary level of similarity (usually >97%) are collapsed (266). More informative analysis

can be achieved through metagenomic shotgun sequencing but this is expensive and generates huge amounts of data that require advanced bioinformatics analysis (267, 268).

1.3.1 The Gut Microbiota - Bacteria

The bacterial gut microbiota comprises over 5000 bacterial species and 3 million genes, and possibly over 35,000 species in the collective human microbiome (269, 270). It is dominated by the phyla *Firmicutes* and *Bacteroidetes*, featuring smaller proportions of *Proteobacteria*, *Verrucomicrobia*, *Actinobacteria*, *Fusobacteria*, and *Cyanobacteria* (261, 271). This consistency of phyla, combined with significant inter-individual variation within the phyla, suggests a selective pressure to maintain the higher taxonomic structure with a functional redundancy at lower levels sufficient to maintain proper function (203, 271, 272).

3.1.1 Colonisation and Composition of the Gut Microbiota

The intestinal tract has been considered sterile at birth, with colonisation beginning immediately through contact with the mother and environmental bacteria, and although recent research has suggested colonisation of the placenta by *Streptococcus agalactiae* in approximately 5% of pregnancies, the possibility remains that this is as a result of sample contamination (273-277). The newborn microbiota is reflective of the delivery method, with babies delivered by Caesarean section having a microbiota characterised by fewer *Bifidobacterium* species compared to vaginal births (278). This colonisation period is crucial in development and lasts for approximately 1-2.5 years (279-281). The microbiota then remains largely stable until old age, in the absence of disruptions such as long-term dietary changes or migration (282, 283). Further changes to the microbiota are observed later in life, such as reduced diversity and symbiotic species and increased enteric bacteria. The

association between these changes and old age, age-related chronic inflammation, changes to diet and shared living spaces is being investigated (284-287).

The upper GI tract contains relatively few microbial inhabitants. The stomach and duodenum contain approximately 10^2 organisms per gram of contents. This rises to 10^4 - 10^7 in the jejunum, finally reaching $\sim 10^9$ colony forming units (CFUs)/mL in the terminal ileum and $\sim 10^{12}$ CFU/mL of primarily anaerobic bacteria in the colon (261, 288). The composition also changes along the length of the GI tract, with *Bacilli* and *Actinobacteria* enriched in the small intestine, while *Bacteroidetes* and *Lachnospiraceae* are enriched in the large intestine (270).

In general, the healthy microbiota is dominated by the phyla *Firmicutes* and *Bacteroidetes*. The ratio between these phyla is affected by dietary habits and geographic location, both of which correlate closely (289). Different studies have reported both *Bacteroidetes* and *Firmicutes* increased in obesity, possibly due to differences in environmental variables, when in reality the effects probably manifest on a lower taxonomic level (290-293). High diversity is also reported as a characteristic of a healthy microbiota. Indeed, diversity is reduced in diseases such as inflammatory disease, obesity and diabetes, while pathobionts such as *Enterobacteria* and *Fusobacterium* are increased (294-298). Diversity in CRC is more variable however, with some studies reporting decreased diversity in the disease state while others report increased diversity due to outgrowth of pathogenic species such as *Fusobacterium*, *Clostridia* and *Peptostreptococcus* suited to the tumour microenvironment (299-305).

1.3.2 The Microbiota and Colorectal Cancer

Research has focussed on the role in the microbiota in colon cancer, given the close apposition between the gut microbiome and colonic epithelium (Table 1.4) (306). The proposed mechanisms by which the microbiota may impact CRC include its effects on proto-

Table 1.4: Bacteria associated with human CRC (modified from Jahani-Sherafat *et al.* (263))

Sample	Bacteria	Reference
Faecal	<i>F. nucleatum</i>	(307-309)
Tumour	<i>F. nucleatum</i>	(310-312)
Faecal	<i>F. nucleatum, C. difficile</i>	(313)
Tumour	<i>F. nucleatum, B. fragilis</i>	(314)
Faecal, mucosal	<i>F. nucleatum, Enterobacteriaceae</i>	(315)
CRC	<i>F. nucleatum, Pan-fusobacterium</i>	(316)
Tumour	<i>Fusobacterium</i>	(317, 318)
Tumour	<i>Fusobacterium, Providencia</i>	(319)
Tumour	<i>Fusobacteria, Firmicutes</i>	(320)
Faeces	<i>Fusobacterium, Atopobium/Porphyromonas</i>	(321)
Faeces	<i>Fusobacterium, Porphyromonas</i>	(322)
CRC	<i>Fusobacterium, ETBF</i>	(323)
Tumour	<i>Fusobacterium, Roseburia</i>	(324)
Tumour	<i>Fusobacterium, E.faecalis, ETBF</i>	(325)
CRC	<i>Fusobacterium, Leptotrichia, Campylobacter</i>	(326)
Faecal	<i>Bacteroides/Prevotella</i>	(327)
Faeces	<i>Enterococcus faecalis</i>	(328)
Faecal, mucosal	<i>Bacteroidetes, Prevotella</i>	(329)
Tumour, mucosal	<i>E. coli</i>	(330)
Meta-analysis	<i>H. pylori</i>	(331)
Bloodstream	<i>S. gallolyticus, C. perfringens, C. septicum, Peptostreptococcus</i>	(332)
Bloodstream	<i>S. bovis</i>	(333)

oncogenic pathways such as immune regulation/inflammation, metabolism, proliferation and apoptosis, and the production of microbial metabolites which also have pro- and anti-tumorigenic associations (334). There is precedence for the involvement of bacteria in GI cancer. *Helicobacter pylori* is a constituent of the stomach microbiota which is present in half the global population (335). Nonetheless, it is the strongest known risk factor for gastric cancer and its links to this disease are well established (336).

However, only associative evidence for the role of the microbiota in human CRC exists. The microbiota is altered in the colon of CRC patients and in the tumour tissue compared to healthy controls, with adenomatous polyps representing an intermediate step between the two states (301). Studies investigating these alterations showed that the colonic mucosa of carcinoma patients, but not controls, was colonised by adherent and invasive *E. coli* (330, 337). Moreover, a high-risk CRC group had increased carcinogenic microbial metabolites, including hydrogen sulphide and cresol, in their faeces compared to a low-risk group despite having similar diets, with the difference ascribed to their different levels of enzymatically-active anaerobic bacteria (338). The gut bacteria can also modulate the production of mucus in the intestinal lumen, which in itself can play an important role in CRC by regulating the interaction of the gut bacteria and luminal contents with the colonic epithelium (339-342). Similar to the aforementioned rodent studies, *Lactobacillus* have been shown to reduce faecal and urinary mutagenicity induced by fried meat consumption and to reduce faecal β -glucuronidase, β -glucosidase, nitroreductase and glycocholic acid hydrolase activity (343-348). The microbiota has also been investigated as a potential predictive biomarker for human CRC. Two meta-analyses of human faecal shotgun sequencing studies identified microbial taxonomic signatures with sensitivity to and specificity for CRC which was comparable to common non-invasive clinical screening tests (349, 350). Models based on the functional gene content of the faecal microbiome were also generated, and enrichment of

the *bai* operon, which is involved in microbial bile acid metabolism, was demonstrated at both the genomic and transcriptomic levels (349, 350).

In contrast, evidence for the role of the gut bacteria in promoting CRC in animal models is more widespread. Germ-free (GF) animals completely lack microbial colonisation and tumour formation is reduced in these animals (351-354). Results from the APC^{MIN} mouse model of familial CRC in GF conditions are more ambiguous (355, 356). Faecal microbial transfer (FMT) from CRC patients or animal models of CRC to GF or conventional mice, either APC^{MIN} or administered AOM with or without DSS, increases tumorigenesis in these animals (357-361). Antibiotic treatment also affected tumorigenesis; reducing it in inflammatory models of CRC, but had neutral to harmful effects in APC^{MIN} mice (362-366). It was protective, however, in APC^{MIN} mice with compound mutations in DNA repair or interleukin receptor genes (367-369). Moreover, administration of bacteria associated with cancer risk, for example *Streptococcus bovis* or *Fusobacterium nucleatum*, to susceptible animals was shown to increase proliferation, inflammation and tumorigenesis (370, 371). Furthermore, Onoue *et al.* observed decreased numbers of ACF in DMH-treated GF rats compared to conventional rats. Tumour multiplicity was also increased in gnotobiotic (GB) rats colonised by enterococci compared to GB rats without enterococci, with the tumour numbers in the former group significantly decreased by inclusion of probiotic strain *Bifidobacterium breve* (372). A similar result was achieved by Horie *et al.* concerning adenomas, with the lowest incidence observed in rats mono-associated with probiotic *Lactobacillus acidophilus* (373).

1.3.2.1 Probiotics: Bacteria can also be used to suppress CRC. One such approach involves exploiting prebiotics or probiotic bacteria. Probiotic bacteria are characterised as “live microorganisms that, when administered in adequate amounts, confer a health benefit on

the host”, while prebiotics are food compounds which promote the growth and survival of beneficial microorganisms (374-378).

Clinical trials have provided evidence for the beneficial role of prebiotics and probiotics in CRC (Table 1.5). One trial administering a probiotic-prebiotic mixture of inulin, *Lactobacillus* and *Bifidobacterium* to CRC and polypectomised patients showed that the treatment lead to a decrease in colonic epithelial proliferation, decreased abundance of *C. perfringens* and reduced ability of faecal water to induce necrosis in colon cells *in vitro* (379). It also improved epithelial barrier function, reduced patients’ exposure to genotoxins and increased production of IFN γ . Similar studies replicated this effect (380-383). Moreover, administration of a mixture of *Lactobacillus* and *Propionibacterium* to healthy subjects reduced faecal levels of bacterial enzyme β -glucuronidase, which is implicated in the activation of AOM and other carcinogens in the colon, as well as β -glucosidase and urease, while *Bifidobacterium*-fermented milk maintained remission in UC patients (384). A prebiotic mixture also decreased irinotecan-induced diarrhoea and enterocolitis in chemotherapy patients (385). However, a study on the effect of prebiotics in post-operative cancer patients showed no improvement in serum levels of IL6, CRP, fibrinogen, white cell count, differential blood count or any reductions in subsequent complications (386).

In animal studies, *Bifidobacterium longum* has been shown to ameliorate AOM/DMH-induced colon carcinogenesis, an effect that is enhanced by co-administration with the prebiotics inulin and lactulose (387, 388). A similar effect was seen with *Lactobacillus* species, although this effect was absent when probiotic administration was delayed until 9 weeks into DMH-administration, suggesting *Lactobacillus* was only protective in the early stages of tumorigenesis (389, 390). Moreover, a reduction in the numbers of tumours that developed was observed after 20 weeks, and not 36 weeks, suggesting *Lactobacillus* increased the latency or induction period of CRC (390).

Table 1.5: Microbial suppression of CRC (modified from Wollowski *et al.* (391))

Probiotic/Prebiotic	Protective Mechanism	References
<i>L. casei</i> , omniflora, or yogurt	Mutations in the Ames test decreased	(392, 393)
Strains of <i>Lactobacillus</i> and <i>Bifidobacterium</i> , cellular components and metabolites of LAB	DNA damage in colon cells decreased (antigenotoxicity)	(394, 395)
Fermented milk with <i>L. acidophilus</i> , <i>B. bifidum</i> , <i>Streptococcus lactis</i> , and <i>Streptococcus cremoris</i> ; lactulose	Procarcinogenic enzyme activity decreased: β -glucuronidase, nitroreductase, azoreductase, and detoxifying enzyme activity increased; Glutathione-S-transferase (GST)	(396-399)
<i>L. acidophilus</i> , <i>S. cremoris</i> , cell wall of LAB	Binding of mutagens	(400-402)
Milk fermented with <i>L. acidophilus</i>	Excretion of mutagens decreased	(403)
Milk fermented with <i>L. acidophilus</i> and <i>Bifidobacterium</i>	Immune stimulation increased	(404)
Fermentation of prebiotics	SCFA increased, pH decreased, probiotics increased	(405, 406)
Butyrate	Proliferation of transformed cells decreased, apoptosis of transformed cells increased	(407-409)

The ability of probiotics to effect early stage cancer development could be due to their function as anti-mutagenic agents. For instance, *Lactobacillus casei* gavage attenuated DNA damage induced by MNNG in rat colonic and gastric mucosa, while in another study, a selection of lactic acid bacteria (LAB) inhibited the genotoxic effects of MNNG and DMH in the rat colon (394, 395). In both studies, heat treatment eliminated the protective effect of the bacteria suggesting that viable bacteria are required for this effect, although the peptidoglycan fraction and whole freeze-dried *L. acidophilus* were anti-genotoxic. Arimochi *et al.* also demonstrated a reduction in ACF in AOM-treated rats after administration of *L. acidophilus* and *Clostridium perfringens* (410). In particular, *L. acidophilus* reduced the half-life of O₆-methylguanine adducts, suggesting a role in repair by DNA methyltransferase. Other potential mechanisms include the ability of LAB to bind dietary mutagens which limits their ability to interact with the colonic epithelium (400, 411). For example, toxic compounds are detoxified by glucuronidation in the liver, but bacterial β -glucuronidase activity may hydrolyse these molecules and liberate carcinogens. The activity of this enzyme was shown to be reduced in AOM- and DMH-treated rats following gavage with the probiotic *B. longum*; an effect that was enhanced by co-administration with prebiotic inulin, possibly as a result of acidification of the intestinal environment and displacement of bacteria expressing β -glucuronidase (412-414).

Probiotic and commensal bacteria can also provide health benefits by competing with more harmful organisms and preventing them from becoming established in the GI tract (415). LAB have been shown to inhibit the growth of coliforms in the GIT and return *E.coli*-infected rats to a normal microbiota composition while reducing β -glucuronidase activity (416). Probiotics can also produce antimicrobial compounds that inhibit enteric pathogens (417, 418).

LAB have also been demonstrated to modulate the immune response. *L. reuteri*, for example, suppressed inflammatory TNF α and CCL-2 production by lipopolysaccharide (LPS)-

activated monocytes and macrophages in children with Crohn's Disease by inhibiting c-Jun and AP-1 (419-423). A similar anti-inflammatory effect was also observed in rat pups (424). As well as inhibiting pro-inflammatory responses, bacteria may also mediate inflammation to suppress tumorigenesis. *L. casei* strain Shirota administered into the intrapleural cavity of tumour-bearing mice induced the production of pro-inflammatory IFN γ , IL1 β and TNF α , which in turn inhibited tumour growth and increased survival (425).

The microbiota may also play a role in the response to cancer chemotherapy and immunotherapy, and these have been discussed in section 1.1.1.8.

The microbiota can also mediate CRC risk by the production of short chain fatty acids (SCFAs). SCFAs, particularly butyrate, have been associated with positive health effects and protection against colon carcinogenesis (426). The majority of butyrate is produced by bacteria in *Clostridium* clusters XIVa and IV, particularly *Roseburia/Eubacterium rectale*-related bacteria in cluster XIVa and *Faecalibacterium prausnitzii* relatives in cluster IV (427-430).

1.3.3 Microbial Metabolites

Another key interaction between the host and the microbiota is through the production of microbial-derived metabolites (431). The next section will address microbial metabolism of bile acids.

1.4 Bile Salts

Bile salts are endogenous steroid molecules stored in the gallbladder for post-prandial release into the duodenum to aid lipid digestion. They are derived from cholesterol and are the major route of cholesterol elimination from the body. Food entering the duodenum triggers the production and secretion of cholecystokinin (CCK) from epithelial enteroendocrine cells. CCK then stimulates contraction of the gallbladder and the relaxation of the sphincter of Oddi, allowing bile and pancreatic juices to flow from the ampulla of Vater into the duodenum to aid digestion. Bile in the gallbladder is composed of about 92% water, 6% bile salts, 0.3% bilirubin, 2% fats, and 200mEq/L inorganic salts (432, 433). The major unconjugated bile acids are presented in Table 1.6.

1.4.1 Bile Salt Structure and Function

Bile salts consist of a bile acid conjugated to a taurine or glycine residue. Bile acids are synthesised in hepatocytes via cytochrome P450-mediated oxidation of cholesterol in a multi-step process, with the rate limiting step catalysed by the addition of a hydroxyl group to the carbon-7 position of the steroid nucleus by CYP7A1 or CYP7B1 (434-436). Bile acids are composed of a sterane core comprising three 6-carbon rings and one 5-carbon ring, with the 5-carbon ring featuring a 5- or 8-carbon side chain emanating from 17-carbon position (Figure 1.5). They can display a number of hydroxyl groups at various positions and the number and orientation of these hydroxyl groups define the different bile acids. The primary human bile acids are cholic acid (CA) and chenodeoxycholic acid (CDCA). CA has hydroxyl groups in the 3-, 7- and 12-carbon position of the steroid ring, each in the α -orientation. CDCA is identical except for missing the 12-carbon hydroxyl group. Bile acids are conjugated to a taurine or glycine residue by Bile acid-CoA:amino acid N-acyltransferase (BAT) to form bile salts (437).

Table 1.6: Major unconjugated bile acids.

	Bile Acid	Abbreviation
Individual Bile Acids	Dehydrocholic Acid	DHCA
	Lithocholic Acid	LCA
	Ursodeoxycholic Acid	UDCA
	Chenodeoxycholic Acid	CDCA
	Hyodeoxycholic Acid	HDCA
	Deoxycholic Acid	DCA
	Cholic Acid	CA
	Hyochoic Acid	HCA
	Muricholic Acids (α , β , ω)	$\alpha/\beta/\omega$ -MCA

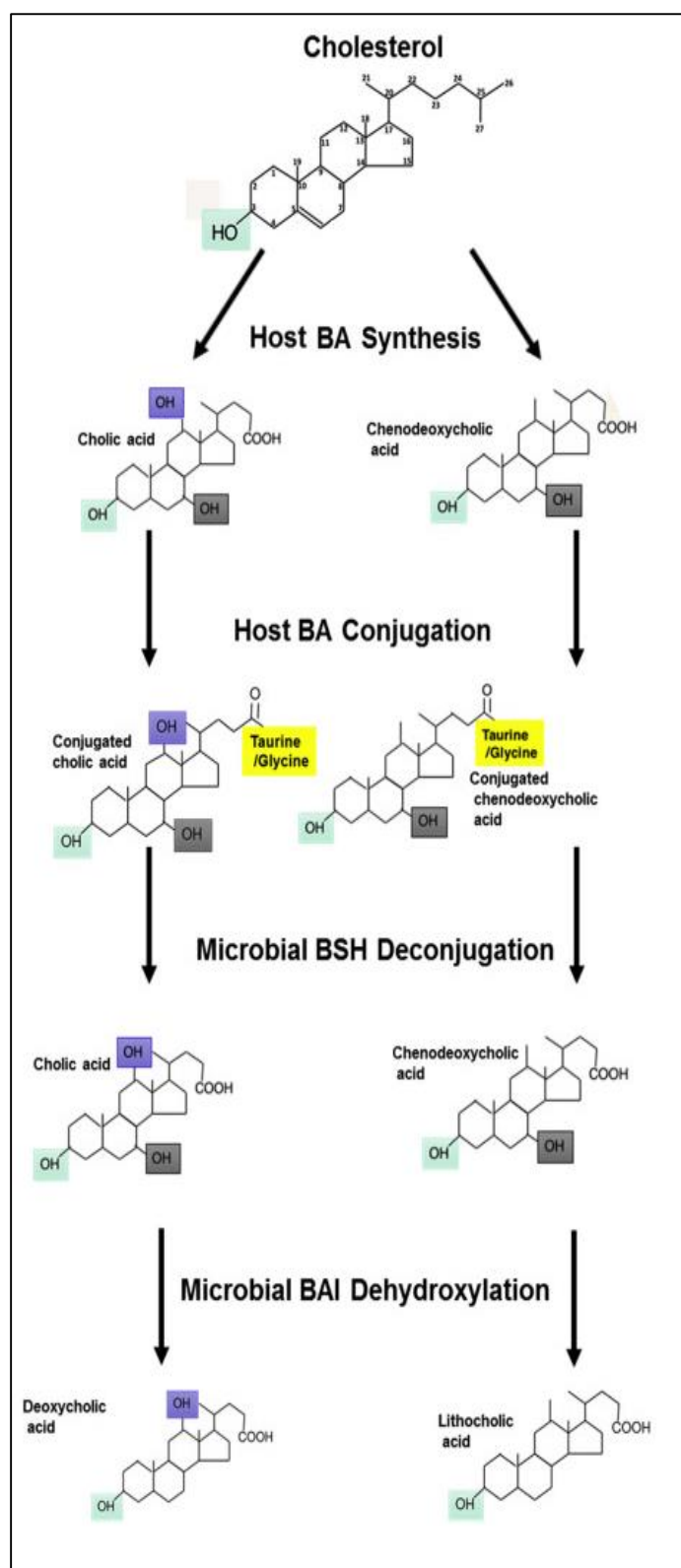


Figure 1.5: Cholesterol, primary and secondary bile acids. Cholic acid (CA) and chenodeoxycholic acid (CDCA) are the primary human bile acids which are formed from cholesterol in the liver by a multi-step pathway. After conjugation and release into the duodenum, 95% are reabsorbed at the terminal ileum but 5% escape to the large intestine to be modified by the microbiota. This includes deconjugation and 7 α -dehydroxylation to form deoxycholic acid and lithocholic acid from CA and CDCA respectively. Figure modified from Long *et al.*, 2017 (438).

Once released into the duodenum, the amphipathic bile salts emulsify lipids forming micelles. This increases the efficiency of pancreatic lipase-mediated hydrolysis of triglycerides into two fatty acids and a monoglyceride which can then be absorbed from the jejunum. This absorption process is believed to be protein-dependent, but passive diffusion or a combination of both is possible (440, 441). Bile salts themselves are not taken up by the enterocyte in this process but are complexed to plasma proteins and reabsorbed from the distal ileum into the bloodstream by active transport. They are then returned via the portal vein to the liver by the process of enterohepatic circulation (442). Upon reaching the liver, they are efficiently absorbed by active transporters on the sinusoidal surface of hepatocytes and secreted into bile canaliculi for return to the gallbladder.

The relative hydrophobicities of bile acids are relevant to their biological effects. Bile acids with greater hydrophobicity are more cytotoxic and more closely associated with disease (443-445). Bile acids show a gradient of increasing hydrophobicity proceeding $UDCA < CA < CDCA < DCA < LCA$ (446, 447). The biplanar sterane core of bile acids has a hydrophobic face (convex, β face) and a less hydrophobic face (concave, α face) (448). Hydroxyl groups which project from the core in the β -orientation therefore do more to reduce the hydrophobicity of the bile acid by obscuring its hydrophobic β face, for example UDCA and MCA which have β -orientated hydroxyl groups in the 7- and 6-carbon positions respectively. Conjugation also decreases the hydrophobicity of bile salts as the amino acid moiety terminates in a ionic polar residue which contributes a strong hydrophilic effect (448). This relative hydrophobicity also contributes to bile acid transport since conjugated bile acids require active transport to cross cellular membranes while free bile acids can be taken up by passive diffusion.

1.4.2 Bile Acid Regulation

Bile acid homeostasis is regulated by Farnesoid X Receptor (FXR; also known as Bile Acid Receptor (BAR)) which is a nuclear receptor expressed by liver hepatocytes and small intestine enterocytes (449-451). Upon activation, FXR forms a dimer with retinoid X receptor (RXR), translocates to the nucleus and directly binds DNA to moderate gene expression. It is activated by all four major human bile acids, with reducing affinity for CDCA>DCA=LCA>CA (452). Activation of FXR was enhanced in conjugated bile acids in cells expressing bile acid transporters required to transport these molecules across the cellular membrane (452-456). Hydrophilic bile acid α -MCA does not activate FXR (449), while β -MCA did not activate FXR in human HepG2 hepatocellular carcinoma cell line which express FXR and organic anion-transporting polypeptides (OATPs) required for hepatic uptake of hydrophobic bile acids (450, 457). However, conjugates of MCAs act as FXR antagonists with IC₅₀ values of 28mM and 40mM for T- α MCA and T- β MCA respectively (458-461). UDCA may also act as a weak FXR antagonist (462). CA does not appear to activate FXR in humans but does in mice which lack CDCA, the most potent endogenous human FXR agonist (463, 464).

Bile acid synthesis is the primary route of cholesterol elimination in the body. It progresses through two major pathways. The classic pathway produces CA and the alternative pathway produced CDCA. Sterol 12 α -hydroxylase (CYP8B1) mediates the production of CA and the alternative pathway produces CDCA when CYP8B1 is deficient (465). Cytochrome P450 7A (CYP7A1) catalyses the rate-limiting step in classical pathways for bile acid biosynthesis. Regulation of these enzymes allows maintenance of bile acid homeostasis.

Ileal FXR inhibits *CYP7A1* by up-regulating FGF19 in the intestine, from where it is circulated to the liver to activate its receptor FGFR4 which complexes with β -Klotho on the surface of hepatocytes (466, 467). *CYP7A1* can also be inhibited by FXR expressed in hepatocytes by a

mechanism dependent on small heterodimer protein (SHP) (468), and can be suppressed by cytokines which are produced as a result of rising concentration of bile acids (469).

1.4.3 Bile Acid Transport

1.4.3.1 Ileal Transporters

Apical sodium-dependant bile acid transporter (ASBT) is found on the apical brush border of ileal enterocytes and transports bile acids from the lumen across the membrane into the cell (Figure 1.6) (470). FXR down-regulates ASBT in mouse enterocytes via the FXR target gene small heterodimer protein (SHP) which interacts with liver receptor-1 (LRH-1) to prevent it binding ASBT response elements and promoting transcription (471). In contrast, if rats lack an LRH-1 responsive element within the ASBT promoter, this gene is not inhibited by FXR. Human ASBT is also down-regulated by SHP interfering with retinoic acid receptor (RAR)/RXR activation in a similar fashion to mice (472), with ASBT deficiency causing bile acid malabsorption, resulting in steatorrhea and reduced plasma cholesterol (473, 474).

Ileal bile acid binding protein (I-BABP), on the other hand, is localised to the enterocyte cytoplasm and is believed to shuttle bile acids from ASBT at the apical membrane to organic solute transporter (Ost) α/β at the basolateral membrane. It is also expressed in the terminal ileum (475). Bile acids up-regulate the expression of I-BABP *in vitro* and *in vivo* by forming a FXR/RXR heterodimer (476, 477).

To complete transport across the cell, Ost α and Ost β form a heterodimer at the basolateral membrane to shuttle bile acids across into portal circulation (478). Their expression is up-regulated by FXR signalling (479, 480). Ost α and Ost β are expressed in the intestine and kidney in mice, as well as the liver in humans (481).

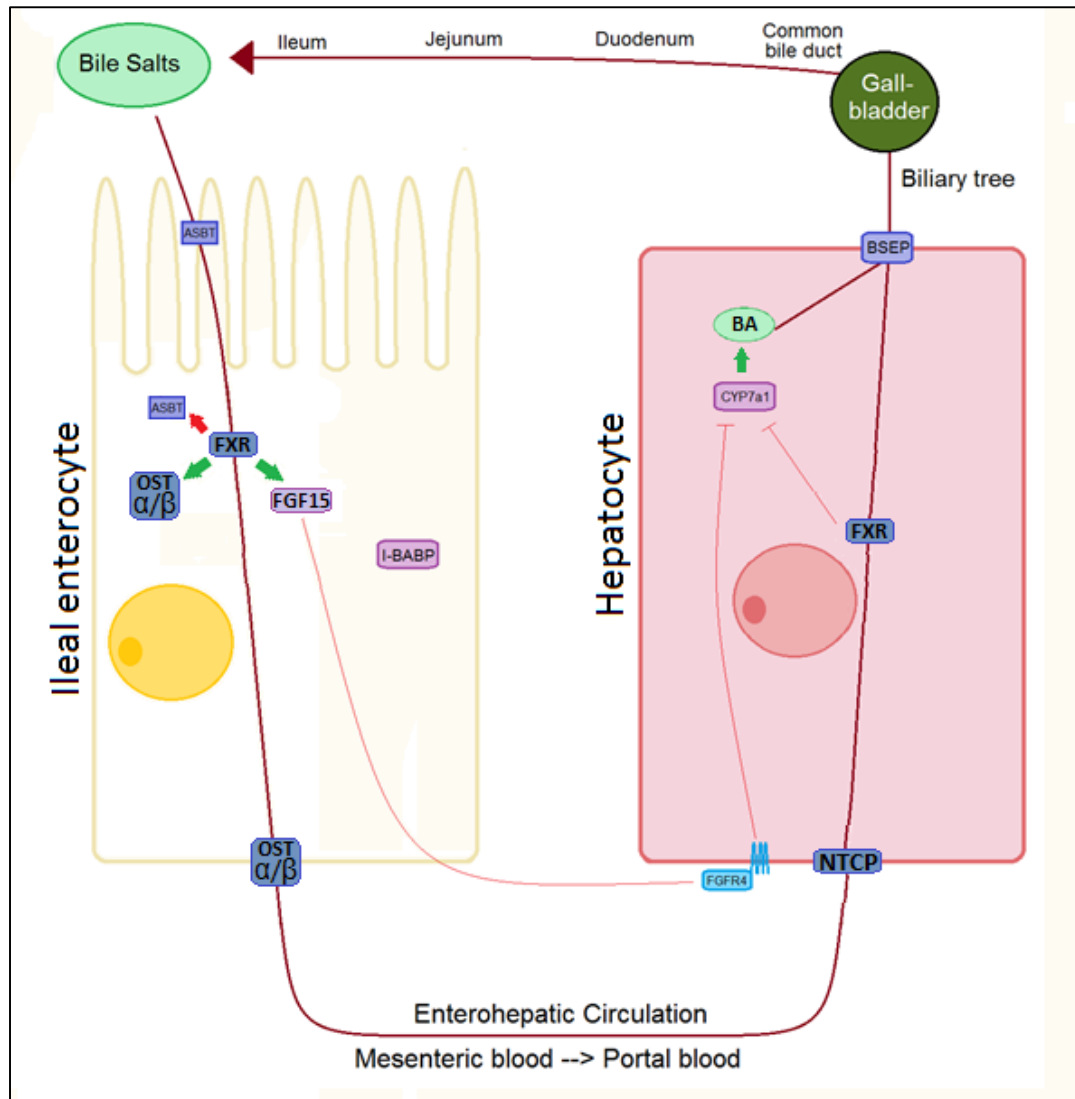


Figure 1.6: Enterohepatic circulation. Bile salts are produced in the hepatocyte and stored in the gallbladder in bile. Fat entering the duodenum triggers the secretion of cholecystokinin (CCK) from epithelial enteroendocrine cells. CCK then stimulates contraction of the gallbladder and release of bile into the duodenum via the common bile duct. Bile salts then aid the digestion and absorption of dietary fats in the small intestine before being transported across the apical membrane of terminal ileal enterocytes by Apical Sodium-dependent Bile acid Transporter (ASBT), possible aided by Ileal Bile Acid Binding Protein (I-BABP). Here, bile salts interact with Farnesoid X Receptor (FXR), which translocates to the nucleus to up-regulate Organic Solute Transporters α and β (Ost α/β) and Fibroblast Growth Factor 15 (FGF15), and down-regulate ASBT. Bile salts are then transported across the basolateral enterocyte membrane by the Ost α/β heterodimer into the mesenteric and portal blood which transport it back to the hepatocyte. It is transported across the basolateral hepatocyte membrane by Na⁺-Taurocholate Co-transporting Polypeptide (NTCP) where it can interact with hepatic FXR to down-regulate bile acid (BA) synthesis by inhibiting CYP7A1. Bile salts are then transported from the hepatocyte back into the biliary system by Bile Salt Export Pump (BSEP). Bile acid synthesis can also be suppressed by ileal FGF15 which translocates to the hepatocyte to bind FGFR4 and inhibit CYP7A1 (466).

1.4.3.2 Hepatic Transport

Na⁺-taurocholate co-transporting polypeptide (NTCP) and organic anion-transporting peptides (OATPs) are responsible for the uptake of conjugated and unconjugated bile acids, respectively, from the portal blood at the hepatocyte basolateral membrane (482, 483). FXR inhibits the expression of NTCP via the induction of SHP (484). The bile salt export pump (BSEP) transports recycled or newly synthesised bile acids across the apical membrane into the bile canaliculi for transport and storage in the gallbladder (485, 486). BSEP is also a target gene of FXR (487).

1.4.4 FXR and CRC

Bile acids have also been associated with cancer via FXR signalling (488). *FXR* expression is down-regulated in human colorectal tumours and colon cancer cell lines (489). Moreover, *Fxr*^{-/-} mice are predisposed to cancer, displaying increased IL1 β , IL6, β -catenin, cyclin D1, and c-myc at three months of age, and hepatocellular adenoma, carcinoma and hepatocholangiocellular carcinoma after 12 months (490, 491). These phenotypes are not simply the result of elevated bile acids as a result of FXR deficiency as they were not ameliorated by co-administration of bile acid-sequestrant cholestyramine (492). Furthermore, administration of the bile acid T- β MCA, which is an FXR antagonist bile acid, increases stem cell proliferation by activating Wnt, impairs intestinal integrity, accelerates tumour growth, induces dysplastic morphology and chromosome instability, and increases levels of serum IFN γ , IL6 and IL17 in APC^{MIN} mice, *in vitro* and *ex vivo* in an FXR-dependent manner (493). In addition, FXR agonists promoted apoptosis, down-regulated the intestinal stem cell genes *Lgr5*, *Olfm4* and the Wnt-dependent cancer stem cell gene *Ascl2*, and also inhibited Wnt signalling (492). FXR agonists also delayed tumour progression, reduced tumour multiplicity, proliferation and serum cytokines, and improved intestinal morphology,

differentiation, barrier function and bile acid homeostasis (493). The APC^{MIN} phenotype can also contribute to bile acid dysregulation via FXR, as these mice display increased CpG methylation in the *Fxr* promoter which silences *Fxr* expression (494). *Fxr* has also been demonstrated to modulate the microbiota. FXR antagonism increased the proportion of *Bacteroidetes* compared to *Firmicutes*, a ratio which may contribute to the pathogenicity of obesity in mice fed a high fat diet (458, 495). FXR can also suppress expression of pro-inflammatory cytokines (496) to the extent that a synthetic FXR ligand protected mice from DSS-induced colitis (497). Impaired FXR function has also been implicated in hepatocellular carcinoma due to dysregulation of bile acid homeostasis and pro-inflammatory signalling (498).

1.4.5 Microbial Bile Salt Metabolism

Although most bile salts are reabsorbed in the distal ileum, around 5% escape to the large intestine where they can be modified by intestinal bacteria (499). This loss is balanced by *de novo* bile acid synthesis from cholesterol in the liver. These bile acids undergo deconjugation and 7 α -dehydroxylation by the gut bacteria, as well as a number of other minor modifications (500).

1.4.5.1 Deconjugation

Deconjugation of bile salts involves hydrolysis of their amino acid moiety to form free bile acids. This is performed by bacteria expressing bile salt hydrolase (*bsh*), a family of enzymes that are widespread in the colonic microbiota. *Bsh* enzymes have been cloned for a number of bacteria (501-503). Their function may be detoxifying bile salts since Bsh activity appears to be proportional to the toxicity of conjugated bile acids to the bacteria, at least in

Lactobacilli (504). This is complicated by the fact that deconjugated bile acids are more toxic than their conjugates (505). However, unconjugated bile acids which undergo subsequent 7 α -dehydroxylation can precipitate and bind to fibre to be excreted, or may be passively reabsorbed across the colonic membrane, reducing their concentrations in the lumen (506). Liberated taurine can also act as a source of sulphur which is utilised by a number of bacteria including *Bacteroides* (507, 508). Dehydroxylation to DCA was also promoted when the initial substrate was T-CA rather than CA, and glycine may provide a similar function to some bacteria (509). *Bsh* from *B. longum* is also co-transcribed with a component of the nitrogen regulation cascade, suggesting nitrogen scavenging from the liberated amino group could be an incentive for deconjugation (510). A *Bsh* enzyme from *Listeria monocytogenes* was identified as a virulence factor, ostensibly by facilitating colonisation in the presence of bile salts (511, 512). Several *bsh* genes have been identified and can differ in their substrate specificity (513), and bacterial strains can express several different *bsh* alleles (514).

1.4.5.2 Dehydroxylation

Deconjugated bile acids are susceptible to hydrolysis of the hydroxyl group at their 7-carbon position by the 7 α / β -dehydroxylase pathway by other microbes. From CA, this forms deoxycholic acid (DCA) with hydroxyl groups at the 3- and 12-carbon position. From CDCA, it forms lithocholic acid (LCA) with a hydroxyl group at the 3-carbon position only. DCA then accumulates in the bile acid pool via passive absorption by the colonic epithelium as the human liver is unable to hydroxylate DCA, as has been observed rodents (515). While some LCA is also reabsorbed, it is sulphated in the liver and secreted into the intestine where it is poorly soluble and subsequently excreted in the faeces (516). As a result, the composition of the human bile salt pool secreted from the gallbladder is ~35% CA and CDCA, ~25% DCA and <5% LCA, while in mice the majority of CDCA is converted into muricholic acid (MCA) (517).

However, DCA and LCA each make up about a third of faecal bile acid, with their precursors CA and CDCA only making up around 2% each, demonstrating the efficiency of this dehydroxylation by the gut bacteria despite only a small portion of *Clostridia* having this capability. The benefit of this pathway to the bacteria involved is the utilisation of bile acids as electron acceptors, with the 7 α / β -dehydroxylation pathway producing a two-electron reduction (506).

Bacteria which dehydroxylate bile acids do so with genes expressed on the *bile acid inducible* (*bai*) operon which has been characterised in a number of *Clostridium* species (518-520).

1.4.5.3 Other Modifications

The colonic microbiota can also reversibly epimerise the hydroxyl groups of bile acids between the α - and β -position by the enzyme hydroxysteroid dehydrogenase (HSDH) through an oxo-intermediate (521). This can produce bile acids such as hydrophilic ursodeoxycholic acid, an epimer of CDCA with the hydroxyl group at the 7-carbon position in the β -orientation (522). Specific HSDH enzymes also exist for hydroxyl groups at other positions on the bile acid sterane core (523). Bacteria may benefit from this reaction by generating adenosine triphosphate (ATP) or by reducing the toxicity of bile acids, for example CDCA to UDCA (524-526). UDCA also inhibits HSDH in *Clostridium absonum*, suggesting this is the intended product. This reaction can be performed on conjugated and unconjugated bile acids (527). Sulphatase activity has also been detected in a subset of gut bacteria, including *Clostridium*, *Peptococcus*, *Fusobacterium* and *Pseudomonas* (528), which can remove a sulfo-group in the 3 α - or 3 β -position on bile acids. Toxic bile acid LCA and other xenobiotics are sulphated in order to aid their excretion in the faeces, mediated by bile acid receptor Pregnane X Receptor (PXR) (529, 530). A reduction in desulphation activity was also observed in IBD patients and may contribute to a pro-inflammatory character of the bile

acid pool in these patients (531). Esters of bile acids are also found in the faeces and are believed to be the product of esterification by *Bacteroides*, *Eubacterium* and *Lactobacillus* species in the gut bacteria (528, 532).

1.4.6 Other Bile Acid Receptors

The family of bile acid receptors includes cell membrane and nuclear receptors with a variety of functions and ligand specificities.

1.4.6.1 Sensing X Receptor/Pregnane X Receptor (SXR/PXR)

Human SXR and its rodent homolog PXR are receptors activated by the bile acid LCA and its 3-keto metabolite. These receptors are involved in the detoxification of various toxic xenobiotics such as LCA which are risk factors for cancer (533). The role of PXR in cancer is unclear. In one study, activation of PXR increased cell growth, invasion and metastasis in a human colon cancer cell line (HT29) and a xenograft model, an effect which was eliminated by PXR knockdown (534). It also promoted the expression of apoptosis inhibitors Bcl-2 and Bcl-xL in human and rat hepatocytes, possibly related to its protecting role in detoxification, and suppressed p53 (535, 536). However, PXR is also lost on many colon tumours. Moreover, ectopic expression of PXR inhibited cell growth in HT29 cells and in a xenograft model by regulating p21 (WAF1/CIP1) and E2F/Rb pathways, in exact contrast to the finding in Wang *et al.* (537). This suggests PXR has pro- and anti-tumorigenic roles depending on the context of its activation and expression (538).

1.4.6.2 Constitutive Androstane Receptor (CAR)

CAR has a similar role as PXR in the hydroxylation, conjugation, and excretion of toxic compounds including LCA, which acts as its ligand (539). It also affects bile acid composition, shifting synthesis toward the formation of less toxic trihydroxy bile acids, decreasing hepatic bile acid concentrations and increasing faecal elimination, by which it may protect against LCA-induced liver injury (540, 541). As well as its cancer-protective role in eliminating possible carcinogens, CAR enhances the expression of tumour suppressor genes WT1 and MGMT. However, CAR-mediated suppression of apoptosis in the presence of chronic xenobiotic stress can increase the risk of hepatic carcinogenesis (542, 543).

1.4.6.3 Vitamin D Receptor (VDR)

VDR is a receptor for LCA and is significantly more sensitive to this secondary bile acid than other nuclear receptors (544). It is also activated by the metabolites of LCA; 3-keto-LCA, G-LCA, and 6-keto-LCA (544).

Vitamin D, also a VDR agonist, has been shown to inhibit tumorigenesis in *N*-nitroso-*N*-methylurea (MNU)-treated rats, up-regulate CYP3A in CRC cells *in vitro*, and has been associated with a reduced risk of CRC in humans in observational studies (545, 546). Vitamin D reduces proliferation in colonocytes, induces differentiation in a calcium-dependent manner, induces apoptosis, inhibits invasion and metastasis and maintains genomic integrity in cooperation with p53 (547-551). It also has a role in immune modulation via T-cell and B-cell suppression, stimulation of monocytes to macrophages and inhibition of dendritic cells (552-556).

1.4.6.4 Takeda G Protein-Coupled Receptor 5 (TGR5)

GPBAR1/TGR5 is a cell surface G protein-coupled receptor sensitive to bile acids. Upon activation, it internalises and activates ERK/MAPK signalling (557). It is highly expressed in human spleen and resting monocytes, with very low expression in rat and mouse (557). TGR5 shows greater affinity for conjugated bile acids, with a decreasing potency for LCA>DCA>CDCA>CA (453, 454, 557). While TGR5 has been associated with gastric carcinoma, no direct link has yet been made with CRC (558).

1.4.6.5 Liver X Receptor (LXR)

LXRs are a family of nuclear receptors. LXR α is primarily expressed in liver, intestine, adipose tissue, and macrophages while LXR β is expressed ubiquitously (559, 560). Their primary ligands are oxysterols but are also activated by hyodeoxycholic acid (HDCA) (559, 561, 562). LXRs are involved in the conversion of cholesterol to bile acids in the presence of high cholesterol concentrations by promoting expression of CYP7A1 (559, 561). LXRs suppress apoptosis in macrophages and are involved in apoptotic cell clearance and the maintenance of immune tolerance. LXR-deficient macrophages displayed defective phagocytosis and pro-inflammatory response to apoptotic cells, towards which they develop auto-antibodies (563).

1.4.7 Bile Acids and CRC

Secondary bile acids are hydrophobic, cytotoxic molecules and evidence suggests they play a role in colon cancer. For example, numerous epidemiological studies have highlighted higher faecal bile acid content in populations with increased CRC rates (564-570). Moreover, DCA has been shown to be higher in patients with colorectal adenomas and was first

proposed as a carcinogen in 1940 based on its induction of tumours in mice (571, 572). Bile acids were initially classified as tumour promoters rather than tumour initiators, as studies primarily demonstrated their action when co-administered with chemical carcinogens such as MAM or MNNG (573-576). However, the role of bile acids as aetiological agents of cancer in their own right is now emerging (577).

A diet high in fat and low in fibre is a known risk factor for colon cancer and this diet was also associated with increased secondary bile acids, as well as increased glucuronidase deconjugation (578).

Interestingly, GF rats are generally resistant to chemical carcinogen-induced colorectal cancer (579). However, rats treated with MNNG and DCA displayed colonic adenocarcinomas, suggesting microbial production of DCA could play a role in tumorigenesis and may explain the resistance to CRC observed in GF animals (580).

1.4.7.1 Mechanisms of Bile Acid-induced Carcinogenesis

Bile acids can increase cancer risk by a number of mechanisms. These include the induction of DNA damage via the production of reactive oxygen and nitrogen species, the compromising of DNA repair, the selection for apoptosis-resistant cells and the promotion of inflammation.

1.4.7.1.1 COX: COX-1 is constitutively expressed in many mammalian tissues whilst COX-2 is expressed during inflammation. COX-2 has been shown to play a role on tumour growth and progression, with COX inhibitors such as non-steroidal anti-inflammatory drugs (NSAIDs) and COX-2-specific drugs shown to reduce the risk of colon cancer (581-585). Interestingly, the dihydroxy bile acids DCA and CDCA were shown to up-regulate COX-2 and its downstream

inflammatory product PGE₂ in a protein kinase C (PKC)-dependent manner, whilst activating c-Jun and AP-1 (586, 587). In contrast, neither CA nor conjugated bile acids had any effect. DCA-induced expression of COX-2 is also reliant on intact NF-κB in HCT116 cells (586).

1.4.7.1.2 Reactive Oxygen Species: Bile acids generate ROS via a detergent effect on membrane enzymes which produces arachidonic acid (588). These in turn act as a substrate for COX and lipoxygenase (LOX) which produce ROS during the formation of prostaglandins and leukotrienes (588-590). Activation of this pathway also stimulates the proliferation of the colonic epithelium (588).

ROS have been shown to induce DNA damage, promote neoplastic growth, induce nucleotide substitutions and encourage microsatellite instability (439, 591-593). Oxidative damage has been described as the primary cause of spontaneous mutation in mammalian cells and although over 20 resultant DNA lesions have been described, only a small number have been characterised (594, 595).

Studies have shown that the hydrophobic bile acids, DCA and CDCA, indirectly activate protein PKC, enhance proliferation and produce ROS (596). PKC activation and subsequent ROS generation was inhibited by retinoic acid. Both DCA and LCA were shown to induce DNA damage in a dose-dependent manner *in vitro* and this was inhibited by both α-tocopherol and retinol, which may involve an antioxidant effect in the case of the former and reduced PKC activation or membrane stabilisation in the latter (597). Intra-colonic administration of DCA also induced proliferation and ornithine decarboxylase (ODC) activity, an effect that was abolished by superoxide dismutase, suggesting a role for reactive oxygen in the stimulation of colonic proliferation by bile salts (589). *In vitro*, DCA and LCA induced DNA damage that was inhibited by endonuclease III treatment, suggesting oxidative DNA damage, at concentrations associated with pathological conditions such as CRC (598).

1.4.7.1.3 Reactive Nitrogen Species: DCA has been shown to activate inducible nitric oxide synthase (iNOS) in a PKC-dependent manner *in vitro* (439, 599). Overexpression of iNOS leads to increased levels of nitric oxide (NO) and other RNS which promote pro-tumorigenic states such as apoptosis resistance and DNA damage (600). Consistent with this, DCA-induced colitis is attenuated in *Nos2*-knockout mice, and exposure of a human colon cancer cell (HCT116 cells) to DCA resulted in S-nitrosylated proteins, which are a biomarker for nitrosative stress that can result in apoptosis resistance (601, 602).

1.4.7.1.4 Apoptosis: Bile acids appear to induce apoptosis in the short term but select for apoptosis-resistant cells in the longer term (603). Oxidative damage caused by ROS induces apoptosis (604). DCA induced apoptosis in HCT116 cells by releasing cytochrome c from mitochondria, an effect that was inhibited by UDCA downstream of cytochrome c release (605). A study on another human colon cancer cell line (SW480 cells) suggested apoptosis was triggered by DCA via the induction of mitochondria-mediated apoptosis, and was independent of the death receptor CD95-mediated apoptotic pathway which is often down-regulated in CRC (606). Moreover, Payne *et al.* showed DCA-induced apoptosis could be prevented in HCT116 cells by inhibition of mitochondrial complexes I-V, implicating mitochondrial crosstalk with apoptotic signalling pathways and mitochondrial-nucleolar crosstalk in the development of apoptosis resistance (607). The ability of bile acids to induce apoptosis appears to be related to their hydrophobicity, with the most powerful effect displayed by the most hydrophobic bile acids, DCA and CDCA (608).

These findings are counter-intuitive to the common understanding of tumorigenic cells as displaying apoptosis resistance. In fact, colon cancer patients' normal tumour-adjacent tissue displays resistance to bile salt- and bile acid-induced apoptosis, and this is mediated by an up-regulation of the anti-apoptotic protein Bcl-xL (603, 609-613). This appears to occur

particularly in the top third of colonic crypts, suggesting adenomas may arise in fields of apoptosis-resistant cells. Indeed, rats chronically fed CA displayed apoptosis resistance and a similar result was observed with HCT116 cells *in vivo* (614, 615). A study in the HT29 human colon cancer cell line implicates NO in apoptosis resistance (616). A proteomic study of HCT116 cells stimulated with DCA identified 18 over-expressed anti-apoptotic proteins and 11 under-expressed pro-apoptotic proteins, again highlighting the role of this bile acid in apoptosis resistance (617).

One mechanism by which bile acids may induce resistance to apoptosis is via inhibition of p53. Tumour-suppressor p53 plays an important role in the context of DNA damage by stimulating the repair of damaged DNA and by inducing apoptosis in irreparable cells. DCA inhibits the p53 response to DNA damage in a dose-dependent manner *in vitro*, likely by proteasomal degradation of p53 via a pathway involving extracellular signal-regulated kinase (ERK) signalling (618). This effect was not observed for CA or UDCA. *In vitro*, HCT116 cells are more susceptible to DCA-induced apoptosis than HT29s, which is consistent with p53-mediated apoptosis resistance mediated by bile acids, as HCT116 cells contain an intact p53 gene while HT29s do not (586, 619). Additionally, a variant of cytosolic bile acid shuttle protein I-BABP, whose expression is controlled by NF- κ B instead of nuclear bile acid receptor FXR, has also been shown to mediate the survival of HCT116 cells at physiological DCA concentrations (620).

1.4.7.1.5 Compromised Repair and Aneuploidy: Bile acids can also induce chromosomal abnormality such as aneuploidy and micronucleus formation (621, 622). DCA, LCA, CDCA and CA each induced mitotic chromosome aneuploidy in yeast, while T-DCA and G-DCA did not (623). Oxidative stress is a well-established source chromosomal instability and this is a plausible route of bile-acid induced DNA damage and increased CRC risk (624, 625).

LCA was demonstrated to inhibit the repair activity of DNA polymerase β which could exacerbate the consequences of bile acid-induced DNA damage (626). A proteomic study of CRC cell lines induced with DCA identified ten alterations in ten proteins involved in DNA repair and cell cycle checkpoints (617).

1.5 Aims and Objectives

The role of the microbiota in human health and disease is complex. It is possible that changes in the gut microbiota in the pre-neoplastic colon could drive or inhibit tumorigenesis and evidence for this has been provided by animal studies. However, it is also possible that tumorigenesis occurs entirely as a result of host processes independent of the microbiota, and so far human studies have failed to provide hard evidence to the contrary. This question of “cause or consequence” is an important unanswered problem in the study of the role of the microbiota in disease. Similarly, after cancer initiation, components of the microbiota may suppress or promote growth, invasion and metastasis but human evidence for this is lacking.

CRC pathogenesis is a process that takes place over decades. This makes it difficult to track the microbiota of a sufficiently large cohort and impossible to combine those data with the invasive colonoscopies and biopsies necessary to provide samples before, during and after tumorigenesis. As such, we rely on animal studies to investigate these effects.

We begin this investigation in **Chapter 3** with a time-course study using the AOM mouse model of sporadic colon cancer. Our objective was to identify the changes that occur in a tumorigenic colon in the host and the microbiota and set them to a timeline. This should provide clarity on which changes are potential causal events occurring earlier in the process from those which occur later. This novel understanding of the nature of host-microbe interaction during tumorigenesis could reveal further research potential, including the possibility of reducing cancer risk via early intervention in the microbiota, which we will address in **Chapter 5**.

We perform a second time-course study in **Chapter 4**, this time with the APC^{MIN} mouse model of familial CRC. Here, we sample the faecal microbiota of our mouse model and wild-type controls before, during and after the onset of tumorigenesis to reveal the temporal order of

host and microbial changes during this process. This has important implications for our understanding of the role of the microbiota and the interaction between host and microbial factors in colon cancer, similar to our AOM time-course study.

We return to the APC^{MIN} mouse for an intervention study in **Chapter 5**. These mice are administered a commensal gut bacteria engineered to overexpress *bsh*, an enzyme common in probiotic bacteria and associated with beneficial health effects. Although this enzyme is widespread in probiotic bacteria, its role in liberating bile salts for modification into secondary bile acids has elicited caution. Our objective is to determine the role of this enzyme in cancer risk and attempt to elucidate its potential mechanisms of action, as well as provide further data on the role of the microbiota in CRC and the feasibility of probiotic gavage as a prophylactic intervention against cancer risk.

1.5.1 Hypotheses

Therefore our hypotheses were:

- That the microbiota is involved in the initiation and progression of CRC, and that changes in the microbiota occur upstream of hyperplastic and neoplastic changes in the host epithelium (overarching hypothesis)
- This hypothesis also applies to **Chapters 3**
- Disease onset has a predominantly genetic basis in APC^{MIN} mice in **Chapter 4**, but we hypothesise that the gut microbiota may nonetheless modulate this process
- We hypothesise that bacteria expressing *bsh* are protective against CRC risk (**Chapter 5**)

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2.0 Materials and Methods

2.1 Reagents

Reagents, their suppliers and catalogue numbers are presented for reference in Table 2.1.

2.2 Mice and Experimental Design

Animal experiments were conducted in accordance with the regulations and guidelines of the Irish Department of Health following approval by the University College Cork Animal Experimentation Ethics Committee (2011/023), licensed under the Cruelty to Animals Act 1876 and the European Union (Protection of Animals Used for Scientific Purposes) Regulation 2012, under licence number B100/4108.

2.2.1 Azoxymethane-Treated Mice and Experimental Design (Chapter 3)

25mg of azoxymethane was reconstituted as a 25mg/mL stock solution in sterile PBS, aliquoted and stored at -20°C until required. Before use, it was diluted in sterile PBS for a 1mg/mL working solution.

C57BL/6J female mice between three and five weeks old were allowed to acclimatise for four weeks on a 12-hour light/dark cycle with access to water and chow *ad libitum*. After acclimatisation, mice were administered an intraperitoneal (i.p.) injection of 10mg/kg azoxymethane each week for five consecutive weeks. Control mice were given matching injections of PBS. Faeces were collected weekly and timed culls were performed at 8, 12, 24 and 48 weeks from the instigation of AOM treatment (Figure 2.1). Mice were culled and tissue was processed as per section 2.2.5.

Table 2.1: Reagents and their suppliers

Reagent	Supplier	Catalogue #
APCmin female mice	Charles River	002020
C57BL/6J female mice	Charles River	000664
Azoxymethane	Sigma Aldrich	A5486
Sucrose	Sigma Aldrich	S-9378
PBS	Sigma Aldrich	D8537
Streptomycin sulphate salt	Sigma Aldrich	S9137
Teklad 18% protein chow	Envigo	2018S
Vacutainer tubes - EDTA (K2)	BD	367525
Formalin	VWR Chemicals	9713-5000
Ki67 antibody	Thermo Scientific	RM-9106
Cleaved caspase-3	Cell Signaling Tech.	9661
GenElute mammalian total RNA	Sigma Aldrich	RTN70
Tetro cDNA synthesis kit	Bioline	BIO-65043
QIAamp Fast DNA Stool Kit	Qiagen	51604
3.5mm glass beads	Thistle Scientific	11079135
RNAlater	Sigma Aldrich	R0901
Ethanol	Sigma Aldrich	51976
β -Mercaptoethanol	Sigma Aldrich	M3148
SensiFAST No-ROX kit	Bioline	BIO-86020
Roche probe library	Roche	4683633001
Custom oligo qPCR primers	Eurofins Genomics	NA
Sodium citrate dihydrate	Sigma Aldrich	W302600
Hydrogen peroxide	Sigma Aldrich	H-1009
Saponin	Sigma Aldrich	S7900
Sodium chloride	Sigma Aldrich	S7653
Potassium chloride	VWR Chemicals	10198
Hydrochloric acid	Sigma Aldrich	84435
Acetic acid	Sigma Aldrich	A6283
Trizma hydrochloride	Sigma Aldrich	T5941
Trizma base	Sigma Aldrich	T1503
Serum	Sigma Aldrich	NA
Secondary antibodies	Dako	NA
Avidin/biotin blocking kit	Vector	SP-2001
Vectastatin ABC kit	Vector	PK-6100
DAB peroxidase substrate kit	Vector	SK-4100
Xylene	VWR Chemicals	28975.360
DPX moutant	VWR Chemicals	360294H
Histo-Clear II	National diagnostics	HS-202
Mayer's haematoxylin	Sigma Aldrich	MHS16
Eosin	Sakura Finetek USA	8702
LB Agar	Sigma Aldrich	L3022



Figure 2.1: Timeline of AOM study. Red lines denote 5 weekly intraperitoneal injections of 10mg/kg azoxymethane in treatment mice and equivalent volume of PBS in controls. Orange lines denote timed culls. Weeks are measured from the date of the first injection. $N=8$ per group, except for AOM Week 12 and PBS Week 24 ($N=7$).

2.2.2 APC^{MIN} Mice and Experimental Design (Chapter 4)

Ten C57BL/6J mice and ten APC^{MIN} mice on a C57BL/6J background were acquired from Charles River. Mice were female, age-matched and housed five per cage on a 12-hour light/dark cycle with access to water and chow *ad libitum*. Mice were individually removed from the cage at ~12pm each day and fresh faeces were collected at 4, 7, 11 and 14 weeks of age. At 14 weeks of age, mice were culled and tissue was processed as per section 2.2.5.

2.2.3 APC^{MIN} Mice and Experimental Design (Chapter 5)

25 APC^{MIN} mice on a C57BL/6J background were acquired from Charles River at four weeks of age. Mice were female, age-matched and housed five per cage on a 12-hour light/dark cycle with access to chow *ad libitum*, and to sterile-filtered drinking water containing 10g/L sterile-filtered streptomycin.

Mice were treated with 100μL by gavage of 15% sucrose/PBS (PBS group, *n*=5), 5x10⁹ CFUs K12 *E. coli* (EC group, *n*=10) or 5x10⁹ CFUs K12 *E. coli* modified to express bile salt hydrolase (*bsh*) (EC-BSH group, *n*=10). Treatment began at 4 weeks of age with gavage on two consecutive days with top-up doses at 8 and 12 weeks of age (Figure 2.2).

Successful colonisation was confirmed by analysis of faecal colony forming units (CFUs) of *E. coli* and *E. coli* engineered to overexpress *bsh*. Faecal pellets were suspended in PBS (25mg/mL) for 30 minutes before a serial dilution was prepared. 10μL dilute was inoculated in triplicate onto plates of LB agar with streptomycin and incubated at 37°C overnight. Colonies were counted the following day and colony forming units (CFUs) per millilitre were determined.

At 14 weeks of age, mice were culled and tissue was processed as per section 2.2.5.



Figure 2.2: Chapter 5 timeline. Streptomycin antibiotic was provided in drinking water throughout the experiment. Mice were gavaged with PBS, *E. coli* K12 or an isogenic *E. coli* strain engineered to overexpress *bile salt hydrolase* (*bsh*) on two consecutive days at 4, 8 and 12 weeks of age. Mice were culled at 14 weeks of age.

2.2.4 Bsh cloning

Bsh from a *Lactobacillus salivarius* strain had previously been cloned into *E.coli* MG1655 using splicing by overlap extension PCR (1). Expression was under the control of the P44 promoter. Transposon integration was carried out as described by McGrath et al. (2).

Successful construction was confirmed by PCR downstream from the *glmS* region, and by sequence analysis (GATC Biotech).

2.2.5 Tissue Collection and Processing

Mice were culled by decapitation and blood was collected. Spleens were removed, weighed and flash frozen in liquid nitrogen. Gallbladders were sealed with surgical suture and stored on ice. Livers were weighed and divided into three equal proportions for storage in formalin, RNAlater and flash freezing in liquid nitrogen. Small intestine length was measured and two ~1cm portions were taken from the proximal and distal ends for storage in RNAlater or flash freezing in liquid nitrogen. The cecum was weighed and stored on ice. Mesenteric and epididymal fat was removed, weighed and stored on ice.

In AOM mice, the remaining length of small intestine was formalin-fixed and divided into three equal portions which were Swiss-rolled and wax-embedded. The colon was processed similarly to the small intestine but was opened longitudinally, washed with PBS, examined for hyperplasia or neoplasia and mounted on microscope slides before immersion in formalin. Formalin-fixed colon tissue was stained with methylene-blue and examined for ACF (3), before being Swiss-rolled and wax embedded.

In APC^{MIN} mice, the remaining small intestine tissue was divided into three equal lengths, opened longitudinally, rinsed with PBS, and polyps were enumerated under a dissecting microscope. Each portion of small intestine was then Swiss-rolled, formalin-fixed and wax-

embedded. The colon was processed similarly to the small intestine without division into different sections.

Subsequently, all tissue which was stored on ice or flash frozen was moved to -80°C. Blood was centrifuged at 2,000xg for 10 minutes and plasma was stored at -80°C. Samples immersed in RNAlater or formalin were stored at 4°C for 24 hours. RNA samples were then decanted and moved to -80°C. Formalin-fixed liver samples were also wax-embedded. Wax-embedded tissue was cut into 4µm sections for mounting onto microscope slides.

2.3 Immunohistochemistry

Immunohistochemistry was performed using the standard procedure on 4µm-sectioned, formalin-fixed, paraffin-embedded slides (4). Sections were deparaffinised in xylene and re-hydrated using an ethanol gradient. Antigen retrieval was performed by microwave-heating in 0.01M sodium citrate pH 6.0. Endogenous peroxidase activity and non-specific binding were blocked with 3% H₂O₂ and 5% normal serum, respectively, prior to blocking with avidin/biotin before overnight incubation with primary antibody. Tissue was exposed to a biotinylated secondary antibody the following day, amplified with an avidin-biotin peroxidase and visualised with 3,3'-diaminobenzidine (DAB). Slides were counterstained with haematoxylin. Negative controls were prepared without primary antibody.

Ki67 primary antibody was used at 1/1000 dilution. The percentage of Ki67-positive cells per crypt was determined by counting the total number of cells and the number of Ki67-positive cells in 10 morphologically-normal crypts in the large intestine.

Activated caspase-3 primary antibody was used at 1/200 dilution. Images were captured at 40x magnification and the number of positive-stained cells per image was determined for

three randomly selected fields in each of three representative mice from each group at Weeks 8 and 24.

2.4 Quantitative Real-Time PCR

Gene transcription was analysed in tissue taken from the distal 1cm of the large intestine in mice treated with AOM (Chapter 3), and from the distal 1cm of the small intestine in APC^{MIN} mice (Chapters 4 and 5). RNA extraction was performed using the GenElute Mammalian Total RNA kit. Frozen tissue was immersed in 500µL lysis buffer containing 1% β-mercaptoethanol and subjected to bead-beating (45 seconds x2 with a 20 second interval using 3.5mm glass beads). Lysate was filtered, bound to a collection column, washed and eluted. Successful extraction was confirmed by determining the RNA concentration of the elute in a NanoDrop 2000 at wavelength 260nm and a 260/280 ratio of between 1.8 and 2.2.

RNA was converted to cDNA using the Tetro cDNA Synthesis Kit. 1-8µL of RNA elute containing ~3µg RNA per sample was incubated for 10 minutes at 70°C with 1µL deoxynucleotide triphosphates (dNTPs, 10mM) and 1µL random hexamer primer (50ng), with diethyl pyrocarbonate (DEPC)-treated sterile water added as necessary to make a total volume of 10µL. A mix of 0.5µL reverse transcriptase (100 units), 2µL reverse transcriptase buffer (5x), 0.5µL RNase inhibitor (5 units) and 7µL DEPC-treated sterile water was subsequently added to the RNA elute mix. This was incubated at room temperature for 10 minutes (initialisation), 37°C for 50 minutes (elongation) and 90°C for 5 minutes (termination).

Primers for qPCR were designed using the Roche Assay Design Centre and ordered as custom oligonucleotides from Eurofins Genomics. qPCR was performed using 1µL cDNA, 5µL SensiFAST No-ROX mix (2x), 0.5µL forward and reverse primers (5pmol), 3µL DEPC-treated

sterile water and 0.1µL Universal Probe Library (UPL, 10µM) hydrolysis probe from the Roche probe library as per Table 2.2, in a LightCycler 480 for 45 PCR cycles. Relative transcription was calculated using the $2^{-\Delta\Delta CT}$ method standardised to the average of the control group ΔCT (5).

2.5 Faecal 16S rRNA Gene Sequencing

Total DNA Extraction: Faecal samples were collected as described in section 2.2 and stored at -80°C. Subsequently, DNA was extracted from one faecal pellet using the QIAamp Fast DNA Stool Kit as per manufacturer's instructions with the addition of a bead-beating step (45 seconds x2 with a 20 second delay using 3.5mm glass beads). Sequencing was performed by Teagasc Moorepark, Ireland.

Amplicon Sequencing: The V3-V4 variable region of the 16s rRNA gene was amplified from each extracted DNA sample according to the 16S metagenomic sequencing library protocol (Illumina, Sweden). Initially, the template DNA was amplified using primers specific to the V3-V4 region of the 16s rRNA gene which also incorporates the Illumina overhang adaptor (Table 2.2). Each PCR reaction contained 2.5µl DNA template, 5µl forward primer (1µM), 5µl reverse primer (1µM) (Sigma, Ireland) and 12.5µl Kapa HiFi HotStart ReadyMix (2X) (Kapa Biosystems, London, United Kingdom). The template DNA was amplified under the following PCR conditions: 95°C for 3 min (initialisation); followed by 25 cycles of 95°C for 30 sec (denaturation), 55°C for 30 sec (annealing), 72°C for 30 sec (elongation); followed by a final elongation period of 5 minutes. A negative control reaction whereby the DNA template was replaced with PCR grade water was employed to confirm lack of contamination and PCR products were visualised using gel electrophoresis (1X TAE buffer, 1.5% agarose gel, 100V) post PCR reaction. Successful amplicons were then cleaned using the AMPure XP purification system (Labplan, Dublin, Ireland). A second PCR reaction was then performed using the

Table 2.2: qPCR Primer Sequences and matching Universal Probe Library (UPL) probes

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')	UPL #
<i>IL1β</i>	agttgacggaccccaaaag	agctggatgctctcatcagg	#38
<i>IL6</i>	gctaccaaactggatataatcagga	ccaggtagctatggtactccagaa	#6
<i>CXCL1</i>	gactccagccacactccaac	tgacagcgcagctcattg	#83
<i>CXCL2</i>	aaaatcatccaaaagataactgaacaa	ctttggttcttccgttgagg	#26
<i>CXCL5</i>	tagagcccaatctccacac	gagctggaggctcattgtg	#67
<i>IL10</i>	cagagccacatgctcctaga	tgtccagctggtcctttgtt	#41
<i>IL12</i>	aaggaacagtgggtgtccag	gttagcttctgaggacacatcttg	#27
<i>TNFα</i>	ctgtagcccacgtcgtagc	ttgagatccatgccgttg	#25
<i>TGFβ</i>	tggagcaacatgtggaactc	gtcagcagccggttacca	#72
<i>I-babp</i>	ggcaaagaatgtgaaatgcag	ccgaagtctggtgatagtgg	#53
<i>Fgf15</i>	ggcaagatatacgggctgat	tccatttcctccctgaaggt	#69
<i>Osta</i>	gctgcccacctctcatactt	ttgaagaaggcgacttgaaa	#3
<i>Ostb</i>	atcctggcaaacagaaatcg	tgacaggtcttctggtgtttct	#5
<i>Jun</i>	ccagaagatggtgtggtgttt	ctgaccctctccccttgc	#11
<i>Sox9</i>	gtacccgcatctgcacaac	ctcctccacgaagggtctct	#66
<i>Myc</i>	cctagtgtgcatgaggaga	tccacagacaccacatcaattt	#77
<i>Axin2</i>	gagagtgagcggcagagc	cggctgactcgttctcct	#96
<i>β-actin</i>	ctaaggccaaccgtgaaaag	accagaggcatacagggaca	#64
16S Ribosomal RNA gene V3-V4 region, forward primer (5'-3'):			
tcgtcggcagcgtcagatgtgtataagagacagcctacggnggcwgcag			
16S Ribosomal RNA gene V3-V4 region, reverse primer (5'-3'):			
gtctcgtgggctcggagatgtgtataagagacaggactachvgggtatctaattcc			

previously amplified and purified DNA as the template. Two indexing primers (Illumina Nextera XT indexing primers, Illumina) were used per sample to allow all samples to be pooled, sequenced and subsequently identified. Each reaction contained 25µl Kapa HiFi HotStart ReadyMix (2X), 5µl template DNA, 5µl index 1 primer (N7xx), 5µl index 2 primer (S5xx) and 10µl PCR grade water. PCR conditions were the same as previously described with the samples undergoing just eight cycles instead of 25. PCR products then underwent the same electrophoresis and cleaning protocols as described above. Samples were then quantified using the Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA) in conjunction with the broad range DNA quantification assay kit (Biosciences, Dublin, Ireland). All samples were then pooled to an equimolar concentration and the pool underwent a final cleaning step. The quality of the pool was determined using the Agilent Bioanalyser prior to sequencing. The sample pool was then denatured with 0.2 M NaOH, diluted to 4pM and combined with 10% (v/v) denatured 4pM PhiX. Samples were then sequenced on the MiSeq sequencing platform using a 2.300 cycle V3 Kit following protocols outlined by Illumina.

Bioinformatic and Statistical Analysis: Two-hundred and fifty base pair paired-end reads were assembled using FLASH (6). Reads were further processed with the inclusion of quality filtering, based on a quality score of >25, followed by subsequent removal of mismatched barcodes and sequences below length threshold using QIIME. USEARCH v7 (64-bit) was used for noise removal and chimera detection as well as clustering into operational taxonomic units (OTUs). PyNAST was used to align OTUs and taxonomy was assigned using BLAST against the SILVA SSURef database release 119.

Composition Analysis: The R package *compareGroups* (v. 3.1) was employed to detect statistically significant differences in abundances of individual taxa between groups using the Mann-Whitney U-test (MWU-test) with multiple corrections. Statistical significance was accepted as $P \leq 0.05$ after false discovery rate (FDR) multiple correction. Compositional alpha

diversity was calculated in QIIME (v. 1.9.1) and statistical differences between groups were detected using the MWU-test. The remaining statistical analyses were all performed in R (v. 3.2.3). The *phyloseq* package (v. 1.10) was used to calculate compositional β -diversity using genus-level relative abundance data. This was visualised by principal coordinate analysis (PCoA) using *ggplot2* (v. 2.1.0). Permutational multivariate analysis of variance (PERMANOVA) was used to test for differences in overall microbiome composition between groups using the *vegan* package's 'adonis' function.

Functional Analysis: PICRUSt was used to investigate the abundances of gene families based on the 16S rRNA gene data and, from this data, infer functional alterations in the microbiome. For this, the *pick OTUs* module was performed at 97% identity in a closed reference way using the Greengenes database (13_8) in QIIME. Data were normalised for 16S rRNA gene copy numbers and the metagenomes were predicted. KEGG Orthologs (KO) were identified from the inferred metagenomes and the R package *compareGroups* was used to identify differentially expressed functions between groups. Functional alpha diversity was calculated using the *vegan* package (v. 1.10) and statistical differences between groups were detected using the MWU-test. The *vegan* package was used to calculate functional β -diversity and this was visualised by principal coordinate analysis (PCoA) using *ggplot2*. Permutational multivariate analysis of variance (PERMANOVA) was used to test for differences in overall microbiome function between groups using the *vegan* package's 'adonis' function.

2.6 Generation of Co-abundance Groups

In order to identify patterns in the variation of the microbiota and investigate associations between these variations and disease states, a set of co-abundance groups (CAGs) were determined by clustering OTUs which tended to co-occur. Initially, OTUs were trimmed to

remove taxa present in less than 20% of samples (Chapter 3) or 50% of samples (Chapters 4 and 5), and all unclassified and non-prokaryotic taxa. A matrix of Kendall's Tau values was then generated for each pair of OTUs and these values were clustered by Ward-linkage according to their Pearson's correlation coefficient and visualised using the *Made4* package in R. Each cluster of taxa was then assigned to a CAG.

2.7 Ultra-Performance Liquid Chromatography – Mass Spectrometry

Faecal and plasma samples were used for analysis of bile acids. UPLC-MS was performed as described by Joyce *et al.* (1). Five microliters of extracted bile acids were injected onto a 50-mm T3 Acquity column (Waters Corp.) and were eluted using a 20-min gradient of 100% A to 100% B (A, water, 0.1% formic acid; B, methanol, 0.1% formic acid) at a flow rate of 400 µL/min and column temperature of 50 °C. Samples were analysed using an Acquity UPLC system (Waters Ltd.) coupled online to an LCT Premier mass spectrometer (Waters MS Technologies, Ltd.) in negative electrospray mode with a scan range of 50–1,000 m/z. Bile acids ionize strongly in negative mode, producing a prominent [M-H]⁻ negative ion. Capillary voltage was 2.4Kv, sample cone was 35V, desolvation temperature was 350 °C, source temperature was 120 °C, and desolvation gas flow was 900 L/h. Principal components analysis (PCA) was performed in Markerlynx (Waters) by limiting the number of elements (N, H, S, C) to be detected in individual analytes. Furthermore, a template of defined known masses was applied to allow the detection of bile acid only. Each analyte was identified according to its mass and retention time. Standard curves were performed using known bile acids, and each analyte was quantified according to the standard curve and normalized according to the deuterated internal standards.

2.8 Statistics

Statistics were performed in SPSS Version 24 and R Version 3.5.0 using the *Made4*, *vegan*, *pairwiseAdonis*, *compareGroups*, *phyloseq* and *ggplot* packages. GraphPad Prism (Version 5) was used for the generation of some graphs. Statistical significance was set to $p < 0.05$. Benjamini-Hochberg FDR adjustment for multiple comparisons was applied where noted, with a false discovery rate set to 5% (7). Normality was determined by a Shapiro-Wilk test (8). Groups were compared by student's T-test or MWU-test. Comparisons across time were made using ANOVA followed by pairwise comparison using Tukey's HSD, or Kruskal-Wallis Rank Sum Test followed by pairwise comparisons using the MWU-test. Permutational ANOVA (PERMANOVA) was used to compare β -diversity and CAGs, using unweighted Unifrac and Euclidean-squared distance matrices respectively. Correlations were examined using Pearson's R and Spearman's R^2 correlation coefficients. In figures, asterisks denote significance such that * is $p < 0.05$, ** is $p < 0.01$, and *** is $p < 0.001$.

2.9 References

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Chapter 3

Concurrent temporal changes in host immune profile, microbiota and bile acids in a chemically-induced murine model of colonic tumorigenesis

3.0 Abstract

Background: The gut microbiota has been shown to be involved in the initiation and development of colorectal cancer (CRC) in animal models. Research into the role of the microbiota in CRC has primarily focussed on changes that exist in and around tumour sites at a single point in time. This has made it difficult to differentiate between changes that promote cancer initiation in healthy tissue from changes that are a result of tumour development. In this study, we tracked changes in the host and microbiota throughout the course of azoxymethane (AOM)-induced colon cancer development to elucidate their contribution to disease. Our aim was to identify changes in each of these criteria and establish their temporal relationships in order to generate a timeline of alterations and define causative and consequential events.

Methods: Mice received five weekly doses of 10mg/kg AOM or PBS control. Timed culls were performed at 8, 12, 24 and 48 weeks afterwards. Faecal microbial composition was determined by 16S rRNA gene sequencing, and faecal and plasma bile acids were measured by UPLC-MS at each time-point. Host response was measured by qPCR in the distal large intestine. Correlation analysis was performed to establish whether any relationships exist between microbial composition and diversity, transcription of inflammatory cytokines and bile acids.

Results: The initial changes were detected in gut microbiota which acquired a pro-tumorigenic profile, defined as an enrichment of bacterial species predominantly associated with increased cancer risk. These changes are suppressed concurrent with the onset of host inflammatory response but are re-established later, possibly due to selection for resistant species. The size and composition of the bile acid pool are also affected and correlation analysis implicates the microbiota in this effect and in cytokine transcription.

Conclusion: Disruption in the large intestine of mice administered AOM allows opportunistic pathogens to impart a pro-tumorigenic profile to the gut microbiota with the potential to contribute to cancer initiation and progression by a mechanism which may involve inflammation and suppression of bile acid signalling.

Acknowledgements: 16S rRNA gene sequencing of the faecal microbiota was performed by Calum Walsh in Teagasc Moorepark (*Materials and Methods* section 2.5, *Amplicon Sequencing* to end of *Functional Analysis*), who also produced the plots in Figure 3.5 and 3.16. UPLC-MS and extraction from faecal and plasma samples for the detection of bile acids was performed by Peter Cronin (*Materials and Methods* section 2.7, *Ultra-Performance Liquid Chromatography – Mass Spectrometry*). Some of this work is presented in his submitted MRes thesis (April 2019). The materials and methods sections for these processes are provided by their respective executors. AOM administration and mouse culling was performed by Pat Casey.

3.1 Introduction

Colorectal cancer (CRC) has a complex aetiology involving the interaction of genetic and environmental factors. Attention has turned to the role of the gut microbiota in this process as it has been shown to be involved in tumorigenesis in animal models (1). The human gut microbiota is altered in CRC. Furthermore, certain microbial patterns are associated with increased cancer risk, but firm identification of mechanisms and specific pathogens involved has proven elusive as species associated with human CRC are not consistent across studies (2, 3).

Investigations to decipher the role of the human gut microbiota in CRC to date have largely examined patients already presenting with CRC (4). Moreover, observational studies have lacked the detailed investigation of the progression from the pre-tumorigenic state through to the adenoma-carcinoma sequence (2). These studies have allowed identification of patterns in the host and microbiota associated with active disease. However, they cannot distinguish between changes that are a result of tumorigenesis from characteristics that existed prior to and during tumour development. Animal models, in contrast, allow deep interrogation of the tumorigenic process over time by allowing timed sampling of large cohorts in controlled conditions.

Previous studies have examined the gut microbiota of mice treated with the carcinogenic compound azoxymethane (AOM) to elucidate changes associated with CRC. These have identified a number of bacterial taxa which have also been observed in human CRC (5, 6). These include increases in pathogenic bacteria that are enriched in human CRC, such as *Fusobacterium nucleatum*, and suppression of species associated with a reduction in cancer risk such as *Lactobacillus* and butyrate-producing *Faecalibacterium prausnitzii* (7-12). *F. prausnitzii* and *Lactobacillus* are also major reservoirs of bile acid-deconjugating enzyme *bile*

salt hydrolase (bsh) in the microbiota, along *Bifidobacterium* and some species of *Enterococcus* and *Bacteroides* (13, 14).

Modulation of the intestinal immune response and inflammation is one mechanism by which the microbiota may affect carcinogenesis (15). Cytokines and chemokines are soluble immune factors produced by tumour cells, stromal cells and tumour-infiltrating immune cells which can promote or suppress cancer initiation and progression by mediating inflammation and the immune response (16-20). This can occur by production of genotoxic compounds such as reactive oxygen species, induction of DNA damage and suppression of apoptosis, and is compounded by the chronic dysregulation of immune response associated with CRC (21). Inflammation in the gut can also affect the composition of the gut microbiota, especially in irritable bowel disease (IBD), resulting in complex reciprocal interactions (22). Moreover, research has suggested a role for the microbiota in the regulation of cytokines and chemokines in mouse models of CRC, especially by their production of short chain fatty acids such as butyrate (23-29).

Another possible mechanism by which the gut microbiota may affect host processes leading to the development of CRC is the production of pro- or anti-tumorigenic bacterial metabolites. Bile salts are endogenous, amphipathic molecules released into the duodenum after feeding to facilitate uptake of dietary fats. Bile salt dysregulation is associated with cancer risk, especially microbial metabolites of bile salts; primary bile acids and secondary bile acids (30). For example, rats administered AOM and fed cholic acid, a primary bile acid, had increased colonic tumours compared to rats treated with AOM alone, and this increase could be ameliorated by co-administration with ursodeoxycholic acid, a less hydrophobic bile acid (31-33). Moreover, a high fat diet is a known risk factor for colon cancer which also increases bile acid excretion and faecal concentration of deoxycholic acid and lithocholic acid

(34, 35). This suggests bile acids and their modifying organisms may represent a potential aetiologic agent of high fat diet-induced colon cancer risk.

Here, we perform a time-course study in a mouse model with a prolonged period of disease onset after AOM administration in the C57BL/6J mouse, in order to increase the temporal resolution of disease progression and allow examination of host and microbial changes during that period. We explore markers of host inflammation, the composition of the gut microbiota and the production of microbial metabolites in the murine colonic epithelium. Our aim was to investigate if there was a relationship between transcription of inflammatory cytokines, microbial composition and bile acids, and set these changes to a timeline. This should allow us to establish a set of causal and consequential events in the process of AOM-induced tumorigenesis as a model for sporadic human CRC.

3.2 Results

3.2.1 AOM-treated mice displayed faecal occult blood and aberrant crypt foci at 24 weeks and colonic adenomas at 48 weeks post-AOM injection

C57BL/6J mice were injected with AOM and changes in the host and microbiota were tracked through four timed culls performed 8, 12, 24 and 48 weeks after instigation of AOM treatment. The appearance of faecal occult blood (FOB) was detected, and the development of aberrant crypt foci (ACF) and adenomas was determined using methylene blue staining. Findings are presented in Table 3.1.

FOB was first detected in one AOM-treated mouse 22 weeks after AOM administration, with ACF detected in the large intestinal epithelium of this mouse at Week 24 (Figure 3.1A). FOB was also apparent in the faeces in three out of eight AOM-treated mice prior to Week 48 and two of these mice harboured at least one colonic adenoma (Figure 3.1B). No signs of neoplastic transformation were observed in mice at Week 8 (0/8) or Week 12 (0/7). As inflammation can lead to shortening of the large intestine, particularly in models of colitis-associated cancer, we measured the length of the large intestine at each dissection. However, no difference between AOM- and PBS-treated mice was detected at any time-point examined (Figure 3.1C; T-test and MWU-test, $p>0.05$).

3.2.2 AOM-treated mice gained less epididymal fat

At Week 8, AOM-treated mice had significantly less epididymal fat than PBS controls (Figure 3.2B; T-test, $p<0.001$). There was a trend towards a reduction in both epididymal and mesenteric fat in AOM-treated mice for the duration of the study, and AOM-treated mice gained less weight than the PBS-treated mice, but this was not statistically significant (Figure 3.2; T-tests and ANOVA, $p>0.05$).

Table 3.1: Incidence of faecal occult blood (FOB), aberrant crypt foci (ACF) and adenomas

		Week 8	Week 12	Week 24	Week 48
PBS	FOB	0/8	0/8	0/7	0/8
	ACF	0/8	0/8	0/7	0/8
	Adenoma	0/8	0/8	0/7	0/8
AOM	FOB	0/8	0/7	1/8	3/8
	ACF	0/8	0/7	1/8	0/8
	Adenoma	0/8	0/7	0/8	2/8

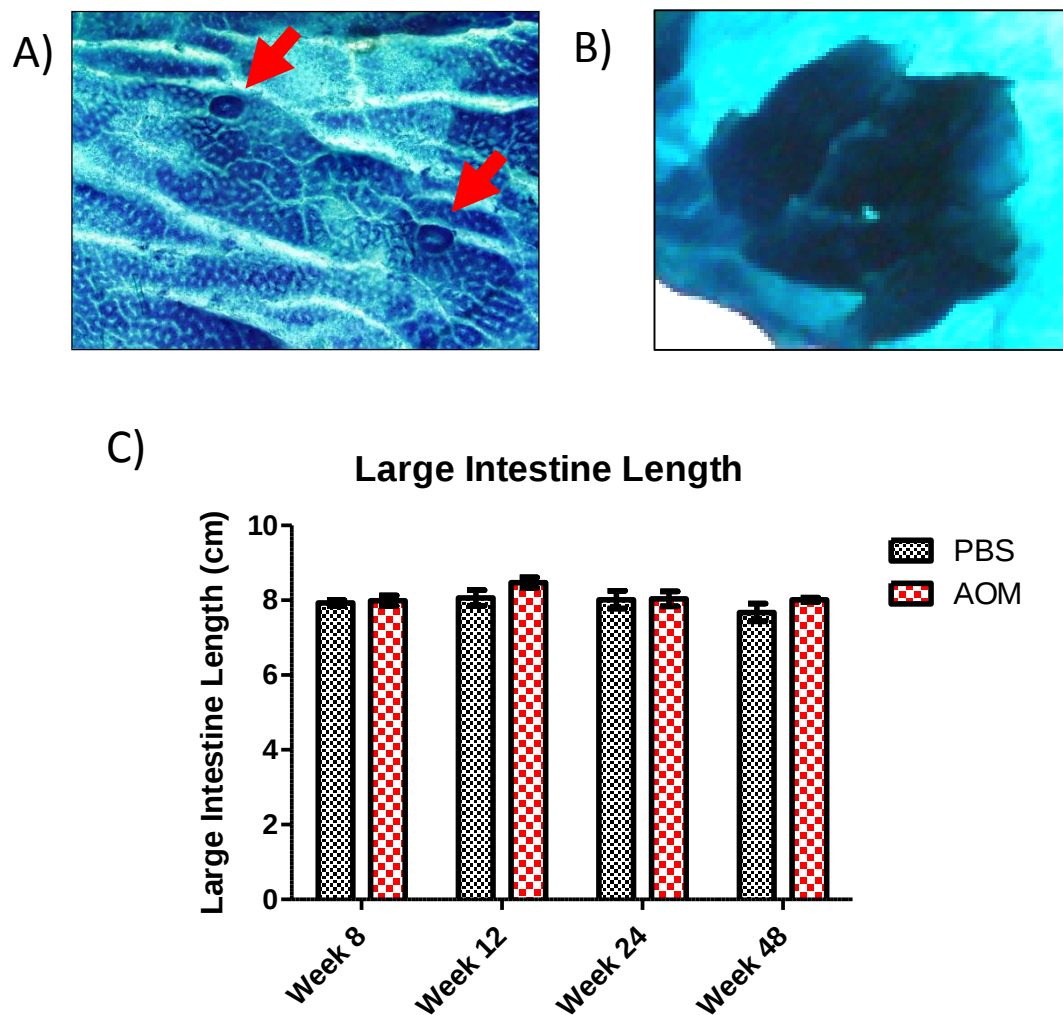
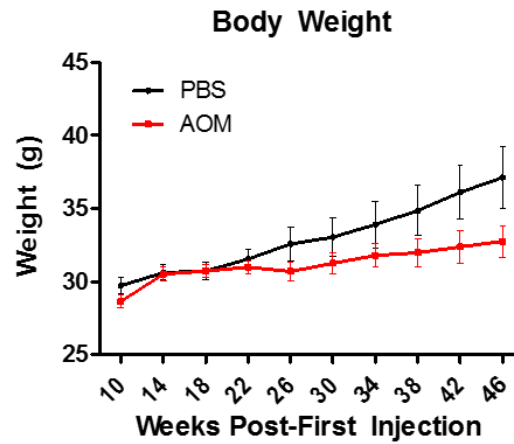
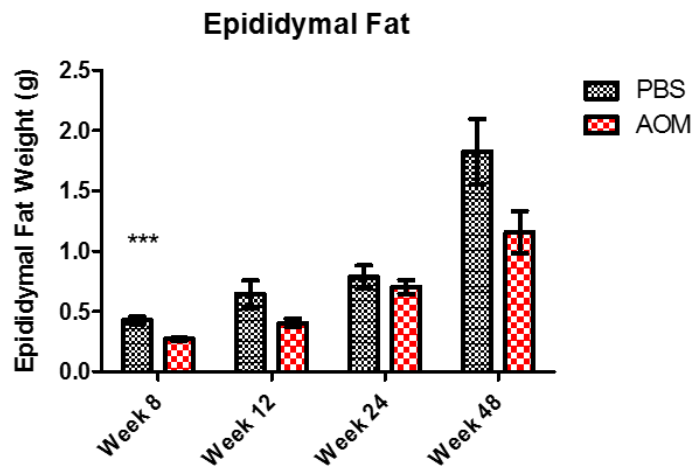


Figure 3.1: Detection of aberrant crypt foci and adenomas by methylene blue staining. Mice were culled by decapitation and colons excised, opened longitudinally and washed with PBS before fixation in 10% formalin overnight. Colons were then immersed in methylene blue for 3 minutes, rinsed in deionised water and photographed. **A)** Representative image of the colonic lumen epithelium stained with methylene blue from the dissection at Week 24 (10x magnification). ACF are highlighted with red arrows. They were identified by their protrusion from the epithelial surface and thickened crypt openings. **B)** Macroscopic image of a mouse colon stained with methylene blue displaying a large adenoma from the dissection at Week 48. Adenomas were detected in two mice at this time-point. **C)** The length of the large intestine was measured at each time-point. Graph represents the mean and SEM. $N=8$ per group, except for AOM Week 12 and PBS Week 24 ($N=7$).

A)



B)



C)

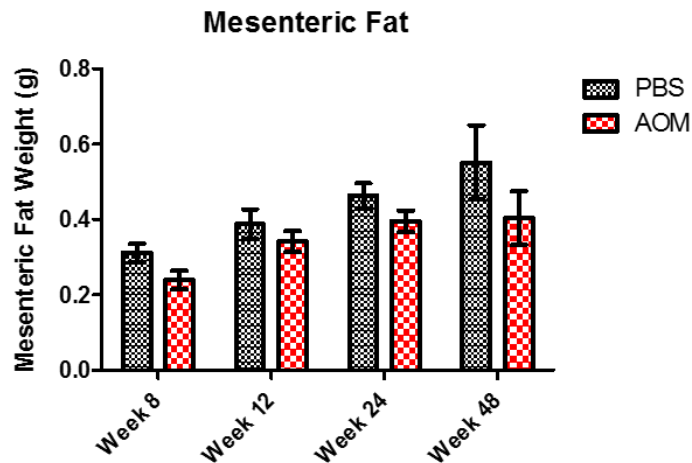


Figure 3.2: Fat gain was affected in mice treated with AOM at Week 8. Mice were weighed weekly over the course of the experiment. At each dissection, tissue samples were taken and weighed. **A)** The difference in body weight between the groups grew as the experiment progressed but this did not reach significance. **B)** The mass of epididymal fat was lower in mice exposed to AOM over the course of the experiment but this was only significant at Week 8. **C)** A similar trend was observed in the mass of mesenteric fat but this was not significant. Graphs present the mean and SEM. $N=8$ per group, except for AOM Week 12 and PBS Week 24 ($N=7$).

3.2.3 Proliferation and apoptosis are unchanged in AOM-treated mice at Week 8 or Week 24

In order to investigate if increased proliferation occurs prior to the development of ACF, intestinal tissue was immunohistochemically stained for Ki67 which detects proliferating cells, and the number of Ki67 positive cells per crypt was examined in PBS- and AOM-treated mice at 8 and 24 weeks post-first injection. These time-points were chosen as disruption to the microbiota began at Week 8 (see Figure 3.5 and 3.6), while the cytokine response peaked at Week 24 (see Figure 3.4). There were no significant differences in the expression of Ki67 or activation of caspase-3 at these time-points, suggesting that AOM does not contribute to tumorigenesis by inducing widespread proliferation of normal epithelial cells, or by suppression of apoptosis at these time-points (Figure 3.3; T-test, $p>0.05$).

3.2.4 Cytokine profile in the large intestine and spleen weight is altered in AOM-treated mice

Inflammation is a risk factor for colon cancer and the tumour microenvironment can modulate inflammatory conditions to promote tumour growth. As such, we measured the transcription of a number of cytokines in the distal large intestine by qPCR. *Cxcl5* was the only inflammatory factor found to be significantly changed at Week 8, where it was decreased in AOM-treated mice compared to PBS controls (Figure 3.4; T-test, $p=0.039$). Spleen weight was also increased in mice administered AOM at Week 8, suggesting an expansion of monocyte cells (Figure 3.4; MWU-test, $p=0.007$). In contrast, numerous cytokines/chemokines were significantly altered at Weeks 12 and 24, with *Il18*, *Il6*, *Cxcl1*, *Cxcl2*, *Cxcl5*, *Il10*, *Il12* and *Tnfa* all found to be increased in AOM-treated mice during this timeframe (Figure 3.4; T-test and MWU-test, $p<0.05$). However, these alterations in cytokine/chemokine transcription was largely absent by Week 48 with only *Il12* significantly increased in AOM-treated mice (Figure 3.4; MWU-test, $p=0.04$).

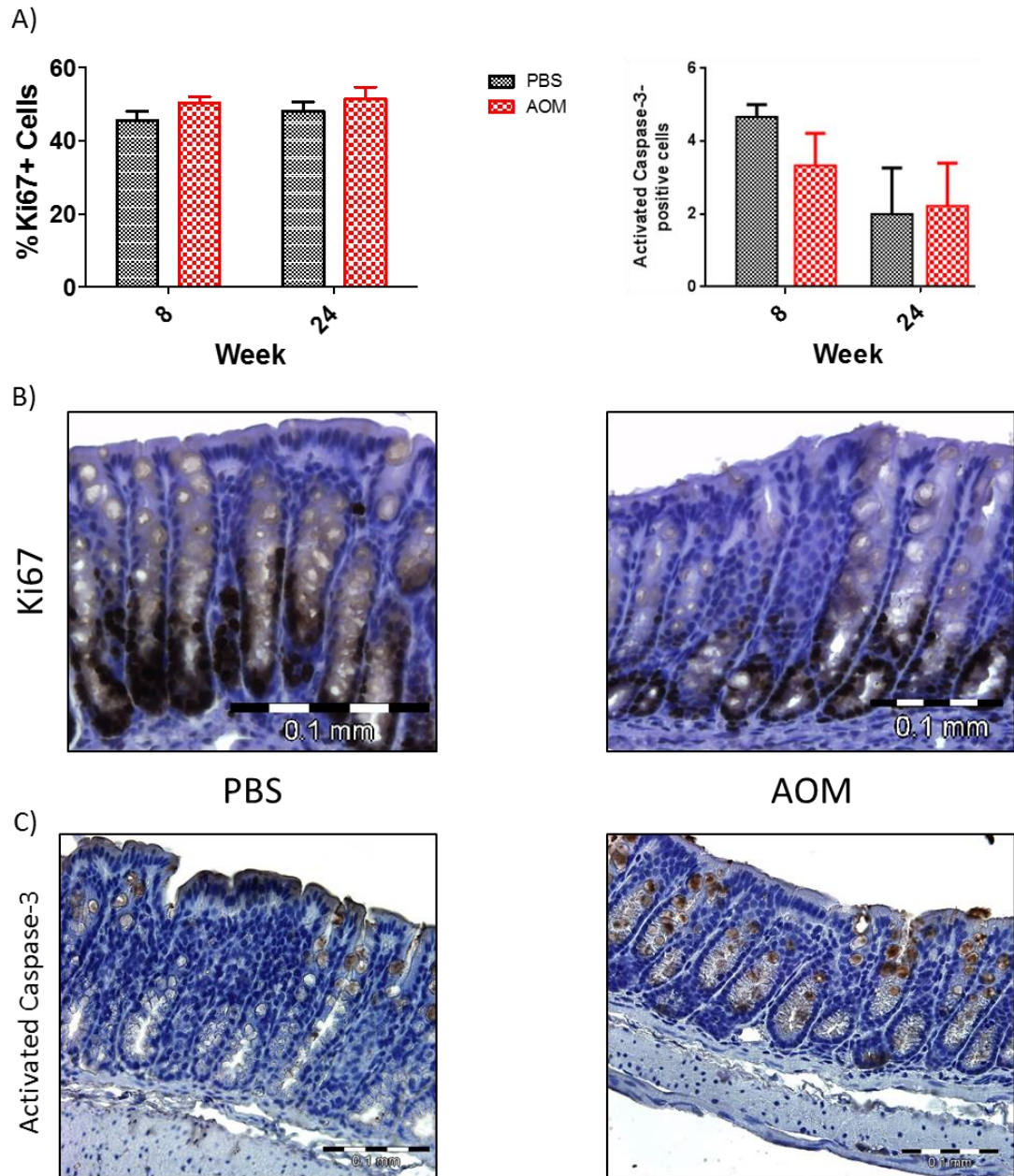


Figure 3.3: Proliferation and apoptosis were unchanged in the colonic crypts of mice administered AOM. Immunohistochemistry was performed with an antibody for proliferation marker Ki67 and an apoptosis marker, activated caspase-3, in the distal colons of PBS- and AOM-treated mice. For Ki67, the total number of epithelial cells were counted for ten crypts as well as the number of cells staining positive for Ki67 at Week 8 and Week 24. For activated caspase-3, three randomly selected microscope fields from three mice in each group were selected and the number of positively staining cells were counted. **A)** The percentage of Ki67-positive cells per crypt and the number of activated caspase-3-positive cells per field were determined in the normal mucosa of mice treated with PBS and AOM at Week 8 and Week 24. The differences were not significant. Graphs present the mean and SEM. N-numbers for Ki67 in PBS- and AOM-treated mice are 6 and 5 at Week 8, and 6 and 7 at Week 24. **B)** Ki67 staining of colon tissue of each group at Week 24 on a background stain of haematoxylin. **C)** Activated caspase-3 staining of colon tissue of each group at Week 24 on a background stain of haematoxylin. Images were taken at 400x magnification. *N*=8 per group, except for AOM Week 12 and PBS Week 24 (*N*=7).

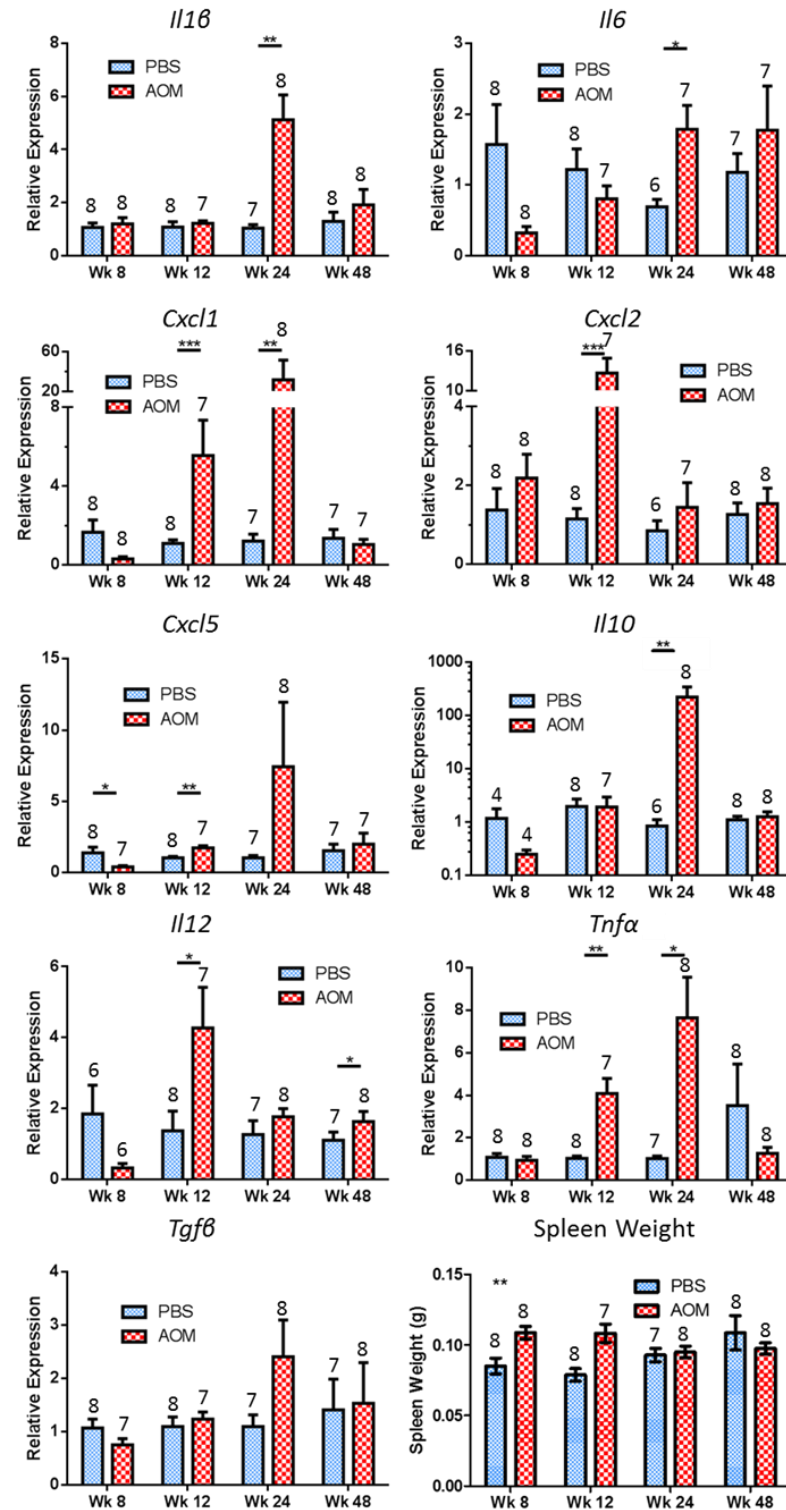


Figure 3.4: Cytokine response is altered in mice administered AOM. The distal 1cm of colon tissue was taken from each mouse at dissection, RNA extracted and converted to cDNA which was analysed by qPCR. A minor down-regulation to cytokine response was observed at Week 8 before pro-inflammatory expression arose at Week 12 and Week 24. This pro-inflammatory profile subsequently subsided by Week 48. Spleens were also removed and weighed at each dissection and a significant increase in mass was observed in mice treated with AOM at Week 8. Graphs present the mean and SEM. N-numbers are presented above bars. Note: *Il10* y-axis is presented in the logarithmic scale.

3.2.5 The faecal microbiota is altered in AOM-treated mice

We collected faecal pellets from each mouse on the day prior to cull and performed 16S rRNA gene sequencing to characterise the microbial community and investigate changes that occur in the gut environment before and during tumorigenesis in response to AOM treatment. *N*-numbers are presented in Table 3.1.

We measured the α -diversity by two metrics. These were Shannon diversity, a mathematical measure of the number of species and their relative abundances, and phylogenetic diversity (PD), which measures the total length of the minimum path spanning each node of a cladogram featuring the detected species but does not consider the relative abundance of those species. At Week 8, phylogenetic diversity (PD) was significantly increased in AOM-treated mice (Figure 3.5A; MWU-test $p=0.03$). At Week 12, the Shannon diversity index was significantly decreased in AOM-treated mice relative to PBS controls (Figure 3.5A; MWU-test, $p=0.04$).

When comparing changes within treatments across time, the Shannon α -diversity index rose steadily in PBS-treated mice. AOM-treated mice showed a similar pattern, except for Week 12 in which a decrease in Shannon diversity occurred that was significantly different from each other time-point (Figure 3.5B; Kruskal-Wallis Rank Sum Test followed by pairwise comparisons using the MWU-test, $p<0.05$ after FDR adjustment).

Beta (β)-diversity, on the other hand, is a measure of between-individual diversity in a community. This also differed significantly between treatments. The *p*-values between AOM- and PBS-treated mice for Weeks 8 to 48 were 0.005, 0.034, 0.012 and 0.004 respectively, corresponding to R^2 values of 0.17, 0.15, 0.19 and 0.24. This suggests a difference due to acute AOM effects at the earliest time-point followed by recovery, before the communities again grow further apart as disease progressed (Figure 3.5C, PERMANOVA of unweighted Unifrac distances, $p<0.05$).

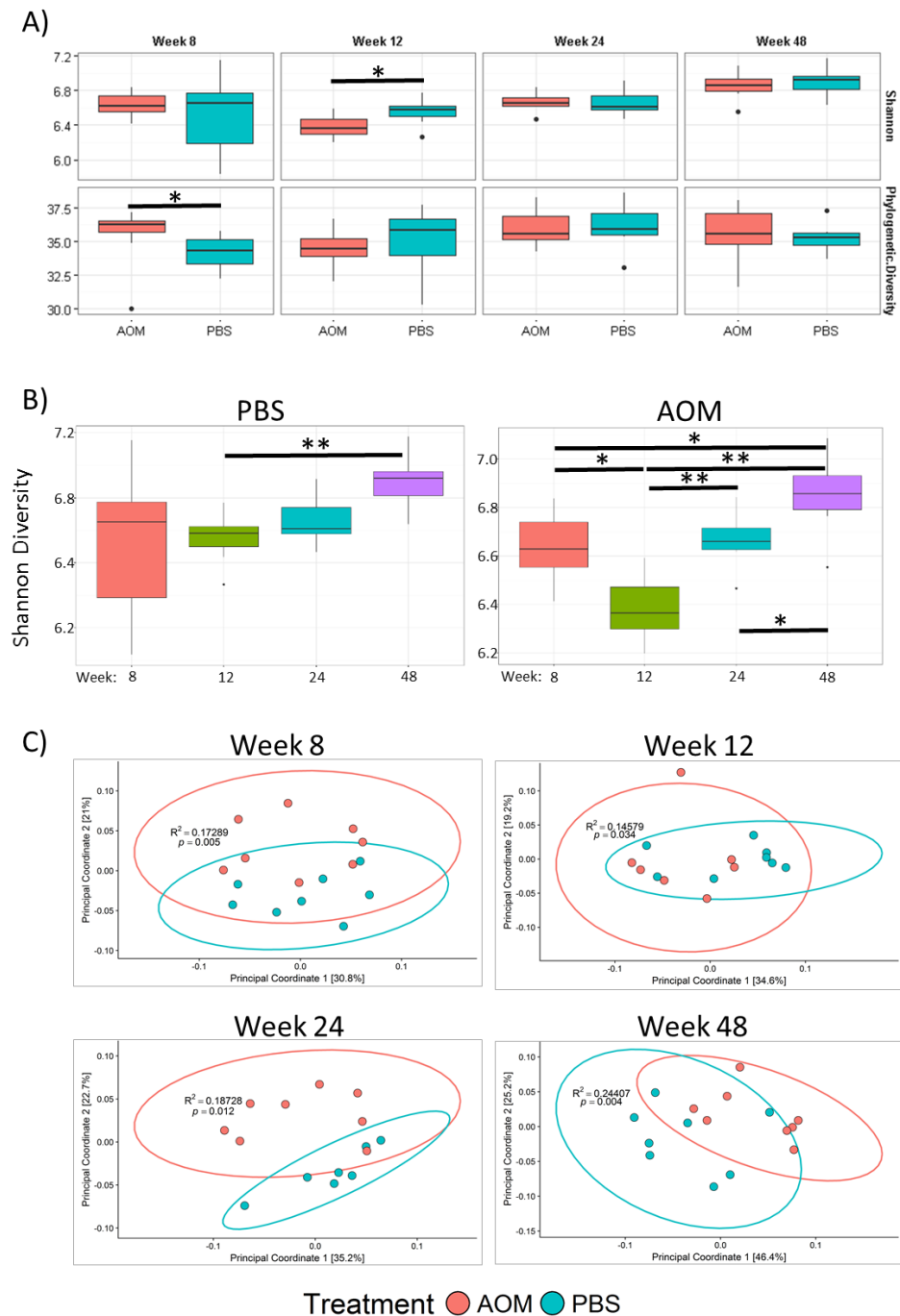
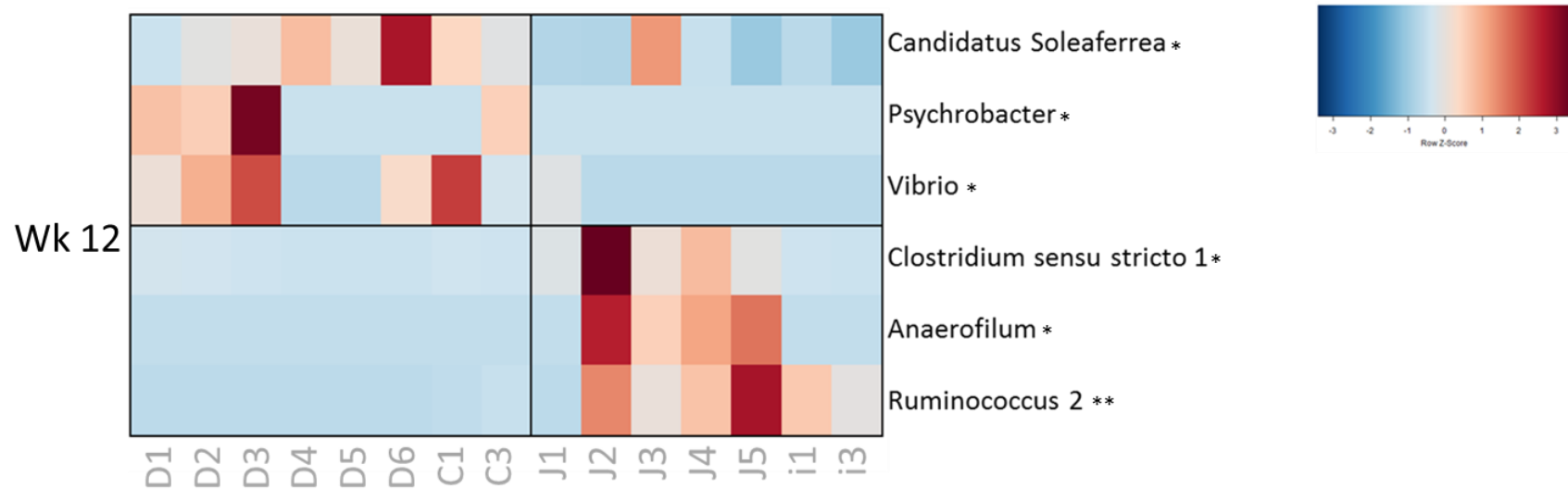
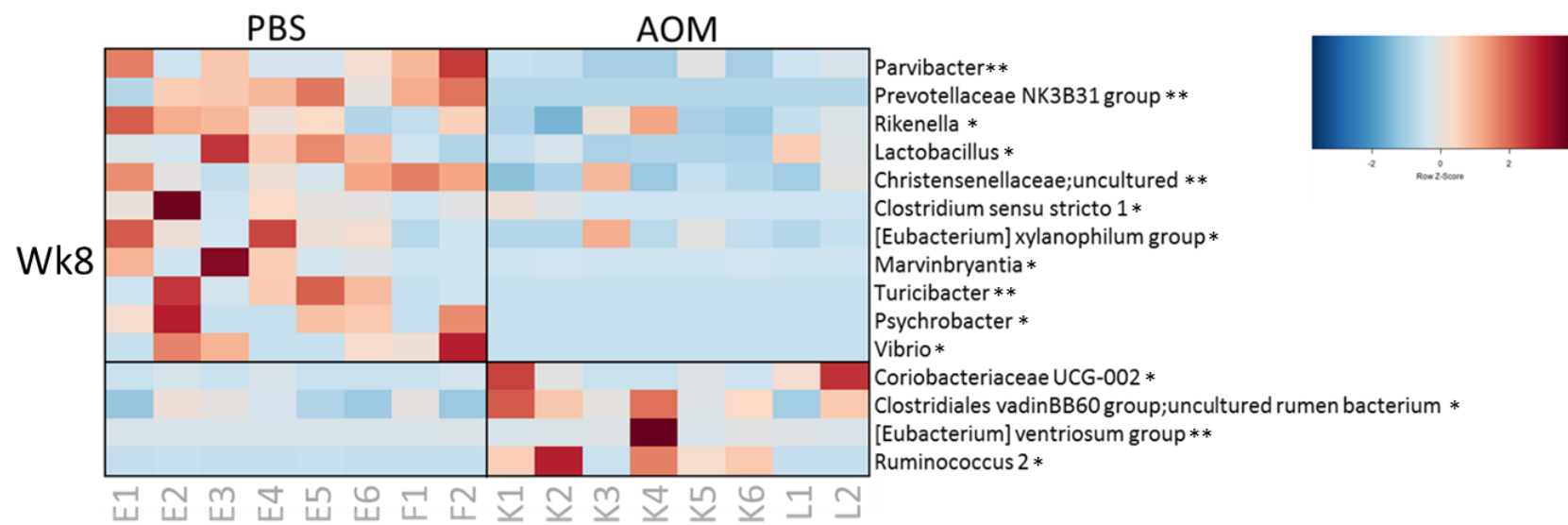


Figure 3.5: Alpha (α)- and beta (β)-diversity are affected in mice administered AOM. Faecal samples were collected throughout the experiment and analysed by 16S rRNA gene sequencing at each dissection to characterise the faecal microbiota. Shannon diversity and phylogenetic diversity were used to interpret the α -diversity while PERMANOVA of unweighted Unifrac distances were used to examine the β -diversity, visualised by principle coordinate analysis. **A)** Phylogenetic diversity was increased in mice administered AOM at Week 8, while Shannon diversity was reduced at Week 12. **B)** Tracking changes in the Shannon diversity across time within each group reveals a significant decrease in the treatment group at Week 12. **C)** We observed a significant difference in the β -diversity between groups at each time-point. $N=8$ per group, except for AOM Week 12 and PBS Week 24 ($N=7$). Figures produced by Calum Walsh at Teagasc, Moorepark.

Next, we considered which individual taxa differed significantly between groups at each time-point. These significantly different taxa are presented in Figure 3.6 (MWU-test with FDR adjustment; $p < 0.05$). At Week 8, eleven taxa were significantly decreased in mice treated with AOM and four were significantly increased. The *Turicibacter* genus was noteworthy at this time-point for being decreased 194-fold in mice administered AOM. The communities were more similar at Week 12. *Clostridium sensu stricto 1* and *Ruminococcus 2* were conspicuous for being 27-fold and 47-fold more abundant after AOM treatment at this time-point. *Marvinbryantia* was 40-fold less abundant in mice treated with AOM at Week 24, when twelve species were significantly increased and ten decreased in the cancer model. The microbial gut communities were most dissimilar at Week 48 however, with nine taxa decreased and fourteen increased in mice exposed to AOM. *Prevotellaceae* UCG-001 was 1208-fold more abundant in control mice despite being present in five mice in the treatment group, while *Parasutterella* was 244-fold less abundant in PBS-treated mice. In addition to these, a number of species were present in one treatment group while being absent or virtually absent on the other. In mice treated with AOM, *Prevotellaceae* NK3B31 group, *Clostridium sensu stricto 1*, *Marvinbryantia*, *Eubacterium ventriosum* group, *Citrobacter*, *Psychrobacter* and *Vibrio* were present in ≤ 2 mice per group at certain time-points despite being well established in the control group. Moreover, these latter two taxa were significantly increased in PBS-treated mice at each time-point while being virtually absent from the AOM-treatment group. In mice treated with PBS, *Eubacterium ventriosum* group, *Ruminococcus 2*, *Anaerofilum*, *Prevotellaceae* NK3B31 group and *Citrobacter* were absent or rare. Several species also differ significantly at more than one time-point. As well as *Psychrobacter* and *Vibrio*, *Marvinbryantia* is increased in control mice at Weeks 8, 24 and 48. *Prevotellaceae* NK3B31 group and *Clostridium sensu stricto 1* are increased in control mice at Week 8 but in treatment mice at two later time-points each, while *Eubacterium ventriosum* group is increased in treatment mice at Week 8 and in control mice twice later.



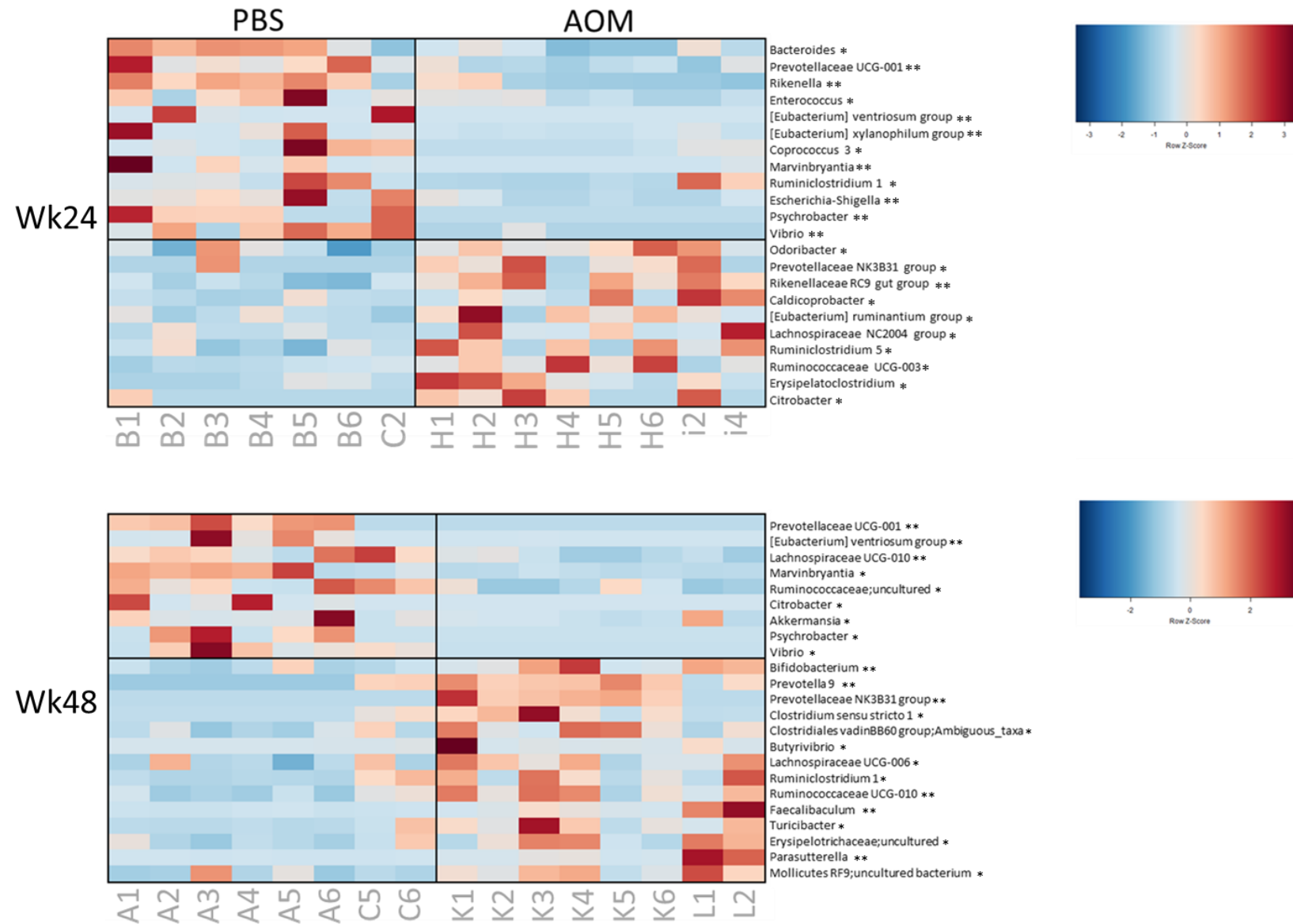


Figure 3.6: Taxa which differ significantly in their abundance between groups. From the data acquired by 16S rRNA gene sequencing of the faecal microbiota, operational taxonomic units were clustered based on 97% sequence similarity taxonomy was assigned using BLAST against the SILVA SSURef database. Statistically significant differences in abundances of individual taxa between groups were determined using the Mann-Whitney U-test with FDR correction for multiple comparisons. Only significantly different taxa are presented here. Taxa in the top of each section are suppressed in mice administered AOM, while species in the bottom are enriched. Data are presented as the z-scores of the abundances scaled by row. N=8 per group, except for AOM Week 12 and PBS Week 24 (N=7).

Eubacterium xylanophilum group, *Prevotellaceae* UCG-001 and *Rikenella* are decreased in control mice at two time-points, while *Ruminococcus 2* is increased in mice administered AOM twice. *Citrobacter*, *Ruminiclostridium 1* and *Turicibacter* also feature twice, but for a different treatment group each time.

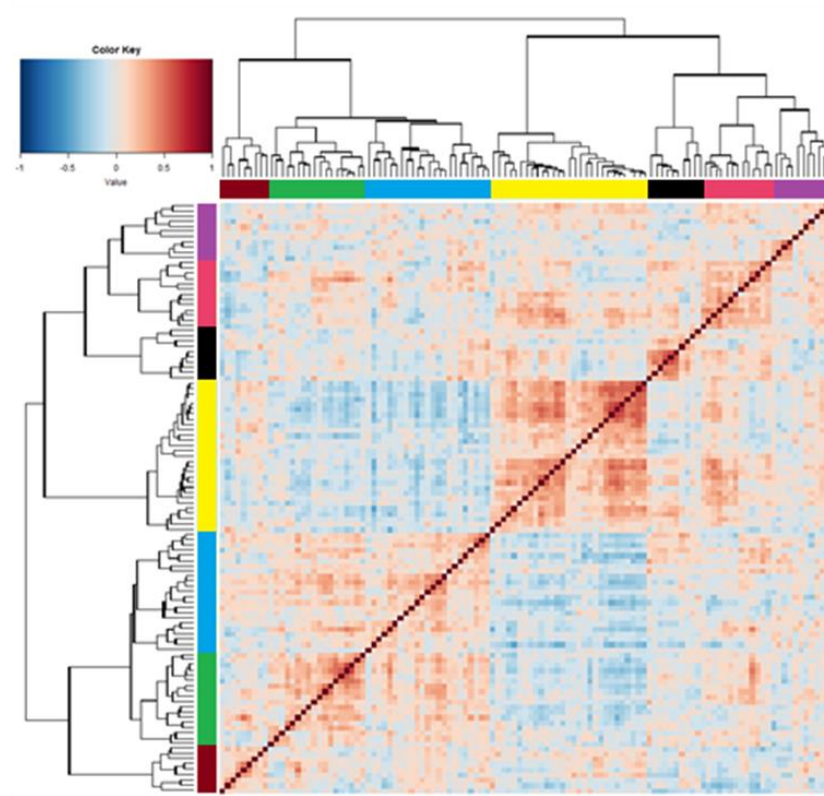
In order to investigate the alterations in the gut bacteria at a higher structural level, we defined a set of bacterial co-abundance groups (CAGs) (Figure 3.7A). PERMANOVA determined that all CAGs were significantly different (Figure 3.7B, $p < 0.05$). The taxon composition of each CAG can be found in Table 3.2.

Seven CAGs were identified, labelled CAG1-7. CAG1 was increased in AOM-treated mice at Week 24 (T-test, $p = 0.032$). CAG2 was increased in AOM-treated mice at Week 48 (MWU-test, $p < 0.001$) while CAG7 was decreased (T-test, $p < 0.001$). There were no significant differences between CAGs in PBS- and AOM- treated groups at Weeks 8 or 12. CAG1 is dominated by the *Rikenellaceae* family which comprise >80% of the abundance of this CAG. *Turicibacter* and *Ruminococcus 1* make up over half of CAG2 with *Bifidobacterium* and an uncultured *Erysipelotrichaceae* adding another 22.5%. An uncultured bacterium from the *Ruminococcaceae* family makes up 84% of CAG7.

3.2.6 Bile acids are altered in AOM-treated mice

In order to investigate the potential role of bile acid modification in our mouse model, we analysed the faecal and plasma bile acid profile of the mice by UPLC-MS. PBS-treated mice had a higher total faecal bile acid content than AOM-treated mice at Weeks 8 and 12 (Figure 3.8; T-test, $p < 0.01$) and as a result, PBS-treated mice had significantly higher volumes of many bile acids while there were no bile acids increased in AOM-treated mice. We therefore compared the fraction that each bile acid made up of the total bile acid volume. The

A)



B)

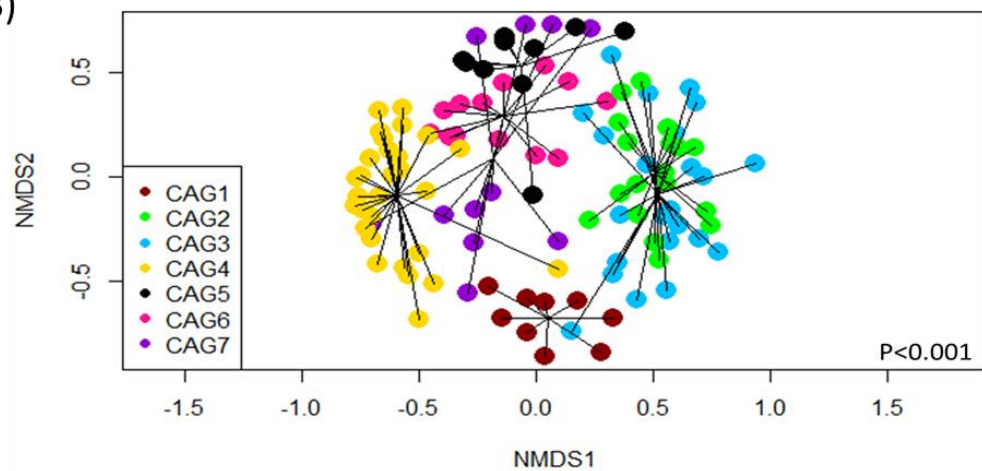


Figure 3.7: Generation of co-abundance groups. The Kendall correlations of OTUs' relative abundances were generated for all 62 mice in the study and clustered by Ward-linkage and Pearson correlation. This defines "co-abundance groups" (CAGs) which describe higher-level structures of the microbiota by highlighting taxa for which changes in abundance correlate. These Kendall correlations were then converted to a distance metric and analysed by PERMANOVA which determined that each CAG was significantly different. Colours represent Pearson's correlation coefficient between pairs of taxa. **A)** A heatplot of Kendall correlation values for each pair of taxa clustered by Ward linkage of their Pearson correlations. Coloured bars along the axes denote the CAGs. The composition of each CAG is presented in Table 3.2. **B)** Non-metric multidimensional scaling (NMDS) of the distance metrics of the CAGs' correlation values.

Table 3.2: The composition of each CAG

CAG1	Prevotella 9
	Alistipes
	Rikenellaceae RC9 gut group
	Ruminococcus 2
	Coprococcus 3
	Papillibacter
	Butyrivibrio
	[Eubacterium] ruminatum group
	Uncultured Firmicutes bacterium
	Prevotellaceae NK3B31 group
CAG2	Lachnospiraceae NC2004 group
	[Eubacterium] coprostanoligenes group
	Clostridium sensu stricto 1
	Turcibacter
	Natranaerovirga
	Streptococcus
	Parasutterella
	Coriobacteriaceae;uncultured
	Clostridiales Family XIII AD3011 group
	Ruminiclostridium 1
CAG3	Ruminococcaceae UCG-010
	Coriobacteriaceae UCG-002
	Faecalibaculum
	Bifidobacterium
	Erysipelotrichaceae;uncultured
	Caldicoprobacter
	Ruminococcus 1
	Anaerobacillus
	Bacteroidales S24-7 group;uncultured bacterium
	Delftia
CAG4	Akkermansia
	Christensenellaceae R-7 group
	[Eubacterium] nodatum group
	Chloroplast;uncultured bacterium
	Bacilli;Ambiguous_taxa
	Candidatus Arthromitus
	Bacteroidales S24-7 group;Ambiguous_taxa
	Erysipelatoclostridium
	Mollicutes RF9;uncultured rumen bacterium
	Mollicutes RF9;Ambiguous_taxa
CAG5	Ruminococcaceae UCG-014
	Mollicutes RF9;uncultured bacterium
	Anaerofustis
	Ochrobactrum
	Enterococcus
	Lactobacillus
	Candidatus Saccharimonas
	Bacteroides
	Parabacteroides
	Rhodospirillaceae;uncultured
CAG6	Odoribacter
	Ruminiclostridium 6
	Candidatus Soleaferrea
	Mucispirillum
	Lachnospiraceae;uncultured
	Blautia
	Tyzzerella 3
	Anaerovorax
	Ruminiclostridium 5
	Anaerotruncus
CAG7	Butyrivibrio
	Clostridiales Family XIII UCG-001
	Peptococcus
	Peptococcaceae;uncultured
	Lachnospiraceae UCG-008
	Faecalibacterium
	Eisenbergiella
	Ruminococcaceae NK4A214 group
	Ruminococcaceae UCG-005
	Lachnospiraceae;uncultured bacterium
CAG8	Tyzzerella
	Intestinimonas
	Ruminiclostridium
	Oscillibacter
	Ruminococcaceae UCG-003
	Bilophila
	Ruminococcaceae UCG-009
	Ruminiclostridium 9
	Christensenellaceae;uncultured
	Rikenella
CAG9	Anaeroplasm
	Porphyromonadaceae;uncultured
	Clostridiales vadinBB60 group;uncultured bacterium
	Clostridiales vadinBB60 group;Ambiguous_taxa
	Lachnospiraceae UCG-006
	Gastranaerophilales;uncultured bacterium
	Clostridiales vadinBB60 group;uncultured rumen bacterium
	Hydrogenoanaerobacterium
	Citrobacter
	Lachnospiraceae UCG-001
CAG10	Coprococcus 1
	Lachnospiraceae NK4A136 group
	Roseburia
	[Eubacterium] brachy group
	Lachnoclostridium
	Lachnospiraceae FCS020 group
	Acetitomaculum
	Escherichia-Shigella
	Enterorhabdus
	Parvibacter
CAG11	Marvinbryantia
	[Eubacterium] xylanophilum group
	[Eubacterium] ventriosum group
	Prevotellaceae UCG-001
	Psychrobacter
	Vibrio
	Ruminococcaceae;uncultured
	Ruminococcaceae UCG-013
	Syntrophococcus
	Desulfovibrio
CAG12	Bacillus
	Senegalimassilia
	Lachnospiraceae UCG-010

significantly different values are presented in Figures 3.9 to 3.12. The list of bile acids measured are available in Table 3.3.

The proportion of hydrophobic bile acids DCA and LCA were decreased in the faeces of AOM-treated mice at Week 8, with DCA still reduced at Week 12. The proportions of five conjugated faecal bile acids were increased at Week 8 including T-HCA, which was still significantly higher than control at Week 12, along with UDCA and DHCA (Figures 3.9 and 3.10; T-test and MWU-test, $p < 0.05$). The concentration of five bile acids are increased in the plasma of mice treated with AOM at Week 12, including the potent FXR antagonist T- $\alpha/\beta/\omega$ -MCA (Figure 3.10; T-test and MWU-test, $p < 0.05$). The specific isomer of this bile acid could not be determined by UPLC-MS. The absolute concentrations of twelve faecal bile acid species were increased in AOM-treated mice at Week 24, of which HDCA and β -MCA were the most abundant, while LCA and G-LCA were reduced (Figure 3.11; T-test and MWU-test, $p < 0.05$). In the plasma at Week 24, T- $\alpha/\beta/\omega$ -MCA remains increased in AOM-treated mice along with six other bile acids (Figure 3.11; T-test and MWU-test, $p < 0.05$). The absolute concentration of only four faecal bile acids differed at Week 48; CA, G-CDCA and G-LCA were decreased in AOM-treated mice, and G-HDCA was increased, while G-LCA was also decreased in the plasma (Figure 3.12; T-test and MWU-test, $p < 0.05$).

3.2.7 Correlation analysis reveals relationships between gut bacteria and bile acid composition

A correlation analysis was performed to determine the relationships between the cytokine and chemokine transcription, α -diversity scores and CAG abundances, and each of those variables' relationships with absolute and normalised faecal and plasma bile acid concentrations.

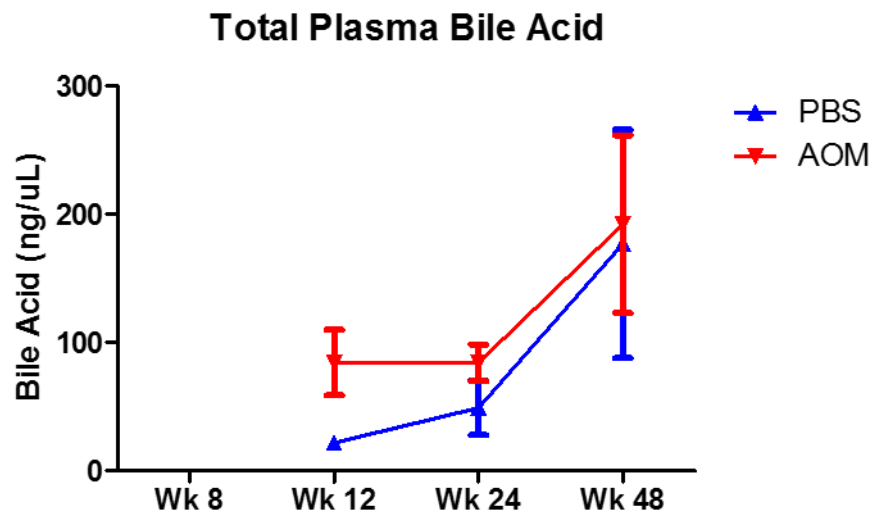
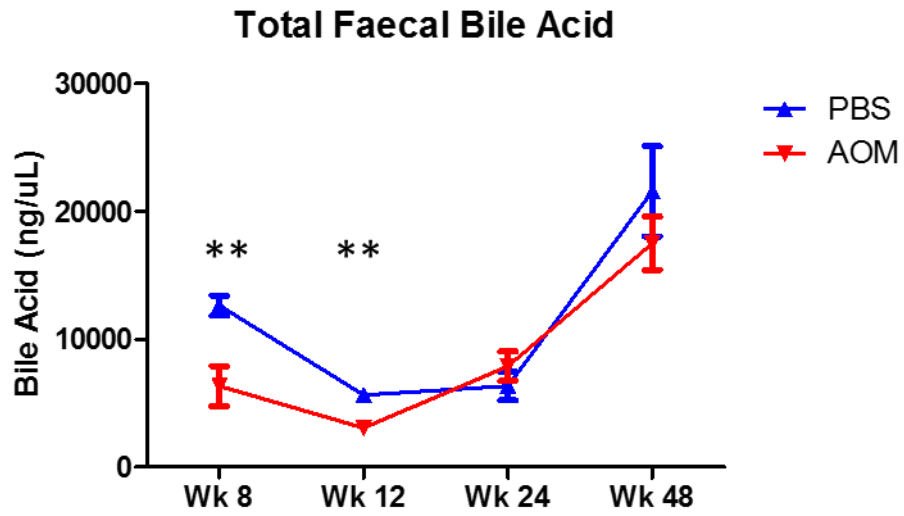


Figure 3.8: Total bile acid concentration is reduced in the faeces of mice administered AOM. Bile acids were determined by UPLC-MS at each dissection. **A)** The total faecal bile acid concentration was significantly reduced at Week 8 and Week 12 in mice administered AOM. **B)** No significant differences in plasma bile acids were detected, although a trend towards lower concentration was observed at Week 12 ($P=0.058$). Graphs present the mean and SEM. *N*-numbers for PBS and AOM faecal bile acids at Week 8 are 7 and 7; at Week 12 are 7 and 6; at Week 24 are 6 and 6; at Week 48 are 7 and 6. *N*-numbers for PBS and AOM plasma bile acids at Week 12 are 5 and 6; at Week 24 are 5 and 7; at Week 48 are 6 and 7.

Faecal Bile Acids

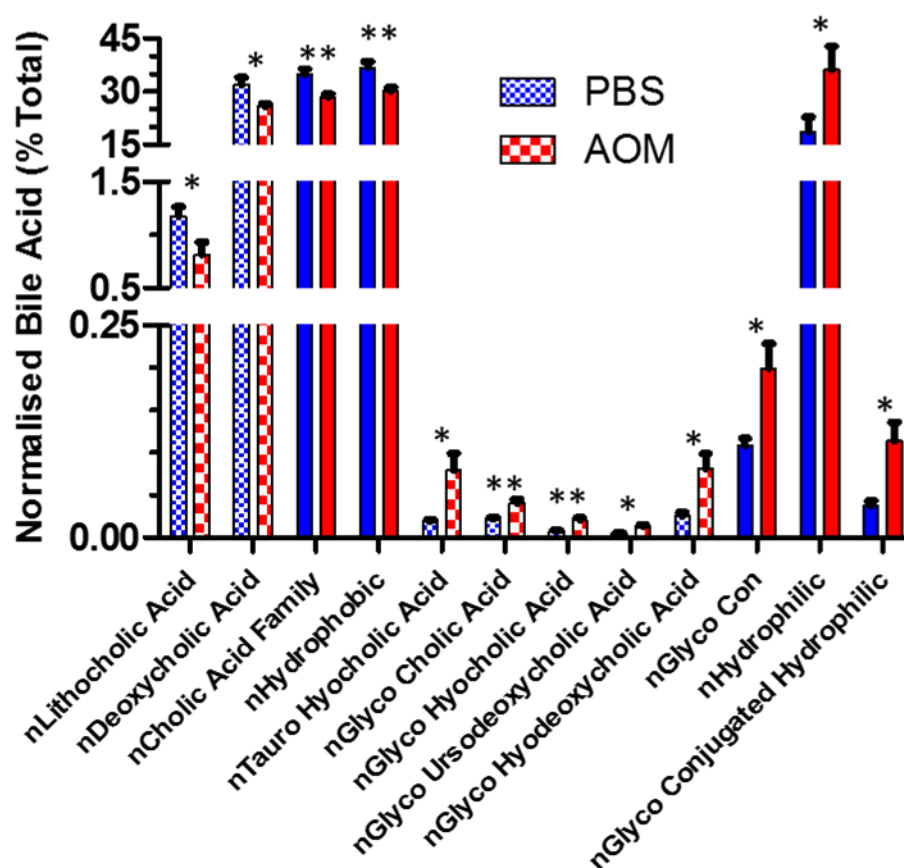


Figure 3.9: Normalised faecal bile acids which differed significantly between groups at Week 8. Faecal bile acids were determined by UPLC-MS at each dissection. Since total faecal bile acid concentration differed significantly between groups at this time-point, we analysed the proportion each bile acid made up of the total faecal bile acid content to unmask treatment effects. Graphs present the mean and SEM. Individual bile acids are represented by chequered bars and families are presented by solid bars. $N=7$ for both groups.

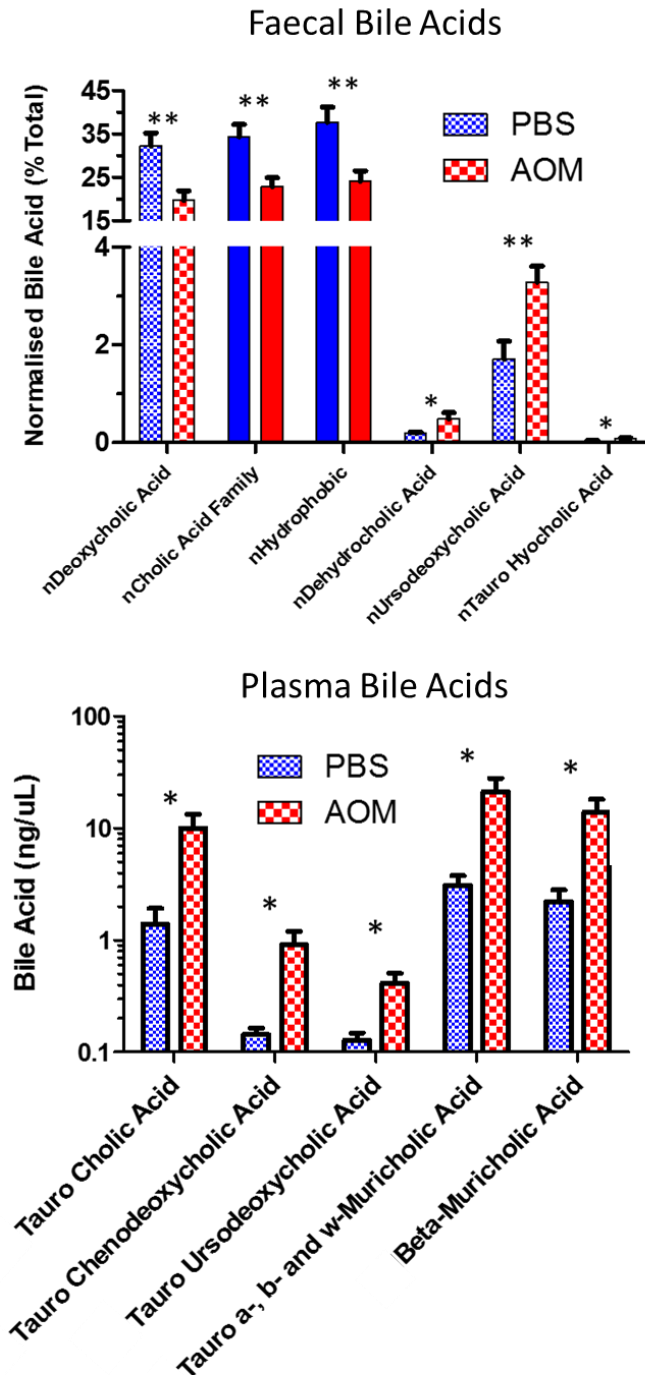


Figure 3.10: Faecal and plasma bile acids which differed significantly between groups at Week 12. Bile acids were determined by UPLC-MS at each dissection. Since total faecal bile acid concentration differed significantly between groups at this time-point, we analysed the proportion each bile acid made up of the total faecal bile acid content to unmask treatment effects. Plasma bile acids are presented as absolute values. Graphs present the mean and SEM. Individual bile acids are represented by chequered bars and families are presented by solid bars. Faecal *n*-numbers for PBS- and AOM-treated mice are 7 and 6, and plasma *n*-numbers are 5 and 6, respectively.

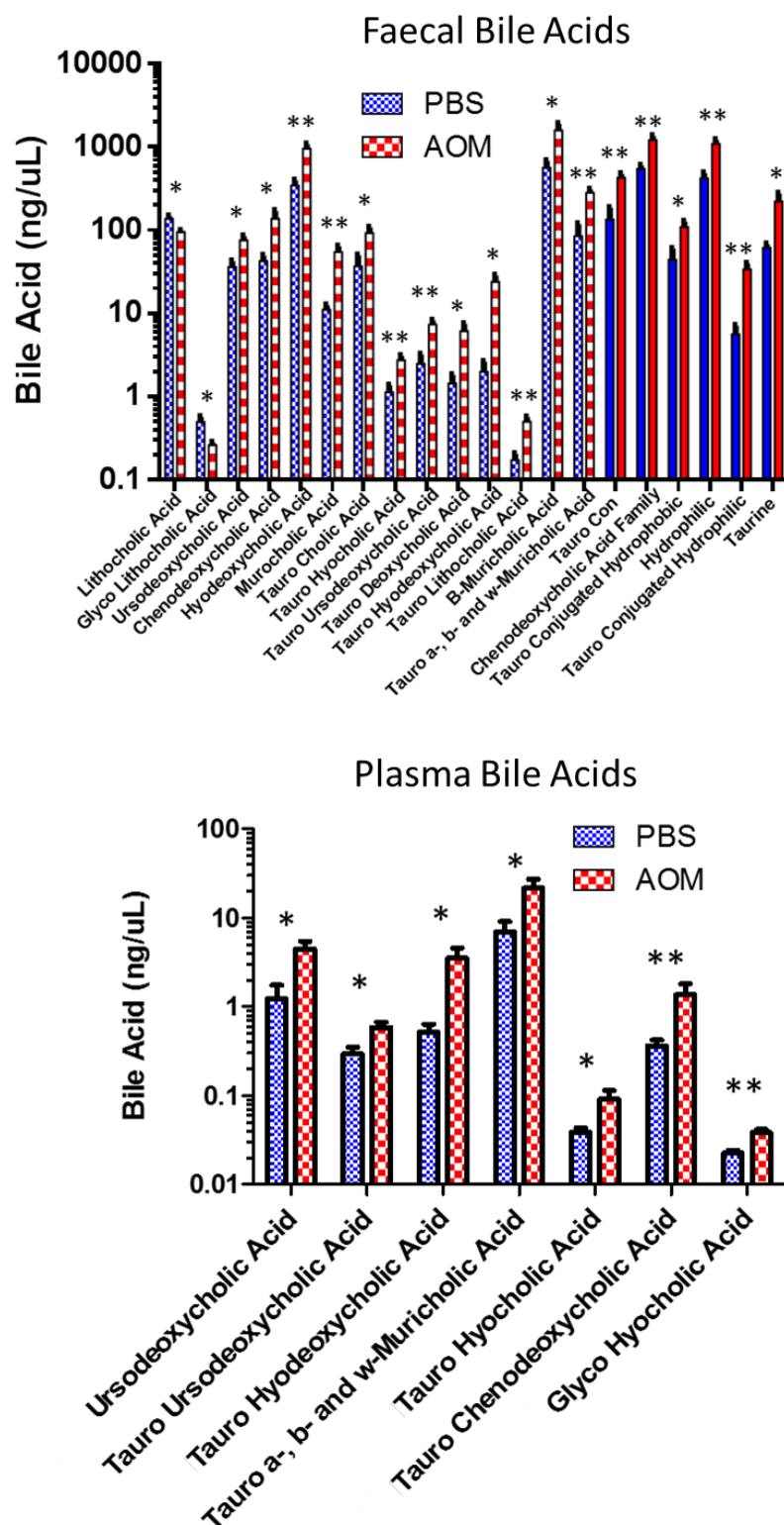


Figure 3.11: Faecal and plasma bile acids which differed significantly between groups at Week 24. Bile acids were determined by UPLC-MS at each dissection. Graphs present the mean and SEM. Individual bile acids are represented by chequered bars and families are presented by solid bars. Faecal *n*-numbers for PBS- and AOM-treated mice are 6 and 6, and plasma *n*-numbers are 5 and 7, respectively.

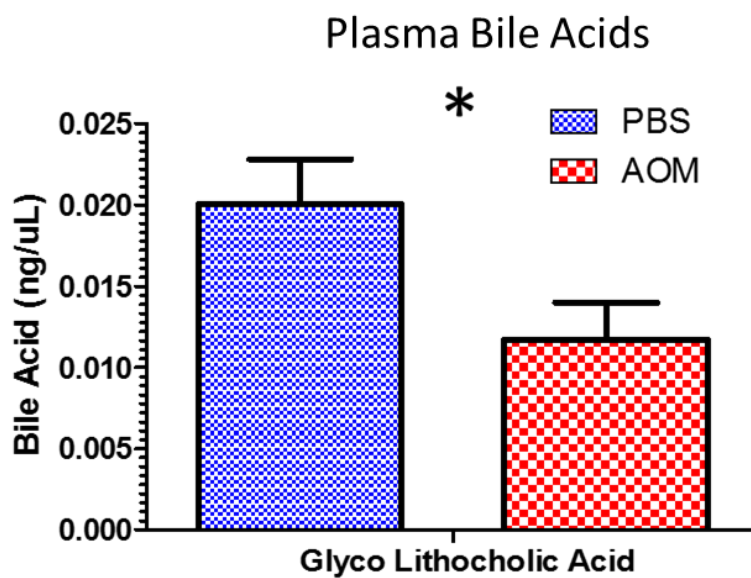
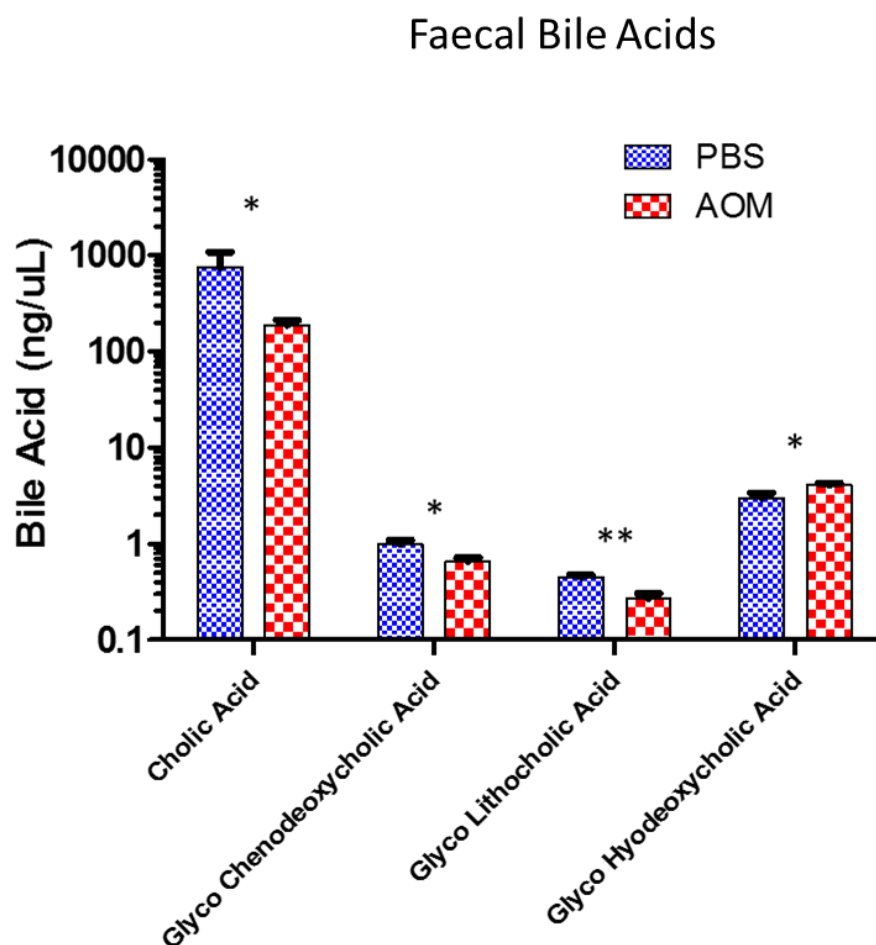


Figure 3.12: Faecal and plasma bile acids which differed significantly between groups at Week 48. Bile acids were determined by UPLC-MS at each dissection. Graphs present the mean and SEM. Faecal *n*-numbers for PBS- and AOM-treated mice are 7 and 6, and plasma *n*-numbers are 6 and 7, respectively.

Table 3.3: Bile acids measured

	Bile Acid	Abbreviation
Individual Bile Acids	Dehydrocholic Acid	DHCA
	Lithocholic Acid	LCA
	Ursodeoxycholic Acid	UDCA
	Chenodeoxycholic Acid	CDCA
	Hyodeoxycholic Acid	HDCA
	Deoxycholic Acid	DCA
	Cholic Acid	CA
	Hyocholic Acid	HCA
	7-Ketolithocholic Acid	7-Keto-LCA
	Tauro-Cholic Acid	T-CA
	Tauro-Hyocholic Acid	T-HCA
	Tauro-Chenodeoxycholic Acid	T-CDCA
	Tauro-Ursodeoxycholic Acid	T-UDCA
	Tauro-Deoxycholic Acid	T-DCA
	Tauro-Hyodeoxycholic Acid	T-HDCA
	Tauro-Lithocholic Acid	T-LCA
	Glyco-Cholic Acid	G-CA
	Glyco-Hyocholic Acid	G-HCA
	Glyco-Chenodeoxycholic Acid	G-CDCA
	Glyco-Ursodeoxycholic Acid	G-UDCA
	Glyco-Deoxycholic Acid	G-DCA
	Glyco-Hyodeoxycholic Acid	G-HDCA
	Glyco-Lithocholic Acid	G-LCA
	Alpha/Gamma (α/ω)-Muricholic Acid	α/ω -MCA
	Beta (β)-Muricholic Acid	β -MCA
	Murocholic Acid	MoCA
	Tauro α -, β -, and ω -Muricholic Acid	T- $\alpha/\beta/\omega$ -MCA
Bile Acid Families	Total Bile Acid Content	Total BAs
	Muricholic Acids	MCAs
	Tauro-Conjugated Bile Acids	T-con BAs
	Glyco-Conjugated Bile Acids	G-con BAs
	Primary Bile Acids	1° BAs
	Secondary Bile Acids	2° BAs
	Free Bile Acids	Free BAs
	Cholic Acid Family	CA family
	Chenodeoxycholic Acid Family	CDCA family
	Hydrophobic Bile Acids	Hydrophobic BAs
	Tauro-Conjugated Hydrophobic	T-Con Hydrophobic
	Glyco-Conjugated Hydrophobic	G-Con Hydrophobic
	Hydrophilic Bile Acids	Hydrophilic BAs
	Tauro-Conjugated Hydrophilic	G-Con Hydrophilic
	Glyco-Conjugated Hydrophilic	G-Con Hydrophilic
	Taurine	
	Deuterated Cholic Acid	CA-d4
	Deuterated Chenodeoxycholic Acid	CDCA-d4

PD correlated positively with *Il6*, *Cxcl1*, *Cxcl5* and *Il12* in AOM-treated mice at Week 8 (Figure 3.13A; Pearson and Spearman correlation, $p < 0.05$ after FDR adjustment), suggesting a relationship between pro-inflammatory cytokine transcription and α -diversity. PD also correlated positively with CAGs 2 and 3, and negatively with CAGs 4 and 7, while the correlations with these CAGs were reversed for Shannon diversity (Figure 3.13B; Pearson and Spearman correlation, $p < 0.05$ after FDR adjustment). PD also correlated positively with the absolute concentration of nine bile acids and taurine at Week 8 (Figure 3.13C; Pearson and Spearman correlation, $p < 0.05$). In contrast, PD correlated negatively with the relative proportions of ten bile acids, but positively with ω/α -MCA at Week 8 (Figure 3.13D; Pearson and Spearman correlation, $p < 0.05$).

This pattern of contrasting correlations is reflected in the relationship between absolute and normalised bile acids and CAGs. CAGs 2 and 3 display positive correlations with the absolute concentration of a number of bile acids, while bile acid correlations with CAGs 4 and 7 are exclusively negative (Figure 3.14A; Pearson and Spearman correlation, $p < 0.05$). Comparing CAGs to normalised bile acids revealed the opposite pattern, except for the correlation between CAGs and MCAs which match across both analyses (Figure 3.14B; Pearson and Spearman correlation, $p < 0.05$).

After Week 8, there were no significant correlations between faecal bile acids and α -diversity after FDR adjustment in mice treated with PBS or AOM. Only one negative correlation between CAG7 and dehydrocholic acid (DHCA) was found in PBS-treated mice at Week 12 (Figure 3.15). DHCA is the lowest abundance free bile acid found in these mice, comprising 0.2% of the total BA content. CAG3 correlates positively with Shannon diversity while CAGs 4 and 6 correlate negatively in PBS-treated mice at Week 8, although there were no conflicting correlations in any CAG between Shannon and PD as we saw in mice treated with AOM at Week 8. CAG4 also correlated positively with PD in PBS-treated mice at Week 24

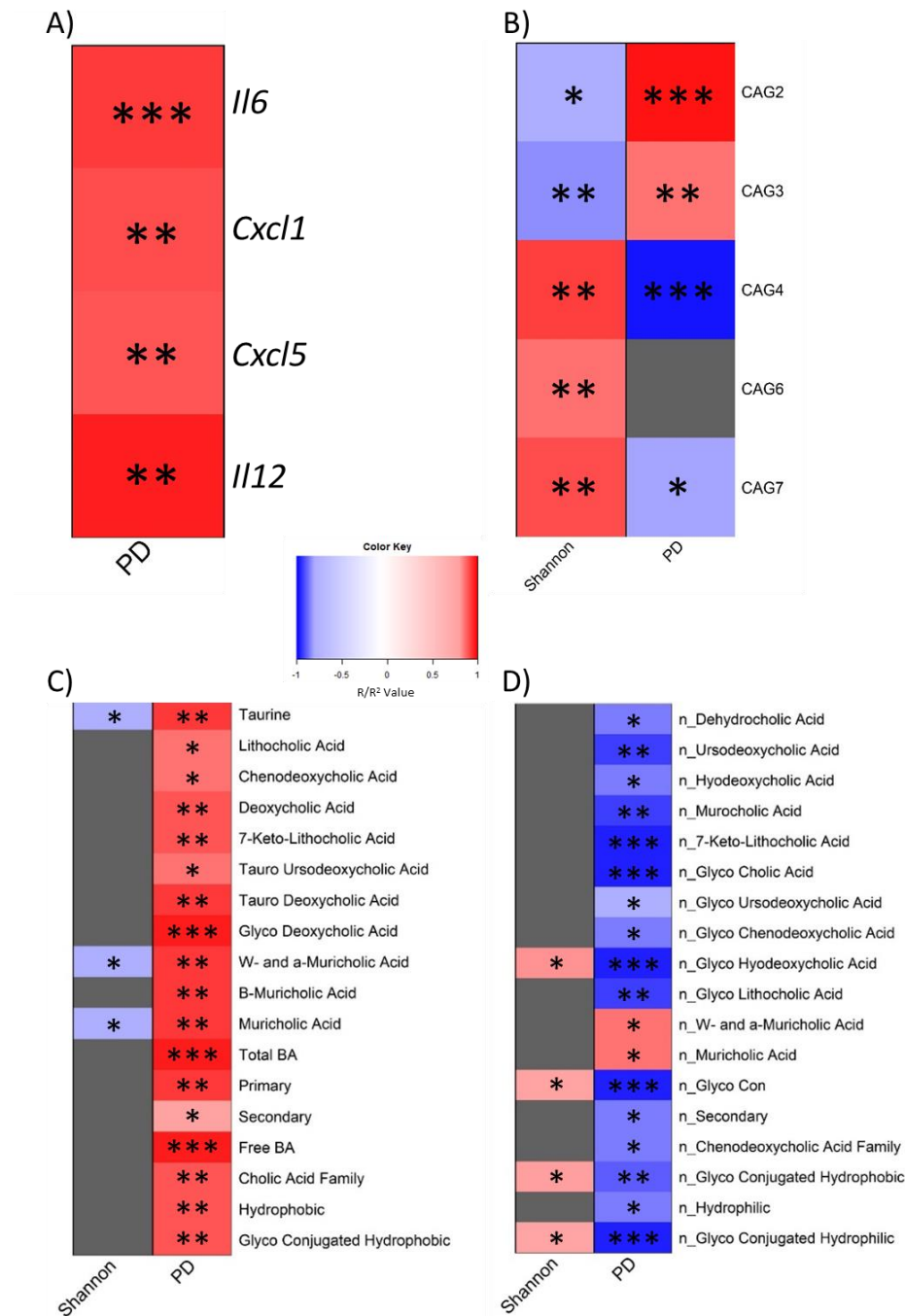


Figure 3.13: Correlations between α-diversity, inflammation, CAGs and bile acids at Week 8. A correlation analysis was performed to examine relationships between α-diversity, inflammation, CAGs and bile acids at Week 8. **A)** Phylogenetic diversity (PD) correlated with a number of pro-inflammatory cytokines. **B)** Shannon diversity and PD showed opposite patterns of correlations with different CAGs 2/3 and 4/7. **C)** A correlation analysis of our α-diversity indices and absolute faecal bile acid values revealed a positive relationship between phylogenetic diversity (PD) and a number of bile acids including the total faecal bile acid concentration. **D)** In order to identify any relationship which exist between PD and faecal bile acids, independent of total bile acid concentration, a correlation analysis was performed between our α-diversity metrics and normalised (n_) faecal bile acids which revealed a negative association with a number of bile acids and a positive relationship between ω/α-Muricholic Acid (MCA) and the MCA family.

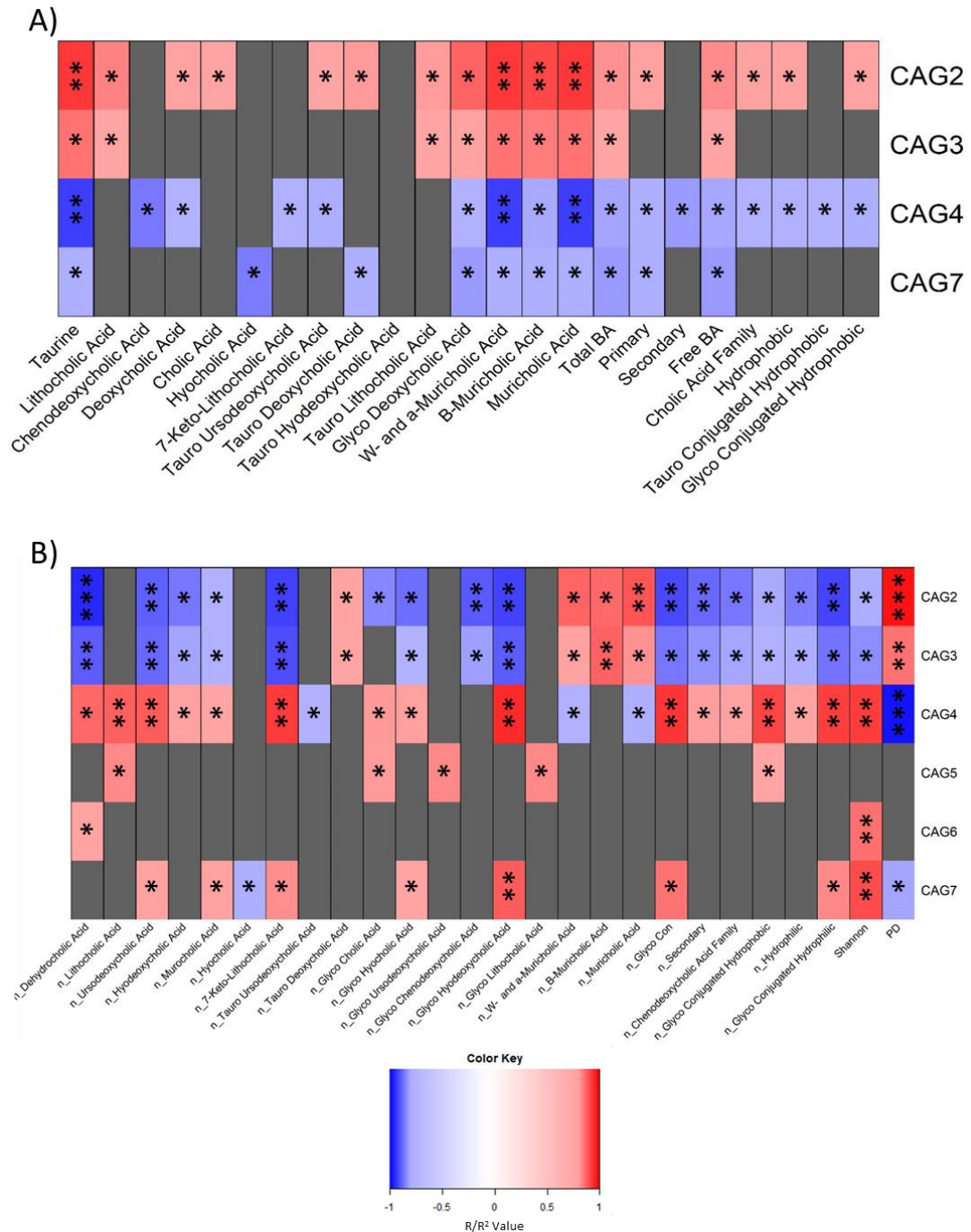


Figure 3.14: Correlations between CAGs and bile acids at Week 8. Bile acids displayed a number of correlations with α -diversity at Week 8. In order to investigate which groups of bacteria may be involved in this effect, a correlation analysis was performed to examine the relationship between absolute and normalised bile acids and CAGs. **A)** CAGs 2 and 3 display positive correlations with a number of bile acids, while bile acid correlations with CAGs 4 and 7 are exclusively negative. **B)** Comparing CAGs to normalised (n_) bile acids revealed the opposite pattern, except for the correlation between CAGs and MCAs.

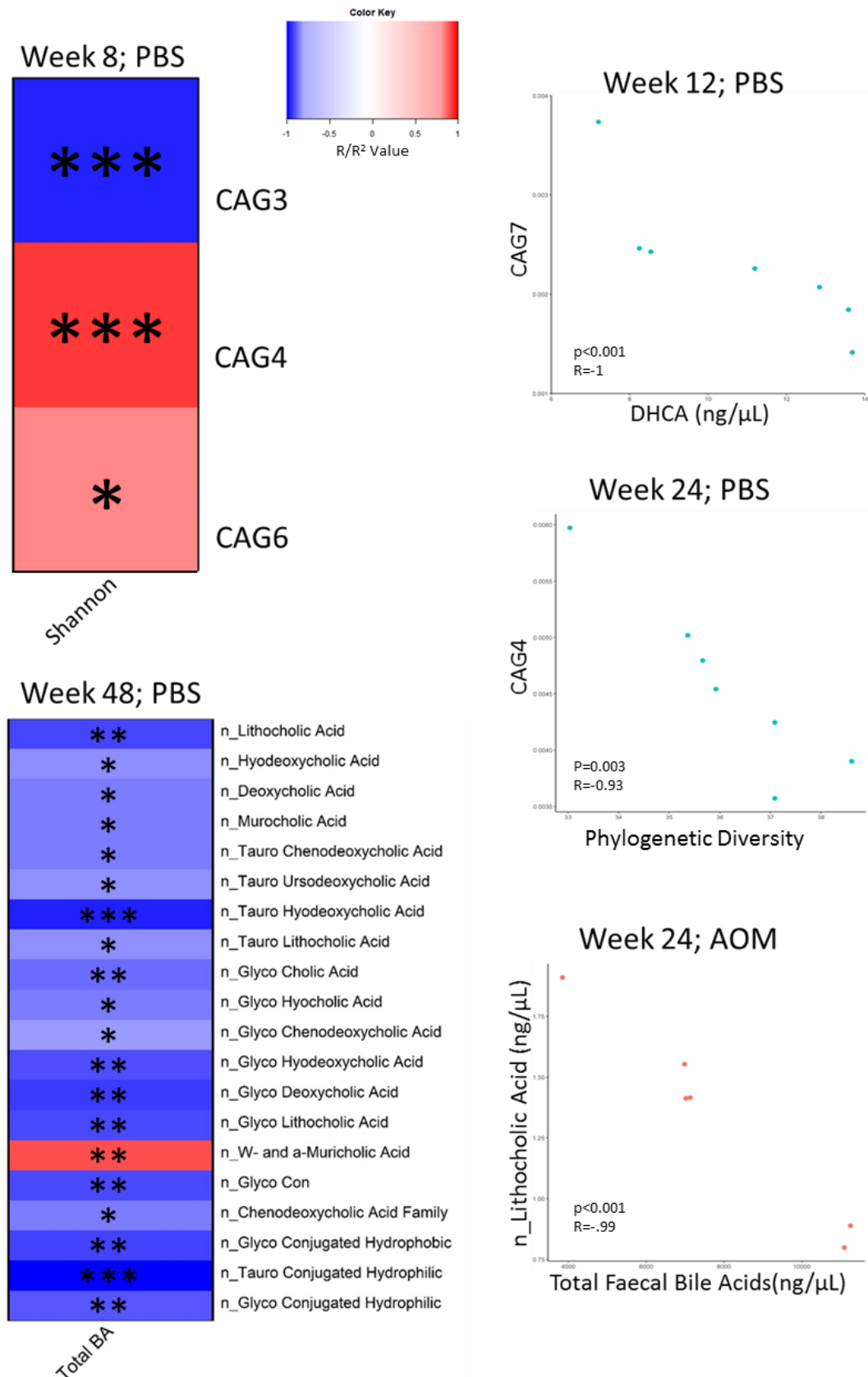


Figure 3.15: Other correlations. Outside of AOM-treated mice at Week 8, the set of significant correlations after correction for multiple comparisons do not present a consistent picture of cancer-associated phenomena (Pearson's R and Spearman's R^2 , $p < 0.05$ after FDR correction).

(Figure 3.15). At Week 48, a large number of normalised bile acids correlated negatively with total faecal bile acid content, including DCA and HDCA, while normalised ω - and α -MCA correlated positively (Figure 3.15).

3.2.8 Neither the inferred functional diversity nor the bile acid-metabolising capacity of the microbiota predicted by PICRUSt differ between treatments

In order to investigate the metabolic capacity of the microbiota and elucidate its potential contribution to disease, we examined the inferred microbial functional content predicted by PICRUSt. This revealed no significant difference in the α - or β -diversities of the inferred functional content between treatments (Figure 3.16; MWU-test and PERMANOVA, $p > 0.05$).

Furthermore, we compared the predicted abundances of microbial gene *bsh* (COG3049) and gene cluster *baiCD* (COG1902), which are involved in the deconjugation and 7 α -dehydroxylation of bile acids, respectively. The abundances of these genes did not differ significantly between groups at any time-point (Figure 3.17; MWU-test, $p > 0.05$ after FDR adjustment).

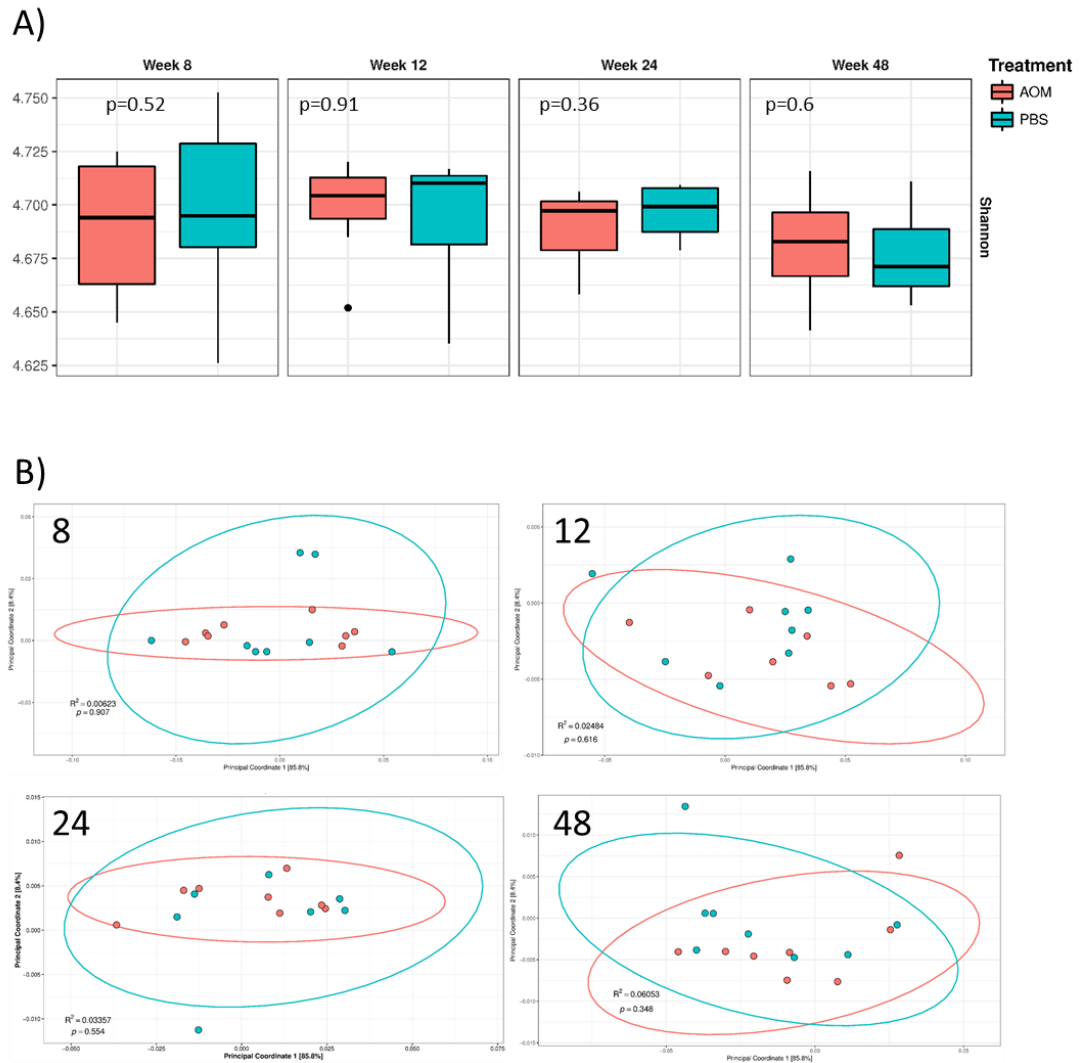


Figure 3.16: PICRUSt α - and β -diversity. A) The Shannon α -diversity of the metagenome predicted by PICRUSt does not differ between treatments at any time-point (MWU-test, $p > 0.05$ after FDR adjustment). **B)** The β -diversity of the metagenome predicted by PICRUSt does not differ between treatments at any time-point (PERMANOVA of unweighted Unifrac distances, $p > 0.05$). $N=8$ per group, except for AOM Week 12 and PBS Week 24 ($N=7$). Figures produced by Calum Walsh at Teagasc, Moorepark.

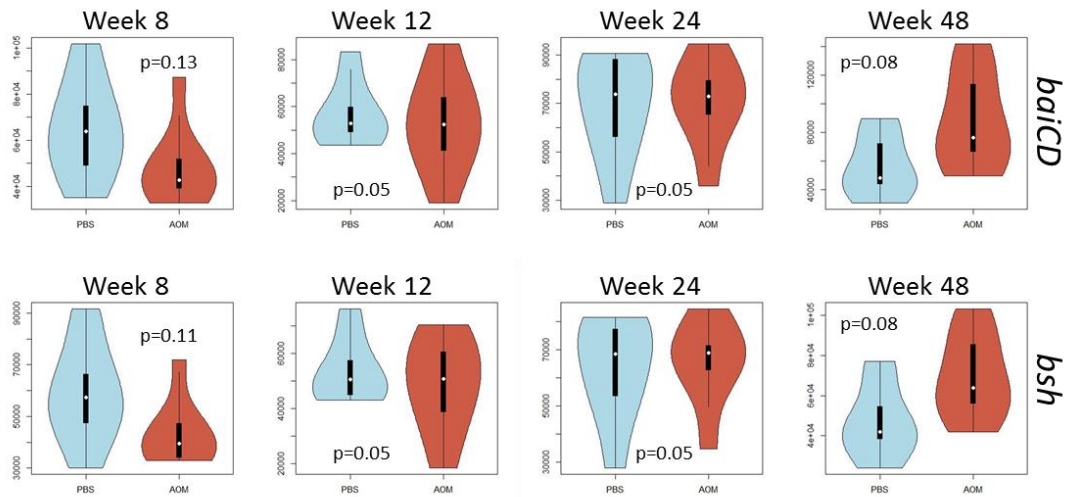


Figure 3.17: Abundance of bile acid-metabolising genes *baiCD* and *bsh* are not significantly different between treatments at any time-point. The microbial genes *baiCD* (COG1902) and *bsh* (COG3049) are involved in the 7 α -dehydroxylation and deconjugation of bile acids, respectively. We examined the abundance of these genes predicted by PICRUSt from our 16S rRNA gene sequencing data and found that they did not differ significantly between groups at any time-point (MWU-test, $p > 0.05$ after FDR adjustment). $N=8$ per group, except for AOM Week 12 and PBS Week 24 ($N=7$).

3.3 Discussion

Research shows that the microbiota is altered in human CRC, but the degree to which the host affects the microbiota and the microbiota alters the host is unclear (36). It is possible that alterations in the gut microbiota can promote or inhibit the development of colon cancer, and evidence for this has been provided by animal studies (2, 3). However, it is also possible that tumorigenesis occurs primarily as a result of alterations in the host independent of the microbiota. Similarly, after cancer initiation, components of the microbiota may suppress or promote growth, invasion and metastasis but human evidence for this is lacking (37). Human CRC pathogenesis is a process that takes place over decades. This makes it difficult to track the microbiota in a sufficiently large cohort and impossible to combine those data with the invasive colonoscopies and biopsies necessary to provide comprehensive samples before, during and after tumorigenesis. This provides the rational for using mouse models of CRC to investigate these changes.

The majority of mice in our model did not develop macroscopic adenomas. C57BL/6J mice do display increased resistance to AOM-induced tumorigenesis (38), and while the basis for this differential sensitivity to AOM in inbred mouse strains has not been established, it appears to lie downstream of DNA alkylation, for example in DNA repair (39, 40). Resistance also appears to involve complex polygenic interactions which suggests that several mechanisms may be involved, including cell proliferation and apoptosis (41, 42). The inflammatory phenotype of these mice, however, coupled with disruption to their microbial community mirrors phenomena observed in western societies exposed to cancer risk factors such as alcohol, red meat and processed food which results in an accumulation mutations in the colonic epithelium. While increased doses of AOM in susceptible models can induce tumorigenesis much more rapidly, it is likely these models do not recapitulate the entire

progression of human CRC. As such, our model may reflect the pro-inflammatory, pre-tumorigenic environment observed in western societies more accurately.

The initial immune response appeared to be down-regulation of cytokine transcription in AOM-treated mice. AOM induces methyl adducts in DNA which are repaired by O⁶-methylguanine-DNA-methyltransferase (MGMT) and when this repair enzyme is overwhelmed, damaged cells are cleared by apoptosis (43). Apoptotic cells may provide anti-inflammatory stimuli by releasing adenosine which is detected by the adenosine A2A receptor on phagocytes (44, 45). This may contribute to the immune suppression observed at Week 8, although studies suggest that the apoptotic response to AOM is concluded within the first five days after AOM administration (43). The activity of repair enzyme MGMT was shown to increase twelve days after AOM administration, and suppression of this enzyme by promoter methylation is associated with the expression of pro-inflammatory cytokines (46, 47), but its renewed activity may not surpass the baseline level observed in control animals (43). In contrast, spleen weight is significantly increased in AOM-treated mice at this time-point. This may be due to production of monocyte-derived macrophages (48, 49), as enlarged spleens have been reported in mice treated with AOM which displayed a dramatic increase in splenic macrophages (50, 51).

The increase in pro-inflammatory cytokine transcription in mice following AOM administration becomes apparent at Week 12 and continues through Week 24. Cytokines increased at these time points include *Il1β*, *Il6*, *Il10*, *Il12* and *Tnfα*. Extensive studies to date have demonstrated a pro-tumorigenic role for IL1β in numerous cancer types, including human colon cancer (17). In our study, *Il1β* was significantly increased at Week 24. Other studies have reported increased *Il1β* transcription in outbred Wistar rats at 7 weeks and at 10 months after administration with AOM alone (52, 53). In contrast, no increase was observed in A/J mice between 3 and 48 hours after injection (54), demonstrating that

transcription is not an acute effect of AOM in the colon. In comparison to the models reported in the literature, our mice appear to have a longer incubation period before induction of *Il18* transcription, and this up-regulation lasts for a shorter period, possibly reflecting the fact that C57BL/6J mice are relatively resistant to AOM-induced carcinogenesis.

Similar to *Il18*, *Il6* was significantly increased in mice administered AOM compared to PBS in our study. IL6 is also a cytokine which is dysregulated in CRC, and elevated levels are associated with poor prognosis in human patients and in AOM-induced mouse models (18, 55-58). Our IL6 data highlights Week 24 as a key stage in the AOM-induced pro-inflammatory cytokine response.

Il10 was also significantly increased in our model at Week 24, where it displayed a 220-fold induction compared to PBS-treated mice. IL10 has been shown to protect against tumorigenesis both by suppressing the pro-inflammatory milieu that can promote tumour progression and by inducing the cytotoxicity of CD8⁺ T cells (59). Indeed, in preclinical tumour models, IL10 induces the rejection of tumours through both its pro-immunity and its anti-inflammatory functions (59). IL10 expression may reflect disease stage, as IL10 was reported to be increased in the serum of mice displaying pre-neoplastic changes in their colons, but not in normal or tumour-bearing mice, 28-30 weeks after administration of two doses of AOM (60). Moreover, IL10 may be modulated by the microbiota. Its transcription was induced by dietary supplementation of prebiotics and probiotics in Fischer 344 rats 32 weeks after exposure to two doses of AOM (61). Our *Il10* data suggest that the anti-inflammatory response was functioning normally in our model, reinforced by the observation that pro-inflammatory cytokine transcription was largely suppressed by Week 48.

We observed an increase in *Il12* in mice administered AOM compared to PBS controls at Weeks 12 and 48. IL12 has been shown to suppress cancer by a number of mechanisms (62-

67). Few studies have tracked the effect of AOM administration alone on IL12. However, it is increased in animals treated with AOM/DSS (68-70) and is, at least partly, regulated by the microbiota (26). Its transcription is also noteworthy at Week 48 as it is significantly higher in the mice harbouring colonic adenomas compared to mice administered AOM which were free of adenomas. This could reflect a continued anti-tumour immune response, or IL12 production by innate immune cells in response to antigen stimulation in adenoma-bearing mice.

Tnfα transcription was increased in our AOM-treated mice at Weeks 12 and 24. TNFα is a pro-inflammatory cytokine which can kill tumour cells, but chronic, low-grade expression can contribute to tumorigenesis (19, 71, 72). While *Tnfα* transcription returned to baseline levels by Week 48, our data indicate a window of at least 12 weeks of up-regulation of this cytokine. In a study featuring sensitive 129S6/SvEvTac mice, *Tnfα* correlated with tumour multiplicity (73). In contrast, no significant increase in *Tnfα* was observed in the serum of Wistar rats eighteen weeks after two doses of AOM, despite a significant increase in the number of ACF compared to controls (50), which implies that this cytokine is not required for ACF formation. TNFα expression is also a target of IL10-mediated immune suppression, and the down-regulation of *Tnfα* observed at Week 48 may be in response to the significant induction of *Il10* measured at Week 24 (74).

TGFβ is protective during the early stages of tumorigenesis but advanced cancers often present with a deletion or mutation of TGFβ receptors (75-78). Alterations to the TGFβ signalling pathway may account for some of the differences in sensitivity to AOM in different mouse strains, as sensitive A/J mice showed a greater suppression of TβR-II mRNA levels compared to AKR/J mice (79). Moreover, conditional knockout of the TGFβ Receptor 2 in the colonic epithelium of FVB mice leads to an increased number of ACF, adenomas and adenocarcinomas, two to six months after twelve doses of AOM (80, 81). However, *Tgfb* did

not differ significantly between treatments at any time-point in our study, although this reflects a differential response in each mouse. At Week 24, two mice in the treatment group transcribe *Tgfb* at levels less than one tenth of that observed in the control group, while five mice exhibit transcription twice as high as control. Transcription largely returned to normal by Week 48 except in one of the two mice harbouring colonic adenomas which has *Tgfb* transcription 6.8-fold higher than control. This simultaneous over- and under-expression of *Tgfb* parallels the differential responses reported in the literature and the complex role of this cytokine in cancer.

Cxcl1, *Cxcl2* and *Cxcl5* were all increased at Week 12 in our model. *Cxcl1* was also up-regulated at Week 24, while *Cxcl5* was suppressed at Week 8 in mice administered AOM, as discussed previously. This is a slightly earlier pattern of transcription than many of the other cytokines we measured, possibly because these chemokines are both produced by, and are chemotactic factors for, neutrophils, which are early response cells in wound healing and cancer (82). There is limited data available on the expression of these chemokines in mice administered AOM alone, but the association between these chemokines and AOM/DSS models of colitis-associated cancer is well established (83-88). There is also evidence that each of these chemokines is regulated by the microbiota, as their expression was reduced by a probiotic cocktail containing four strains of *Lactobacillus*, three strains of *Bifidobacterium* and one strain of *Streptococcus*, which also suppressed tumour formation in the colon of mice treated with AOM/DSS (29). The early pattern of up-regulation in our model also implicates these chemokines in the development of ACF observed at Week 24 and adenomas at Week 48.

Our data reveals a significant up-regulation of pro-inflammatory signalling beginning at Week 12, before detectable neoplastic changes were observed in the colonic epithelium of mice administered AOM. This implies that inflammatory cytokine transcription may have acted as

a tumour-promoter in these mice, and that it is unlikely that neoplastic transformation instigated inflammatory transcription. This is reinforced by the fact that inflammatory transcription had subsided by the time colonic adenomas were observed at Week 48. It also highlights the possible role of chemokines as early response elements in cancer development as each of the chemokines we measured were up-regulated at Week 12, along with *Tnfa* and *Il12*.

Changes to microbial diversity were apparent as early as Week 8 in AOM-treated mice. Human studies on the gut microbiota in cancer often report increased tumour-associated α -diversity as a result of the growth of opportunistic pathogens adapted to the novel tumour microenvironment (89-94). Studies which sample the faecal microbiota tend not to detect a difference between cancer patients and controls however, ostensibly because the faecal microbiota includes bacteria from the entire gastrointestinal tract which dilutes the signal generated by the cancer-associated species (89, 95-100). This is further evidenced by the fact that tissue-associated microbiota has half the diversity of faecal samples (90). Increased α -diversity in the faecal microbiome of CRC patients has been reported, however (2, 101). Studies have also reported a decrease in α -diversity in CRC patients' microbiota in both tissue and faecal samples (102, 103). This decrease in CRC-associated α -diversity was also detected in a retrospective analysis of 156 CRC patients and healthy controls, which further indicated a significant negative correlation between gut microbiota diversity and the stage of CRC (104). Our data suggests different changes associated with disease state. This begins with an increase in diversity following exposure to AOM, followed by microbial suppression, possibly mediated by the immune response (27, 105, 106).

In our data, PD was significantly increased in mice administered AOM at Week 8, while Shannon diversity was significantly decreased at Week 12, which marks the beginning of the cytokine response we observed in these mice. Shannon diversity index considers the

evenness of distribution of species' relative abundances across the population while PD does not. This could explain the increase we observed in PD but not Shannon diversity at Week 8, reflecting an expansion of low-abundance opportunistic pathogens (101). When considering which species might represent these enriched pathogens, we identified six taxa present in AOM-treated mice at Week 8 which were completely absent in PBS-treated controls. These included *Prevotella*, which is often reported as enriched in CRC (36, 107). *Prevotella* is a constituent of the oral microbiome, members of which have previously been associated with CRC (108). These six taxa also include *Chryseobacterium*, which is found in the murine oral microbiome (109). The other taxa are *Subdoligranulum*, and unclassified members of the *Ruminococcaceae*, *Erysipelotrichaceae* and *Bacilli*. A chi-squared test also identified *Ruminococcus 2*, *Faecalibacterium*, and the *Eubacterium ventriosum* group as qualitatively enriched in AOM-treated mice, although correction for multiple comparisons reduces these p-values to insignificance.

Later, an inflammation-induced reduction of these low-abundance opportunistic bacteria could allow their niche to be filled by predominant high-abundance taxa such as *Bacteroidales*, which have been associated with increased CRC risk (110). This would contribute to altered species distribution and suppress Shannon diversity without reducing PD. After Week 12, neither Shannon nor PD display any significant differences between AOM and PBS treatment groups. However, the three mice showing macroscopic signs of disease (ACF or adenomas) were among the four mice with lowest Shannon diversity indices at Week 48.

Beta (β)-diversity demonstrated that the composition of the gut microbiota in AOM-treated mice was significantly different from control at each time-point, with the model explaining between 14.6% and 24.4% of this variation. From Week 24 however, β -diversity becomes more disparate between treatments while α -diversity converges, suggesting that

tumorigenesis affects the specific composition of the gut microbiota more profoundly than the breadth of diversity within the microbiota of individual mice. The gut microbiota is a relatively stable ecosystem, robust against daily exposure to environmental microbes and dietary compounds (111-113). It features a core set of redundant metabolic functions distributed across its component species and recovers quickly from disruptions such as enteric infection and antibiotic exposure (114, 115). We observed a similar process in our α -diversity in response to AOM, but not in our β -diversity, suggesting that α -diversity is a more stable characteristic of the gut microbiota than β -diversity. The α - and β -diversity of the microbial metagenome predicted by PICRUSt also does not differ between treatments at any time-point, highlighting the stability of the metabolic functions provided by the microbiota. The consistent difference in β -diversity between treatments, however, reveals that this stability can be provided by communities of gut bacteria with significantly different compositions, and that the ecological shock of AOM treatment shifted the gut bacteria of these mice into an alternative stable state (116). This has implications for the persistence of disease-associated microbial compositions and treatments intended to restore patients to a “healthy” gut bacteria through treatments such as faecal microbial transfer (117).

In order to investigate if the altered composition of the gut bacteria of mice treated with AOM could contribute to tumorigenesis, we examined the specific species that differed between treatments at each time-point. At Week 8, the trend in taxa suppressed by AOM reflects a reduction in bacteria which produce butyrate, such as *Marvinbryantia* (118-129), and bacteria which are maladapted to inflammatory environments (27, 130-142). Butyrate has anti-inflammatory properties and may protect against CRC (143). Given that there was no increase in inflammation at this time-point, this suggests that changes in inflammation-suppressing microbiota precede induction of a pro-inflammatory immune response. All four species that were enriched in mice administered AOM were associated with cancer risk factors such as processed meat, high-fat diet or high BMI (144-151), while *Clostridiales*

vadinBB60; uncultured rumen bacterium was suppressed by cancer-protective factors such as resistant starch and butyrate (145, 152). However, *Eubacterium ventriosum* group and *Ruminococcus 2*, which are both negatively associated with CRC, were increased in our cancer model (153-157). The totality of the data does suggest that these mice have a cancer- and inflammation-associated microbiota at this time-point however, despite the absence of cytokine response or neoplastic transformation in the colonic mucosa. This would put microbial changes upstream of these effects during tumorigenesis.

By Week 12, the communities become more similar once the cytokine response has begun, which concurs with the idea that α -diversity is increased early in tumorigenesis due to the growth of opportunistic pathogens, but this is reversed later once the immune response begins.

At Week 24, the specific composition of the microbiota becomes more disparate again, even though α -diversities have converged and the cytokine response remains active. Three taxa are reduced at this time-point in AOM-treated mice that were also suppressed at Week 8, *Rikenella*, *Marvinbryantia* and *E. xylanophilum*, which are associated with factors demonstrated to reduce cancer risk (27, 120-123, 133-137, 158-164). Of note, four species are suppressed that are associated with reduced cancer risk and three of these express *bile salt hydrolase*, implicating bile acid metabolism in CRC (132, 165-167). Bacteria that are increased at Week 24 include two species closely associated with increased cancer risk, *Odoribacter* and *Citrobacter* (168-179). *Lachnospiraceae NC2004*, on the other hand, may be mildly protective by producing butyrate (180, 181). Similar to Week 8, these mice present with a pro-carcinogenic microbial profile, this time alongside a concurrent cytokine response.

At Week 48, when the cytokine response has diminished, the specific microbial changes are no longer conspicuously pro-carcinogenic. Three of the seven taxa reduced in AOM-treated mice at this time-point have negative associations with cancer or colitis (27, 121-123, 149,

150, 153, 160-163, 182), while *Akkermansia*, which is also reduced, has pro- and anti-tumorigenic reports in the literature (143, 183-189). In contrast, *Citrobacter* is closely linked to murine CRC and was increased at Week 24 but is decreased in mice administered AOM here (170-179). Taxa that were increased in AOM-treated mice include protective genera such as *Bifidobacterium*, *Prevotellaceae NK3B31*, *Butyrivibrio* and *Faecalibaculum* (131, 132, 190-207), and a number of other species with harmful or ambiguous effects in CRC such as *Clostridiales VadinBB60* and *Parasutterella* (208-212). While the microbiota may have contributed the cancer progression earlier in the model, it does not appear to have a significant pro-tumorigenic bias at this time-point.

Research has focussed on the role of individual bacteria in gut health and CRC risk, but this may overlook larger structural trends in the microbiota. In order to investigate these community trends in our model, we defined a set of co-abundance groups (CAG). These describe a set of bacterial taxa which displayed similar shifts in abundance across the experiment, which may be a result of some degree of interaction or inter-dependence, or a reliance on shared metabolic pathways. CAGs showed the opposite pattern of divergence to our α -diversity data. Of the seven CAGs our data produced, none showed any difference between treatments at Week 8 or 12. This suggests that the microbiota at the structural level of CAGs is robust against the forces that affected α -diversity. Differences were detected at Weeks 24 and 48, with CAG1 increased in AOM mice at Week 24, while CAG2 was increased and CAG7 decreased in these mice at Week 48. CAG1 is dominated by the order *Bacteroidales*, especially the *Alistipes* genus, both of which are associated with cancer-inducing inflammation (213, 214). This reinforces the pro-carcinogenic profile we observed in the species composition of mice treated with AOM at Week 24. CAG2 is more diverse, featuring significant proportions of *Erysipelotrichales*, *Clostridiales* and *Bacteroidales*, and several genera with protective associations in CRC including *Bifidobacterium* and *Prevotellaceae NK3B31*. The predominant species in CAG7, meanwhile, are

Ruminococcaceae; *uncultured* and *Desulfovibrio*, the latter of which is reported as an intestinal pathogen, although there are mixed reports on its role in CRC (215-217). These data also support the observations on species composition of mice treated with AOM at Week 48, in that the communities were significantly different but did not present a conspicuously carcinogenic profile.

One of the primary mechanisms by which the gut microbiota may influence CRC initiation and progression is via modification of host compounds to produce bioactive microbial metabolites, including bile acids. Bile acids have been implicated in the pathogenesis of colon cancer through their potential for cytotoxic effects, and well as by their hormonal signalling via FXR (218, 219). Alterations to bile acid-metabolising species was a characteristic of the microbiota of AOM-treated mice and so we examined the faecal bile acid composition of our mice.

Total faecal bile acid excretion was significantly decreased at Weeks 8 and 12, prior to the development of disease. However, in contrast to studies demonstrating a pro-tumorigenic function for hydrophobic bile acids such as DCA and LCA, these bile acids were reduced in AOM-treated mice before Week 48 (220, 221). These secondary bile acids are the end products of microbial deconjugation by Bsh and dehydroxylation by enzymes in the 7 α -dehydroxylation pathway, and a reduction in bacteria expressing these enzymes could explain this effect. We did see a reduction in *Lactobacillus* at Week 8 and these bacteria are major producers of Bsh in the gut, while *Bacteroides* and *Enterococcus* also feature Bsh-producing species and these taxa are decreased at Week 24 (13). The expansion of Bsh-producing *Bifidobacterium* in AOM-treated mice at Week 48 could also contribute to the resolution of this effect. This idea is reinforced by the increase in conjugated bile acids observed in AOM-treated mice at Week 8 and Week 24, although there were no differences in bile acid metabolism genes predicted by PICRUSt.

As regards the reduction in total faecal bile acids up to 12 weeks post treatment, this may be a consequence of reduced microbial bile acid metabolism, as previously described in germ-free mice (222). This increased the levels of T- β -MCA and subsequently up-regulated ileal ASBT, a bile acid transporter, resulting in a decrease in total faecal bile acids, similar to the effects we observe in our model (223). T- β -MCA is an FXR antagonist and may suppress the protective effect of FXR against cancer (224, 225).

Correlation analysis revealed a complex set of interactions at Week 8 post-AOM exposure. It also highlights the relevance of CAGs as distinct, structural units in the microbiota.

PD was increased in our AOM-treated mice at Week 8 and this correlated with the transcription of several pro-inflammatory cytokines. The mice with highest PD values, on which AOM ostensibly had the largest effect on microbial diversity, also had the highest abundance of CAGs 2 and 3 and lowest abundance of CAGs 4 and 7. In fact, these pairs of CAGs tended to have opposite correlations with α -diversity and faecal bile acids. This is especially noteworthy in the correlations between CAG2/3 and CAG4/7 and normalised faecal bile acids. These are primarily negative for CAG2/3 and positive for CAG4/7, but this is reversed for FXR-antagonist MCAs. A similar phenomenon is observed for the correlations between PD and normalised faecal bile acids, which are universally negative except for a subset of MCAs. This hints at a network of interaction between microbial diversity and composition, bile acid regulation and cytokine signalling at this stage of our model which may contribute to CRC progression.

The initial changes we observe in response to AOM occur in the microbiota, which takes on a pro-tumorigenic and pro-inflammatory profile before any inflammatory or tumorigenic changes occur in the host, potentially placing this as an instigating phenomenon. This occurs alongside an increase in microbial richness and alterations to the bile acid pool which suggest a reduction in microbial bile acid metabolism. Our correlation analysis also reveals a

relationship between the microbiota, inflammation and bile acids at this time-point. The cytokine response and bile acid dysregulation are largely ameliorated by Week 48 when macroscopic adenomas appear, while the faecal microbial profile is no longer conspicuously pathogenic. However, we did not sample the tumour-associated microbiota of the two mice which developed adenomas, and bacteria in the tumour microenvironment could still contribute to disease progression locally. Bile acids did not appear to contribute directly to cancer risk as the faecal concentration was reduced in our model, along with the absolute and relative proportions of the major hydrophobic bile acids, but may affect cancer risk indirectly via repression of FXR signalling as a result of alterations in microbial bile acid metabolism. Our data highlights the acquisition of a pro-inflammatory gut microbial composition as the instigating tumorigenic change detected in our model, and suggests that targeting the microbiota with probiotic and/or antibiotic therapy could be an effective prophylactic defence against CRC.

3.4 References

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Chapter 4

Time course analysis of the gastrointestinal microbiome of APC^{MIN} mice
and its association with bile acid signalling

4.0 Abstract

Background: The gut microbiota has been implicated in the initiation and development of colorectal cancer (CRC). Research into the role of the microbiota in CRC has primarily focussed on changes that exist in and around tumour sites at a single point in time. This has made it difficult to differentiate between changes that promote cancer initiation from those that are a result of tumour development. In this study, we tracked changes in the microbiota throughout the course of tumorigenesis in APC^{MIN} mice, a model of familial CRC, to elucidate their potential contribution to disease. In addition, we measured plasma and faecal bile acids and targeted gene expression at the end-point of the study. Our aim was to establish the temporal development of the microbiota in APC^{MIN} mice in order to generate a timeline of alterations and events that may influence tumorigenesis in these animals.

Methods: The faecal microbiome was analysed by 16S rRNA gene sequencing, and faecal and plasma bile acids were determined by UPLC-MS at 14 weeks of age. Host cytokine response, proliferation index, bile acid signalling and polyp multiplicity was measured at Week 14. Correlation analysis was performed to identify any relationships between microbial composition and diversity, transcription of inflammatory cytokines and bile acids.

Results: The gut microbiota of APC^{MIN} mice differs significantly from controls at each time-point analysed. In particular, we identified bacterial taxa that are consistently altered in APC^{MIN} mice throughout the study. These included a reduction of *Akkermansia*, *Bacteroides* and *Lachnospiraceae* species and an increase in *Eubacterium* species in APC^{MIN} mice relative to control animals. Further taxa (including *Allobaculum* and *Lachnoclostridium*) were prone to significant variation throughout the study, a finding which may indicate sensitivity to the changing histopathological landscape associated with tumorigenesis. Only slight differences were observed in the bile acid pool at end-point, with a reduction in plasma lithocholic acid evident in APC^{MIN} mice relative to control animals. Analysis of FXR-regulated gene expression

in the ileum and correlation analysis suggests a suppression of FXR signalling which has previously been linked to tumorigenesis. The metagenome predicted by PICRUSt also identified disruption to bile acid metabolising pathways at 7 weeks of age.

Conclusions: We identified a number of bacterial taxa in APC^{MIN} mice that are changed at key points during tumour onset and progression. We also implicate bile acid signalling in small intestine polyposis in these mice.

Acknowledgements: 16S rRNA gene sequencing of the faecal microbiota was performed by Calum Walsh in Teagasc Moorepark (*Materials and Methods* section 2.5, *Amplicon Sequencing* to end of *Functional Analysis*), who also produced the plots in Figure 4.3 and 4.13. UPLC-MS and extraction from faecal and plasma samples for the detection of bile acids was performed by Peter Cronin (*Materials and Methods* section 2.7, *Ultra-Performance Liquid Chromatography – Mass Spectrometry*). Some of this work is presented in his submitted MRes thesis (April 2019). The materials and methods sections for these processes are provided by their respective executors. Mice were culled by Cara Hueston.

4.1 Introduction

The onset and development of colorectal cancer (CRC) involves the complex interaction of genetic and environmental factors. Recently, attention has turned to the influence of the gut microbiota on this process (1). Cancer patients display a gut microbial composition that is significantly different to healthy counterparts and certain microbial patterns are associated with increased cancer risk. However, definitive identification of mechanisms and bacterial species involved has proven elusive as taxa associated with human CRC are not consistent across studies (2, 3).

Research into the microbiota in CRC has focussed on patients presenting with active disease, which has left the dynamic interaction of host and microbiota unexplored during the earliest stages of disease onset (4). Animal models provide an inexpensive and predictable tool for the investigation of these stages by allowing timed sampling of large cohorts in controlled conditions. Mice heterozygous for the tumour-suppressor *Apc* gene represent an animal model of familial colorectal cancer (CRC). These mice develop intestinal polyps starting at 7-8 weeks of age, with inflammation becoming apparent at approximately 12 weeks of age (5, 6). Humans with this mutation suffer from Familial Adenomatous Polyposis (FAP) and these patients exhibit dozens of adenomatous polyps in their large intestine with a risk of progressing to malignant CRC approaching 100% in middle age. The microbiota of FAP patients are enriched for pro-tumorigenic bacteria such as *Escherichia coli* expressing colibactin and enterotoxigenic *Bacteroides fragilis* (7), changes which are reflected in APC^{MIN} mice (5). Importantly, APC^{MIN} mice have been utilised to study the role of the microbiota in the progression of CRC and human tumour-associated bacteria have been shown to aggravate tumorigenesis in these mice (8).

One possible mechanism by which the gut microbiota may affect host processes leading to the development of CRC is the production of bacterial metabolites. Aberrant microbial

metabolism of bile salts is associated with cancer risk (9). For example, APC^{MIN} mice fed deoxycholic acid (DCA) had increased tumour multiplicity, and this effect was mediated by the microbiota (10). Bile acids can also modify cancer risk via hormone signalling through the bile acid receptor FXR which suppresses intestinal tumorigenesis *in vivo* (11). High fat diets are also known to increase CRC risk, and also increase bile acid excretion and the faecal concentrations of DCA and lithocholic acid (LCA) (12, 13). This suggests that bile acids and the microorganisms involved in bile acid metabolism may represent potential aetiological agents of high fat diet-induced colon cancer risk.

Disruption of the normal function of the intestinal immune response may also affect CRC risk (14). Cytokines and chemokines are soluble immune factors which can drive tumorigenesis by production of genotoxic compounds such as reactive oxygen species, by induction of DNA damage and by suppression of apoptosis (15). They are produced by tumour cells, stromal cells and tumour-infiltrating immune cells (16-20). There is also evidence that the microbiota is involved in the regulation of cytokines and chemokines in mouse models of CRC, especially by production of short chain fatty acids such as butyrate (21-27).

To our knowledge, the temporal flux of the community structure of the microbiota has not been previously analysed in APC^{MIN} mice. Such an analysis has the potential to reveal microbial species which distinguish the microbiota of such mice from wild-type counterparts and which may be linked to disease progression. We therefore performed a time-course study of the APC^{MIN} mouse, to examine the progression of microbial changes during disease onset. At the end-point of the experiment, we measured polyp multiplicity, markers of host inflammation and the production of microbial metabolites to identify phenotypic markers of disease progression. Data indicate a distinct microbial profile in APC^{MIN} mice which differs from wild-type animals and coincides with enriched α -diversity and reduced transcription of FXR target genes.

4.2 Results

4.2.1 APC^{MIN} mice develop significantly more polyps in their small intestine

APC^{MIN} mice are a model of familial CRC which develop dozens of polyps in the small intestine and a smaller number in the colon. As expected, we observed significantly more polyps in each segment of the small intestine of APC^{MIN} mice than our wild-type control (Figure 4.1A; T-test and MWU-test, $p < 0.001$). All APC^{MIN} mice developed small intestine polyps while none were detected in control. Five APC^{MIN} mice also developed colonic polyps (Figure 4.1A; MWU-test, $p = 0.063$).

4.2.2 Faecal microbial diversity is significantly different in APC^{MIN} mice

We collected faecal pellets from each mouse throughout the experiment and performed 16S rRNA gene sequencing at 4, 7, 11 and 14 weeks of age to characterise the microbial community and investigate changes that occur before and during the process of tumorigenesis in APC^{MIN} mice. Alpha (α)-diversity was measured by Shannon diversity and phylogenetic diversity (PD) (Figure 4.2).

At Week 4, both Shannon and PD were increased in APC^{MIN} mice, and Shannon diversity was also increased at Week 14 (Figure 4.2; MWU-test with FDR adjustment, $p < 0.05$). No differences were observed at Week 7 or Week 11 (Figure 4.2; MWU-test with FDR adjustment, $p > 0.05$).

Beta (β)-diversity is a measure of between-individual diversity in the microbiota which we measured by PERMANOVA of the unweighted Unifrac distances, visualised by PCoA. The

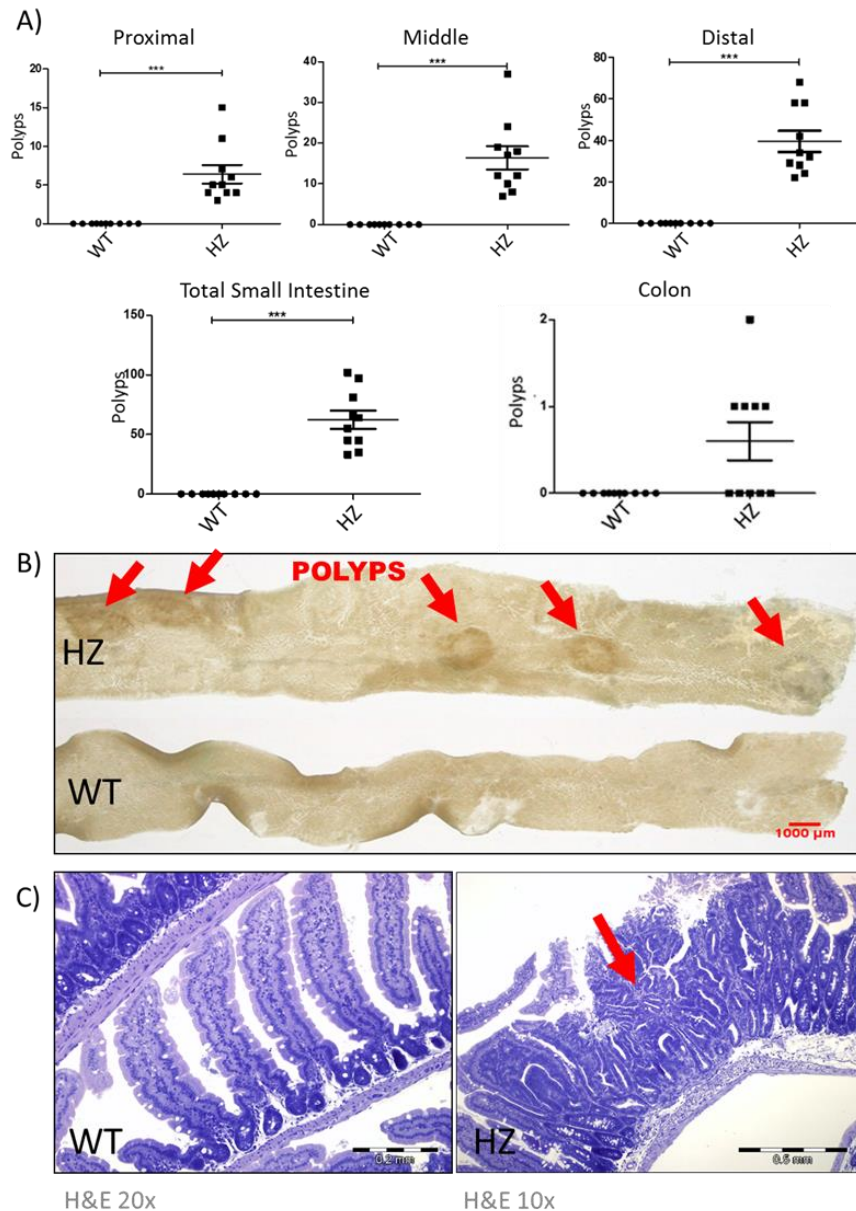


Figure 4.1: APC^{MIN} mice develop significantly more polyps in the small intestine. Mice were culled at 14 weeks of age ($n=10$ per group) and 1cm of their proximal and distal small and large intestine was removed for RNA analysis. The remaining small intestine was divided into three equal portions and the number of polyps in each section was enumerated under a dissecting microscope. The number of polyps in the colon was also determined. **A)** APC^{MIN} mice (HZ) had significantly more polyps than controls (WT) in each portion of their small intestine and significantly more total small intestine polyps (T-test, $P<0.001$). The total number of colonic polyps was not significantly different between groups despite being completely absent in the control group due to the low prevalence of colonic adenomas in APC^{MIN} mice (MWU test, $P=0.063$). **B)** A length of the distal small intestine of an APC^{MIN} mouse and its wild-type equivalent. The APC^{MIN} section displays adenomatous polyps, highlighted by red arrows. **C)** After polyps were enumerated, intestinal sections were paraffin-embedded and sectioned for microscopic analysis. Normal wild-type intestinal villi stained with haematoxylin and eosin (H&E) are presented here, as well as an adenomatous polyp from an APC^{MIN} mouse.

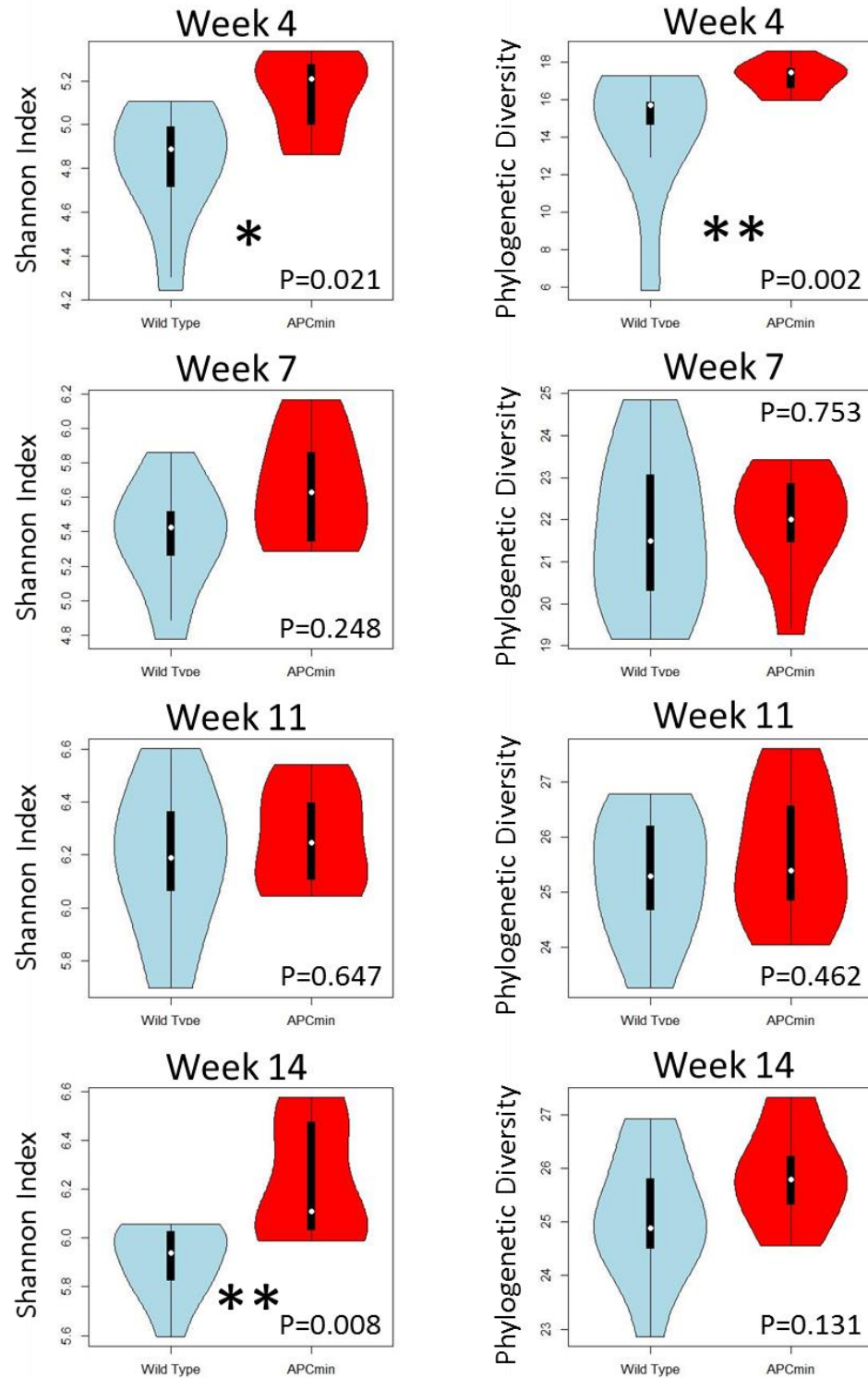


Figure 4.2: Alpha (α)-diversity is significantly altered in APC^{MIN} mice at both Weeks 4 and 14. We performed 16S rRNA gene sequencing for the V3-V4 region of the faecal microbial rRNA and examined the α -diversity by generating the Shannon and Phylogenetic Diversity (PD) metrics. Both of these metrics were significantly different between control and APC^{MIN} animals at Week 4, while only Shannon diversity was different at Week 8. Neither measure of α -diversity was altered between groups at Week 7 or Week 11. $N=8$ per group for Weeks 4-11, $n=10$ per group for Week 14.

communities were significantly different at each time-point, while the genotype explained between 21% and 15% of the difference between groups (Figure 4.3; PERMANOVA, $P < 0.001$).

Finally, we considered individual species differences between groups. The significantly different species are presented in Figure 4.4 (MWU-test with FDR adjustment, $p < 0.05$). At Week 4, eight taxa were decreased in APC^{MIN} mice and twelve were increased. The greatest changes were seen in *Eubacterium ventriosum*, which was 440-fold higher in relative abundance in WT mice, and in *Ruminiclostridium 6* which was 953-fold higher in APC^{MIN}, while *Christensenellaceae; Uncultured*, *Coriobacteriaceae UCG-002* and *Rhodospirillaceae; Uncultured* were present in APC^{MIN} mice but completely absent in WT. At Week 7, nine taxa were decreased and 26 were increased. *Faecalibaculum* was decreased 109-fold in APC^{MIN} mice while *Lachnospiraceae NC2004 Group* was completely absent from APC^{MIN} mice. *Anaeroplasma*, *Anaerovorax*, *Desulfovibrio* and *Eubacterium fissicatena group* were also common in APC^{MIN} mice despite being present in, at most, one member of the control group. At Week 11, eleven taxa were decreased in APC^{MIN} mice and 19 were increased. *Akkermansia* was 120-fold more abundant in WT mice, while *Lachnospiraceae NC2004 Group*, *Lachnospiraceae; Uncultured Bacterium* and *Papillibacter* were present in two or fewer APC^{MIN} mice. *Anaeroplasma* was 278-fold more abundant in APC^{MIN} while ten taxa were common in this group but present in two or fewer WT mice. At Week 14, seven taxa are decreased in APC^{MIN} mice while 17 are increased. *Lachnospiraceae NC2004 Group* is completely absent in APC^{MIN} mice while *Anaerovorax*, *Catenibacterium*, *Eubacterium ruminantium Group*, *Odoribacter* and *Ruminiclostridium 1* are present in, at most, one APC^{MIN} mouse.

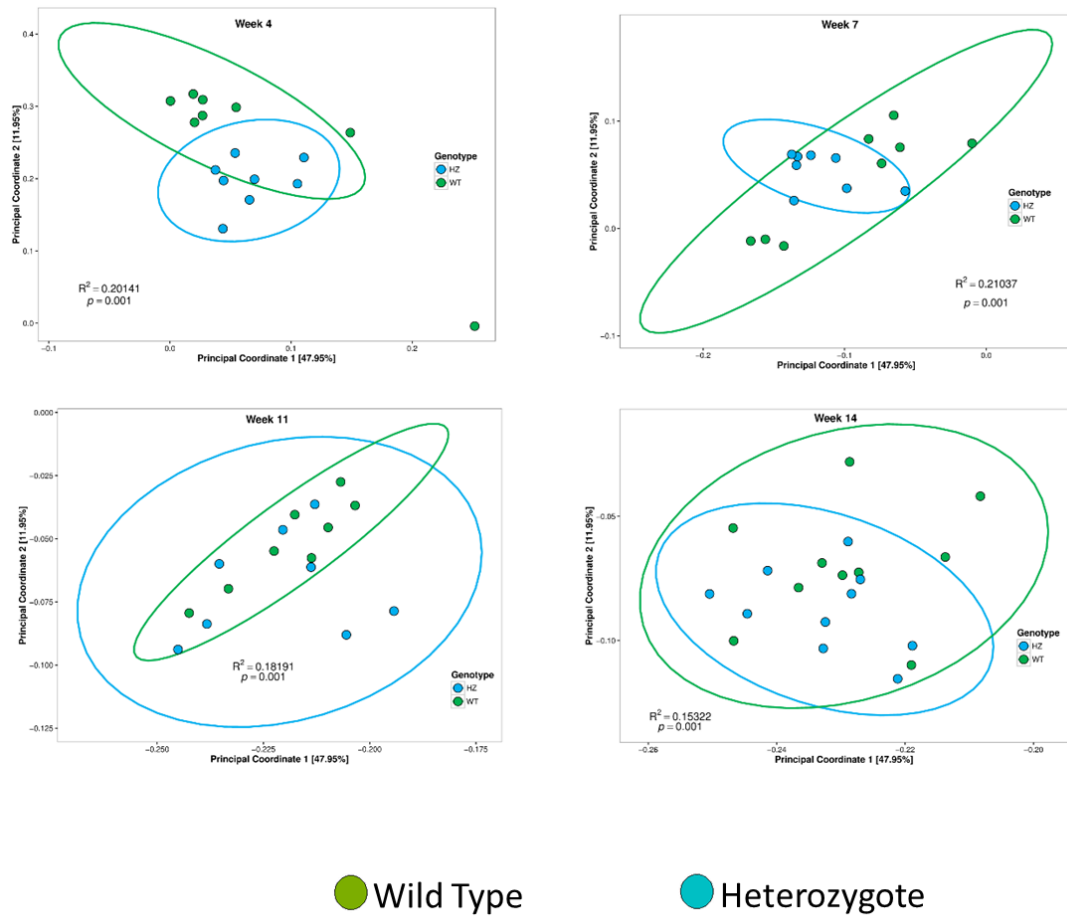
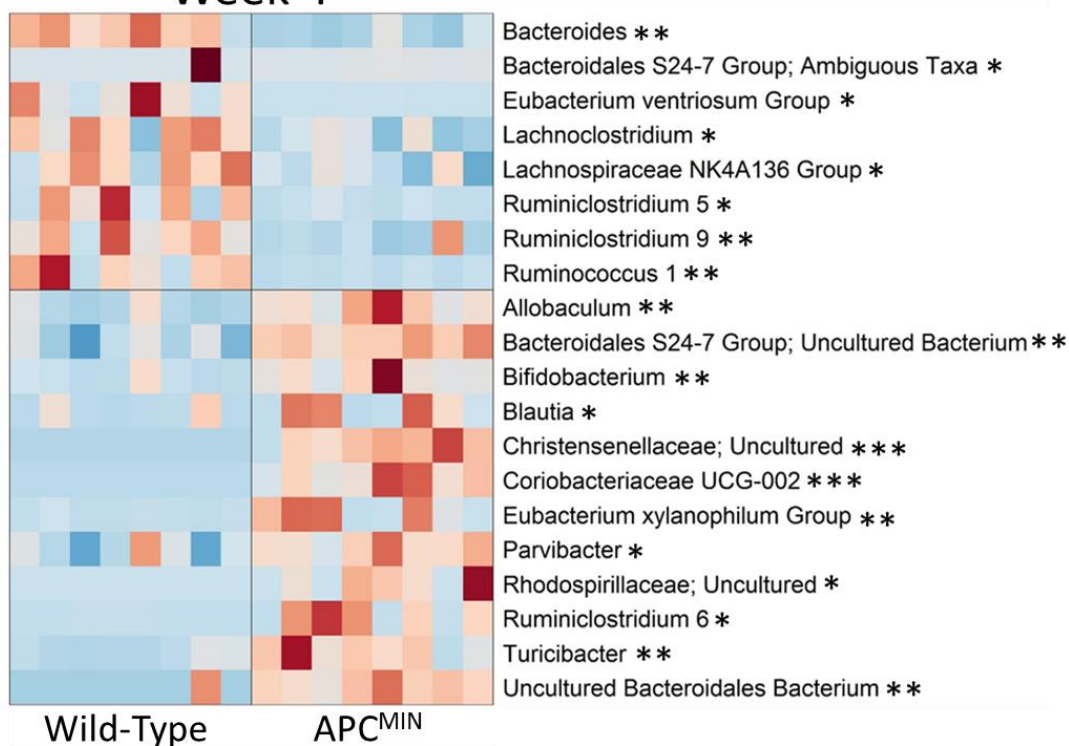
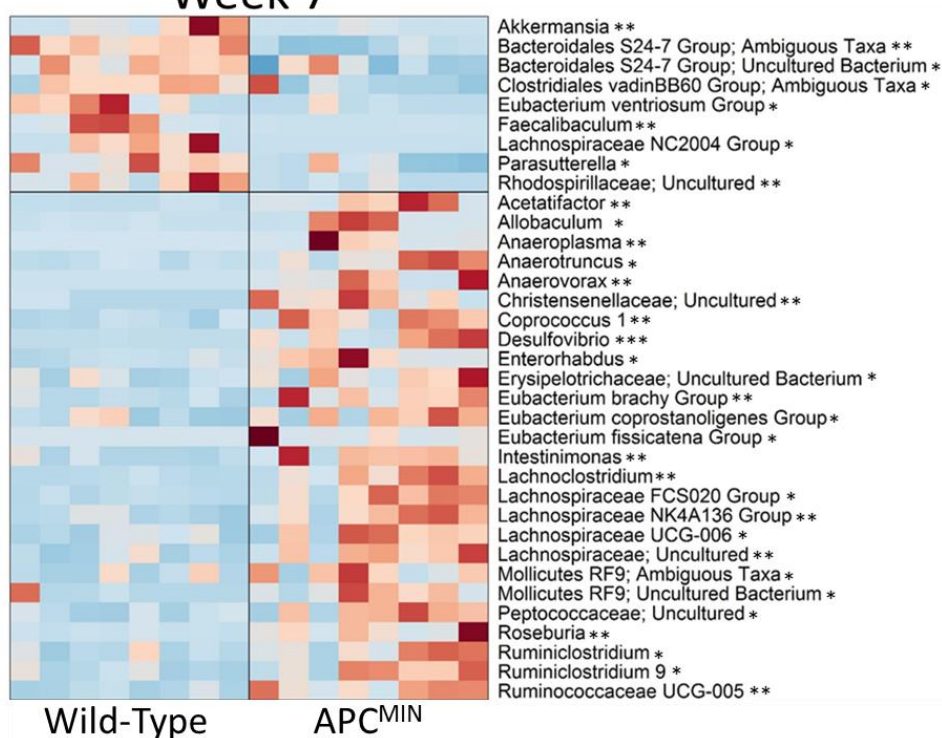


Figure 4.3: Beta (β)-diversity is significantly altered in APC^{MIN} throughout the experiment. We compared the microbial β -diversity between control and APC^{MIN} mice at each time-point by unweighted Unifrac distances, visualised by PCoA. This analysis revealed significant differences between the groups. Figure produced by Calum Walsh at Teagasc, Moorepark.

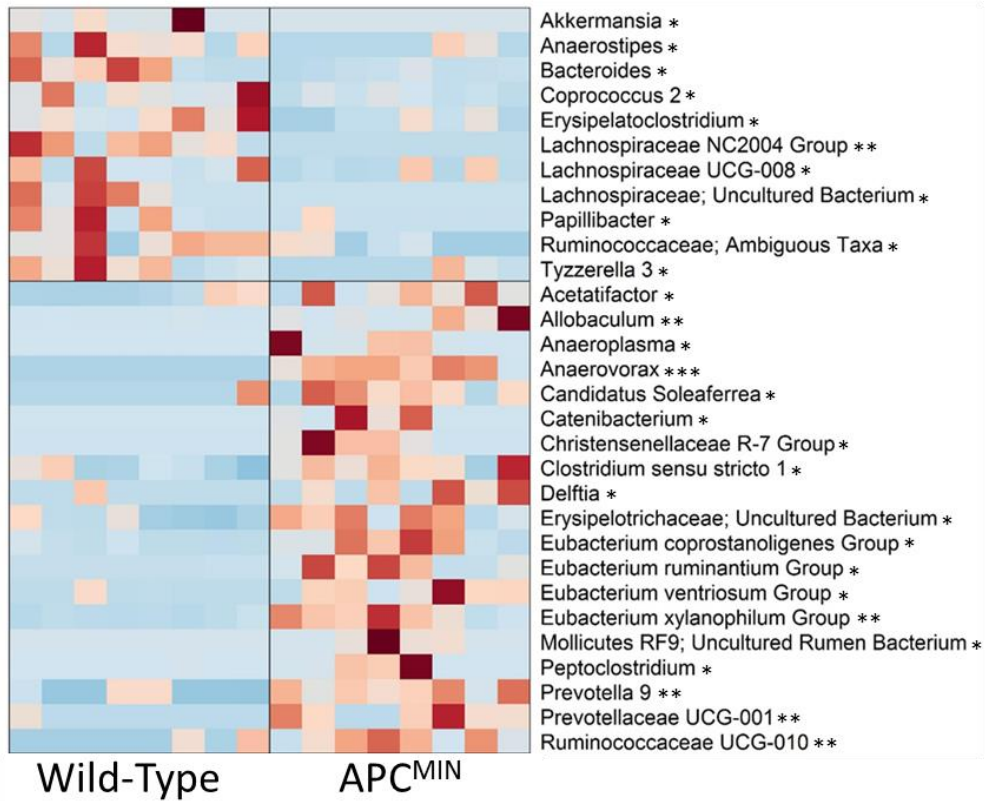
Week 4



Week 7



Week 11



Week 14

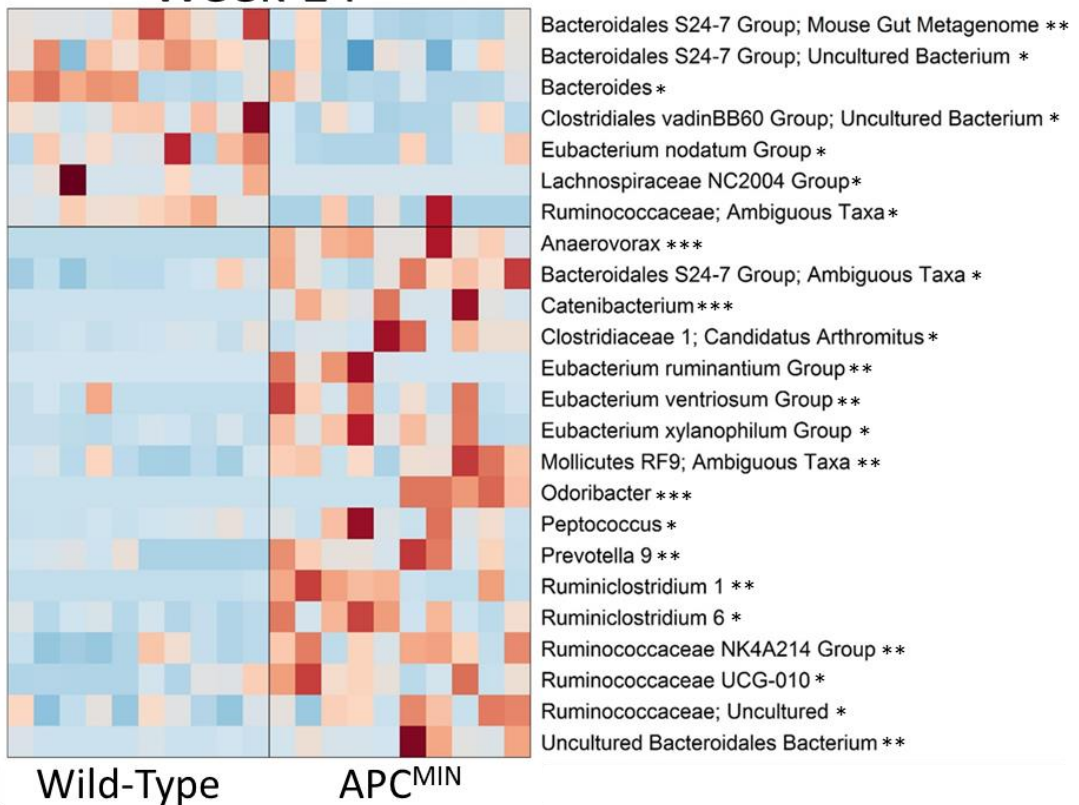


Figure 4.4: Taxa different significantly between groups. We compared the relative abundance that each taxon comprised in each group. This revealed a number of significantly different bacteria (MWU-test with FDR adjustment, $p < 0.05$). Only the significantly different OTUs are presented here. Taxa on the top of each figure are suppressed in APC^{MIN} mice while taxa in the bottom of each figure are enriched in APC^{MIN} mice. Data are presented as Z-scores normalised to the row average. $N=8$ per group for Weeks 4-11, $n=10$ per group for Week 14.

Temporal variations in the microbiota were also considered by highlighting bacteria which differed between APC^{MIN} and WT mice at more than one time-point across the study (MWU-test with FDR adjustment, $p < 0.05$). These taxa are presented in Figure 4.5.

In order to investigate the alterations in the gut bacteria at a higher structural level, we defined a set of bacterial co-abundance groups (CAGs) (Figure 4.6A) (28). These correlations were converted to a distance matrix on which PERMANOVA was performed which determined that all CAGs were significantly different (Figure 4.6B, $p < 0.05$). The composition of each CAG can be found in Table 4.1.

Eight CAGs were identified, labelled CAG1-8. The relative abundance of CAG1 is increased in APC^{MIN} mice at Week 4 and CAG3 is decreased (T-test, $p < 0.01$). CAGs 1 and 7 are decreased in APC^{MIN} mice at Week 7 while CAGs 3, 5, 6 and 8 are increased (T-test and MWU-test, $p < 0.05$). At Week 11, only CAG4 is decreased in APC^{MIN} mice (T-test, $p = 0.02$), while at Week 14 CAGs 1 and 4 are decreased and CAGs 7 and 8 are increased (T-test, $p < 0.05$). Considering CAGs that differ at more than one time-point, CAG1 is increased in APC^{MIN} mice at Week 4, before decreasing in abundance in two later samples. CAG3 is more abundant in WT mice at Week 4 and APC^{MIN} at Week 7, while CAG7 displays the opposite pattern. CAGs 4 and 8 differ at two time-points each but are consistently increased in control group for the former and in the APC^{MIN} group for the latter. CAGs 5 and 6 only differ at Week 7 where they are increased in APC^{MIN}, while CAG2 does not differ at any time-point.

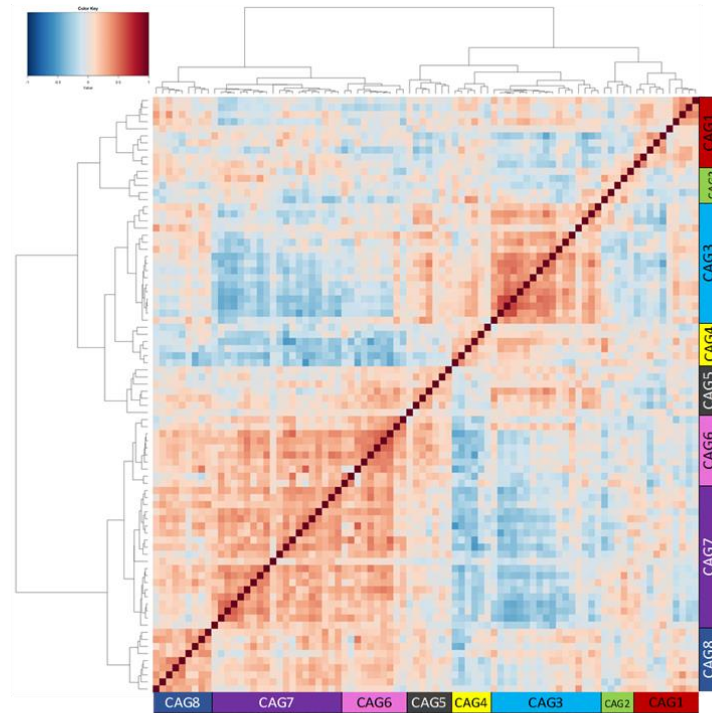
CAG1 is dominated by *Bacteroidales S24-7 group; uncultured bacterium* which makes up more than 90% of this CAG in both groups at each time-point. CAG2 features only five species, with *Eubacterium ventriosum group*, *Clostridiales vadinBB60 group*; *Ambiguous taxa* and *Marvinbryantia* most prevalent. CAG3 contains 17 species. *Lachnospiraceae NK4A136*

A)	Week 4	Week 7	Week 11	Week 14
Christensenellaceae; Uncultured	***	**		
Eubacterium xylanophilum Group	**		**	*
Ruminiclostridium 6	*			*
Uncultured Bacteroidales Bacterium	**			**
Acetatifactor		**	*	
Anaeroplasma		**	*	
Erysipelotrichaceae; Uncultured Bacterium		*	*	
Eubacterium coprostanoligenes Group		*	*	
Anaerovorax		**	***	***
Mollicutes RF9; Ambiguous Taxa		*		**
Ruminococcaceae UCG-010			**	*
Catenibacterium			*	***
Eubacterium ruminantium Group			*	**
Prevotella 9			**	**
Bacteroides	**		*	*
Akkermansia		**	*	
Ruminococcaceae; Ambiguous Taxa			*	*
Lachnospiraceae NC2004 Group		*	**	*

B)	Week 4	Week 7	Week 11	Week 14
Bacteroidales S24-7 Group; Uncultured Bacterium	**	*		*
Rhodospirillaceae; Uncultured	*	**		
Allobaculum	**	*	**	
Lachnoclostridium	*	**		
Lachnospiraceae NK4A136 Group	*	**		
Ruminiclostridium 9	**	*		
Bacteroidales S24-7 Group; Ambiguous Taxa	*	**		*
Eubacterium ventriosum Group	*	*	*	**

Figure 4.5: Taxa which differ at more than one time-point. Understanding the temporal variations of the microbiota over the course of tumorigenesis was a key objective of our study, and identifying taxa which differed at more than one time-point was an important part of this. **A)** Taxa that are enriched at more than one time-point in one group. Taxa enriched in APC^{MIN} mice are marked in red and taxa enriched in wild-type controls are marked in blue. **B)** Taxa that differ at more than one time-point in different groups. Taxa enriched in APC^{MIN} mice are marked in red and taxa enriched in wild-type controls are marked in blue. *N*=8 per group for Weeks 4-11, *n*=10 per group for Week 14.

A)



B)

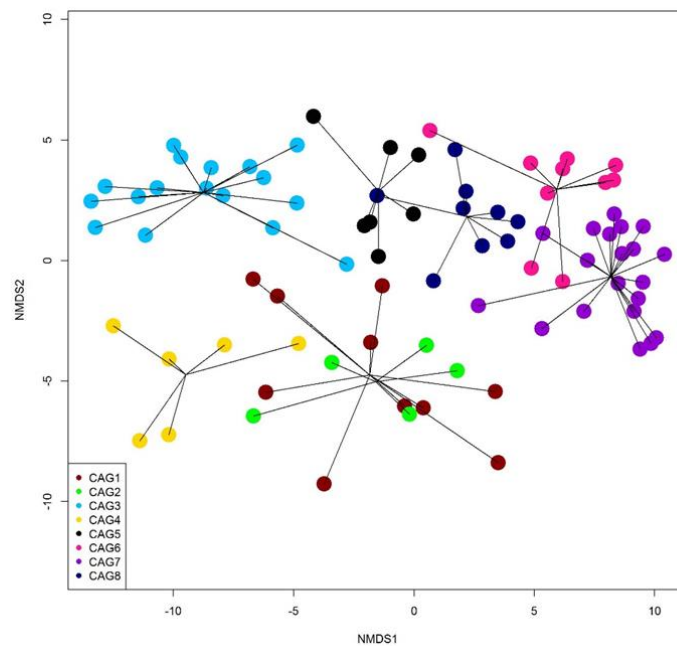


Figure 4.6: Generation of co-abundance groups. The Kendall correlations of OTUs' relative abundance were generated and clustered by Ward-linkage and Pearson correlation. This defines "co-abundance groups" (CAGs) which describe higher-level structures of the microbiota by highlighting taxa for which changes in abundance correlate. These Kendall correlations were then converted to a distance metric and analysed by PERMANOVA which determined that each CAG was significantly different. **A)** A heatplot of Kendall correlation values for each pair of taxa clustered by Ward linkage of their Pearson correlations. Coloured bars along the axes denote the CAGs. The composition of each CAG is presented in Table 4.1. **B)** Non-metric multidimensional scaling (NMDS) of the distance metrics of the CAGs' correlation values.

Table 4.1: CAG Composition

CAG1	Ruminococcaceae UCG-013	CAG6	Ruminococcaceae; Ambiguous taxa
	[Eubacterium] nodatum group		Coprococcus 1
	Clostridiales Family XIII; AD3011 group		Desulfovibrio
	Parvibacter		Enterorhabdus
	Escherichia-Shigella		Roseburia
	Bacteroidales S24-7 group; uncultured bacterium		Clostridiales Family XIII UCG-001
	Coriobacteriaceae UCG-002		Lachnospiraceae UCG-001
CAG2	Parasutterella	Peptococcus	
	Turicibacter	Tyzzerella 3	
	Bifidobacterium	Gastranaerophilales; uncultured bacterium	
	[Eubacterium] ventriosum group	Prevotella 9	
	Staphylococcus	Coriobacteriaceae; uncultured	
CAG3	Clostridiales vadinBB60 group; Ambiguous taxa	Christensenellaceae; uncultured	
	Marvinbryantia	Candidatus Saccharimonas	
	Lactococcus	Clostridium sensu stricto 1	
	Ruminococcaceae UCG-005	Ruminococcaceae UCG-010	
	Lachnoclostridium	Ruminococcaceae NK4A214 group	
	Ruminiclostridium 5	Anaerostipes	
	Ruminococcus 1	Bacteroidales S24-7 group; mouse gut metagenome	
	Peptococcaceae; uncultured	Erysipelatoclostridium	
	Acetatifactor	Delftia	
	Ruminococcaceae; uncultured	Candidatus Arthromitus	
	Ruminiclostridium 9	Lactobacillus	
	Lachnospiraceae NK4A136 group	Streptococcus	
	Oscillibacter	Erysipelotrichaceae; uncultured	
	Lachnospiraceae FCS020 group	Faecalibaculum	
	Ruminococcaceae UCG-009	Rhodospirillaceae; uncultured	
	Intestinimonas	Alistipes	
	Ruminiclostridium	Bacteroidales S24-7 group; Ambiguous taxa	
Anaerotruncus	Ochrobactrum		
CAG4	Ruminococcaceae UCG-003	CAG7	Ruminiclostridium 6
	Lachnospiraceae UCG-006		Anaerovorax
	Blautia		[Eubacterium] xylanophilum group
	Uncultured Bacteroidales bacterium		Anaeroplasm
	Allobaculum		Mollicutes RF9 uncultured bacterium
	Clostridiales vadinBB60 group; uncultured bacterium		Erysipelotrichaceae; uncultured bacterium
	Akkermansia		Ruminococcaceae UCG-014
CAG5	Bacteroides	CAG8	[Eubacterium] coprostanoligenes group
	Tyzzerella		Mollicutes RF9; Ambiguous taxa
	Coprococcus 2		
	Lachnospiraceae UCG-008		
	Lachnospiraceae; uncultured		
CAG6	[Eubacterium] brachy group		
	Butyricicoccus		
	Anaerobacillus		

group comprises >50% of the abundance of this CAG in both groups at each time-point. CAG4 contains only six species, and *Clostridiales vadinBB60 group*; *uncultured bacterium*, *Akkermansia* and *Bacteroides* predominate at different time-points. *Lachnospiraceae*; *uncultured* comprises >80% of CAG5 in each sample. CAG6 is a diverse group, with *Coprococcus 1*, *Roseburia* and *Lachnospiraceae UCG-001* predominating at different times. Likewise, *Alistipes*, *Bacteroidales S24-7 group*; *Ambiguous taxa* and *Lactobacillus* are the most common taxon in CAG7 in different samples. *Ruminococcaceae UCG-014* is consistently the highest abundance taxon in CAG8.

4.2.3 Bile acid composition in APC^{MIN} mice

In order to investigate the potential role of microbial bile acid modification in our mouse model, we analysed the faecal and plasma bile acid profile of each group at Week 14 by UPLC-MS. After correction for multiple comparisons, only the plasma concentration of LCA differed between groups (Figure 4.7; T-test, $p < 0.001$), and this comprised less than 1% of the total plasma bile acid pool. The list of bile acids measured is presented in Table 4.2. Total bile acid concentration was not significantly different between groups (T-test, $P > 0.05$), and there were no differences among the faecal bile acids (T-test and MWU-test, $P > 0.05$).

4.2.4 Cell proliferation is not increased in APC^{MIN} mice

Loss of cell cycle control in APC^{MIN} mice has been shown to precede the development of intestinal polyps (5). In order to determine how far this loss of control extends, we measured the degree of proliferation in normal villi in the ileum of APC^{MIN} and control mice at our end-point at Week 14. The expression of proliferation indicator Ki67 did not differ between groups (Figure 4.8; T-test, $p = 0.66$).

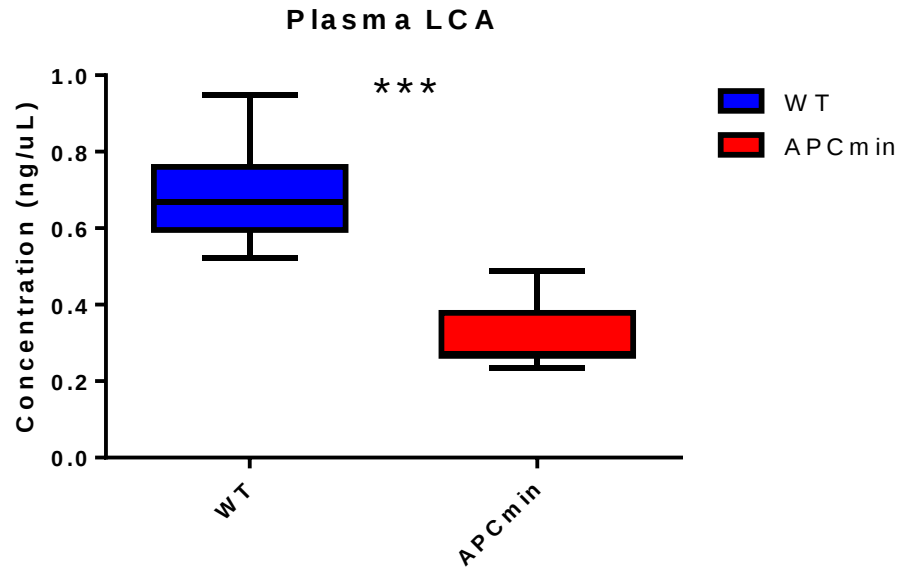


Figure 4.7: Faecal and plasma bile acid composition is largely unchanged in APC^{MIN} mice. Microbial metabolism of bile acids has been implicated in the pathogenesis of colon cancer. To investigate the role bile acids play in our model, we measured the concentration of faecal and plasma bile acids at Week 14 by UPLC-MS. After correction for multiple comparisons, only the concentration of LCA in the plasma was significantly different between groups (T-test and MWU-test with FDR adjustment, $p < 0.05$). Each bile acid measured is presented in Table 4.2. $N=6$ for wild-type, $n=7$ for APC^{MIN}.

Table 4.2: Bile acids measured

	Bile Acid	Abbreviation
Bile Acids	Dehydrocholic Acid	DHCA
	Lithocholic Acid	LCA
	Ursodeoxycholic Acid	UDCA
	Chenodeoxycholic Acid	CDCA
	Hyodeoxycholic Acid	HDCA
	Deoxycholic Acid	DCA
	Cholic Acid	CA
	Hyocholic Acid	HCA
	7-Ketolithocholic Acid	7-Keto-LCA
	Tauro-Cholic Acid	T-CA
	Tauro-Hyocholic Acid	T-HCA
	Tauro-Chenodeoxycholic Acid	T-CDCA
	Tauro-Ursodeoxycholic Acid	T-UDCA
	Tauro-Deoxycholic Acid	T-DCA
	Tauro-Hyodeoxycholic Acid	T-HDCA
	Tauro-Lithocholic Acid	T-LCA
	Glyco-Cholic Acid	G-CA
	Glyco-Hyocholic Acid	G-HCA
	Glyco-Chenodeoxycholic Acid	G-CDCA
	Glyco-Ursodeoxycholic Acid	G-UDCA
	Glyco-Deoxycholic Acid	G-DCA
	Glyco-Hyodeoxycholic Acid	G-HDCA
	Glyco-Lithocholic Acid	G-LCA
	Beta (β)-Muricholic Acid	B-MCA
	Alpha/gamma (α/ω)-Muricholic Acid	α/ω -MCA
	Murocholic Acid	MoCA
	Tauro α -, β -, and ω -Muricholic Acid	T- $\alpha/\beta/\omega$ -MCA
	Total Bile Acids	Total BAs

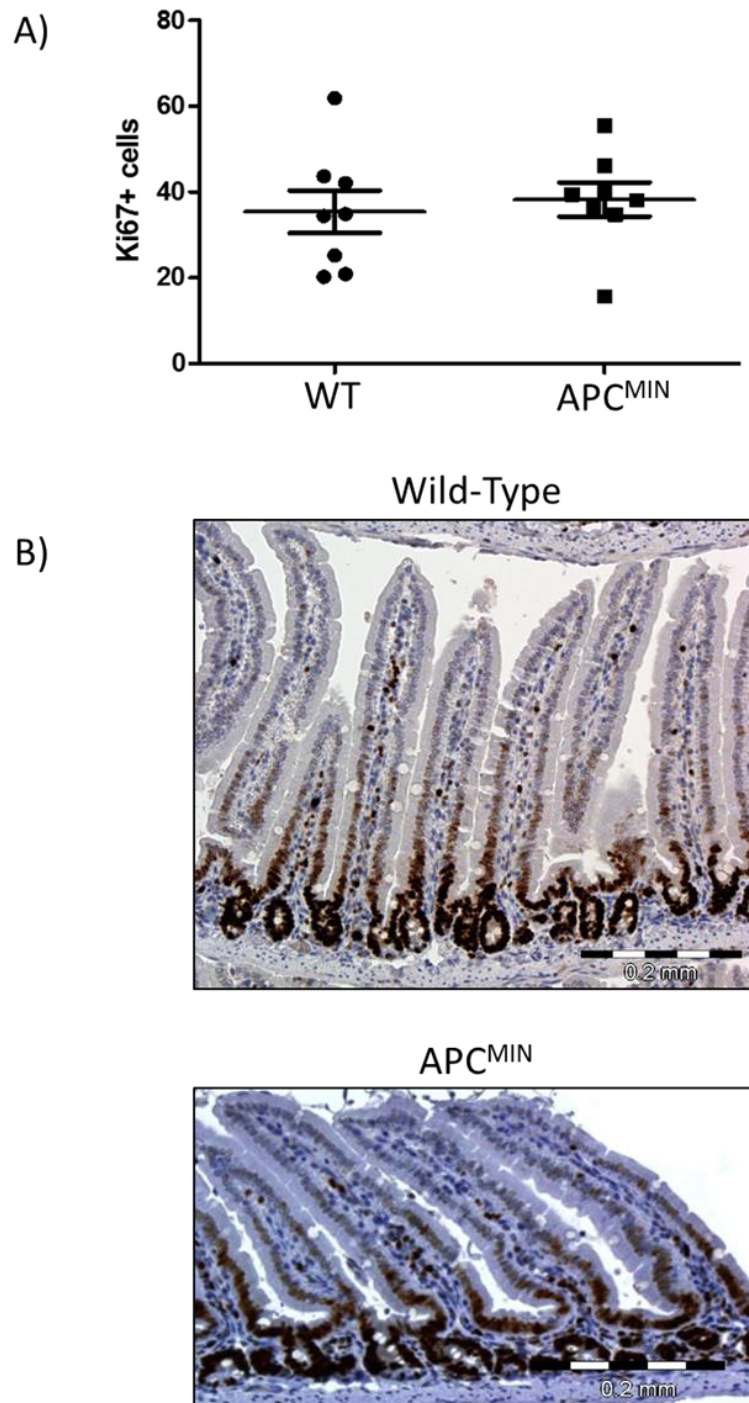


Figure 4.8: Proliferation in normal villi did not differ between groups. Mutation in the tumour suppressor *Apc* gene can lead to constitutive Wnt pathway activation and aberrant cell proliferation. In order to investigate if proliferation is up-regulated in the normal-appearing villi in the small intestine of APC^{MIN} mice prior to the development of polyps, we stained the epithelium for Ki67 and determined the percentage of Ki67+ cells per crypt-villus unit for ten such units per mouse. **A)** There was no significant difference in Ki67 expression in the normal villi of APC^{MIN} mice, which suggests that dysregulated proliferation is not ubiquitous in the small intestine of these mice (T-test, $P=0.66$). **B)** Sections of the distal ileum of wild-type and APC^{MIN} stained for Ki67 on a background of haematoxylin. Magnification 200x.

4.2.5 Weight gain was not affected in APC^{MIN} mice

Cancer-associated weight loss, or cachexia, is an indicator of severe disease in human cancer that is also observed in late-stage APC^{MIN} mice. We tracked the body weight of our mice at weekly intervals throughout the study and measured the mass of their mesenteric and epididymal fat after sacrifice at Week 14. There were no differences in body weight or either type of fat over the course of the study (Figure 4.9; 2-way ANOVA and T-tests, $P>0.05$).

4.2.6 Cytokine and chemokine gene transcription in in APC^{MIN} mice

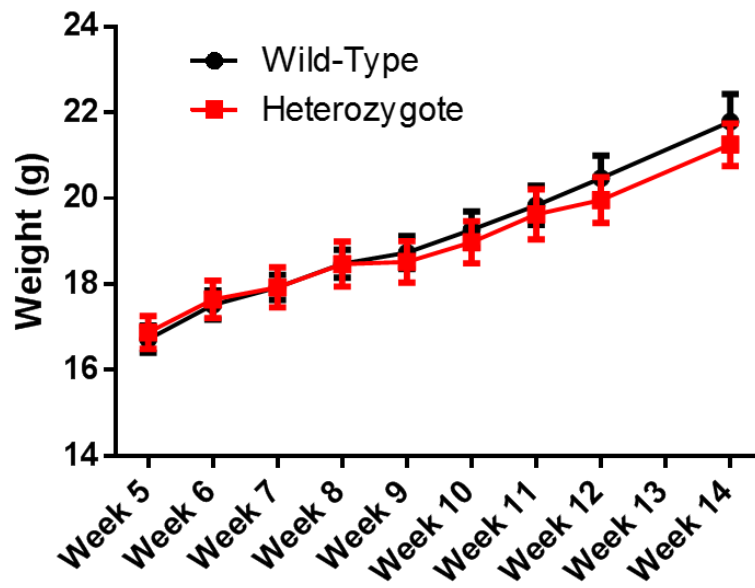
Inflammation is a risk factor for colon cancer and the tumour microenvironment can modulate inflammatory conditions to promote tumour growth (22, 29). As such, we measured the transcription of several cytokines in the distal small intestine by qPCR, as well as measuring the spleen weight and the length of the small and large intestine, which can be used as indicators in intestinal inflammation (Figure 4.10).

The relative transcription of *Il10* was significantly decreased in APC^{MIN} mice (Figure 4.10; T-test, $p=0.045$), while their spleen weight was increased (Figure 4.10; T-test, $p=0.006$). There were no significant differences between relative transcription of *Il16*, *Cxcl1*, *Cxcl5*, *Tgfb* between groups at Week 14 (Figure 4.10; T-test and MWU-test, $p>0.05$).

4.2.7 The transcription of FXR target genes is decreased in the ileum of APC^{MIN} mice

Bile acid receptor FXR regulates the transcription of a number of genes in the ileum and has also been implicated in protection from CRC (11, 30). In order to determine if bile acid signalling is affected in our model, we measured the transcription of four FXR target genes (Figure 4.11).

A)



B)

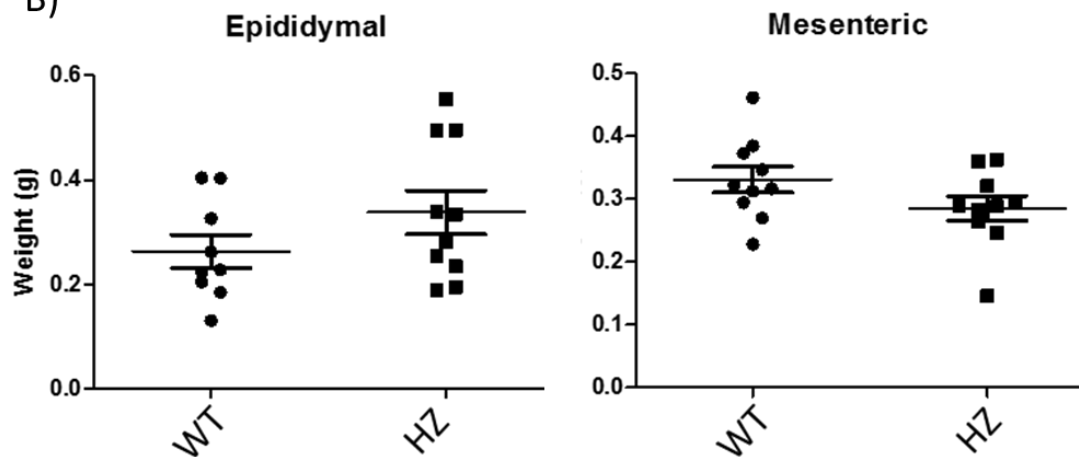


Figure 4.9: Neither body weight nor body fat differed between groups. Weight loss or inhibited weight gain is a sign of severe pathology in human cancer and mouse models of cancer. We tracked the body weight of our mice every week from 5 weeks of age (**A**) and recorded the mass of their mesenteric and epididymal fat after cull (**B**), neither of which differed between groups (Two-way ANOVA and T-tests, $P > 0.05$).

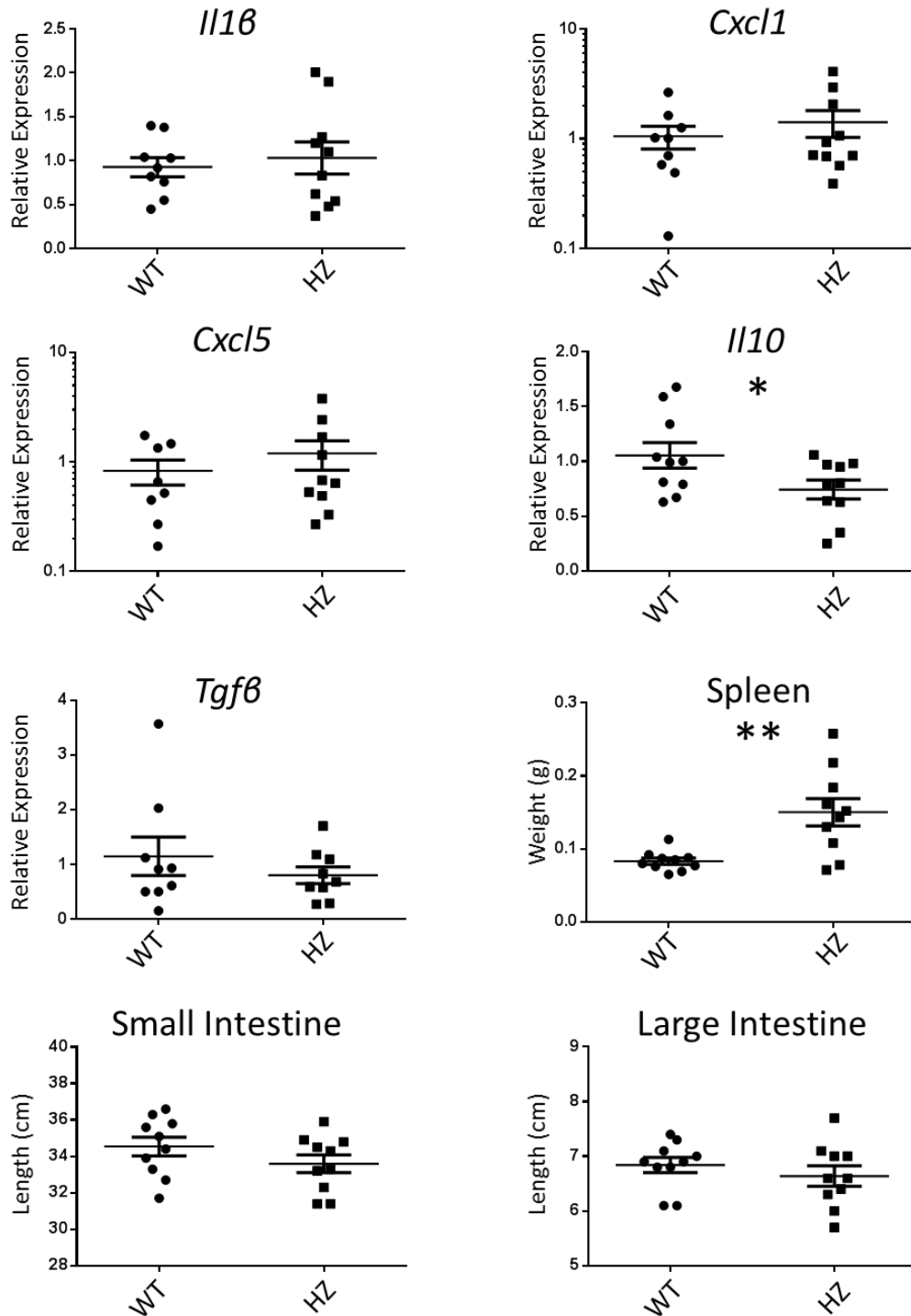


Figure 4.10: Only minor changes in the inflammatory environment are observed in APC^{MIN} mice. Chronic inflammation is a risk factor for colorectal cancer and inflammation in the tumour microenvironment can drive cancer progression. In order to establish if inflammation played a role in the pathogenesis of our model, we extracted RNA from the distal small intestine, the primary site of polyposis in APC^{MIN} mice, and performed qPCR for several cytokines. We also measured spleen weight and the length of the small and large intestine, as inflammation can lead to a shortening of these organs. The expression of IL10 was decreased in APC^{MIN} mice, while their spleen weight was increased. No changes were observed in any other measure.

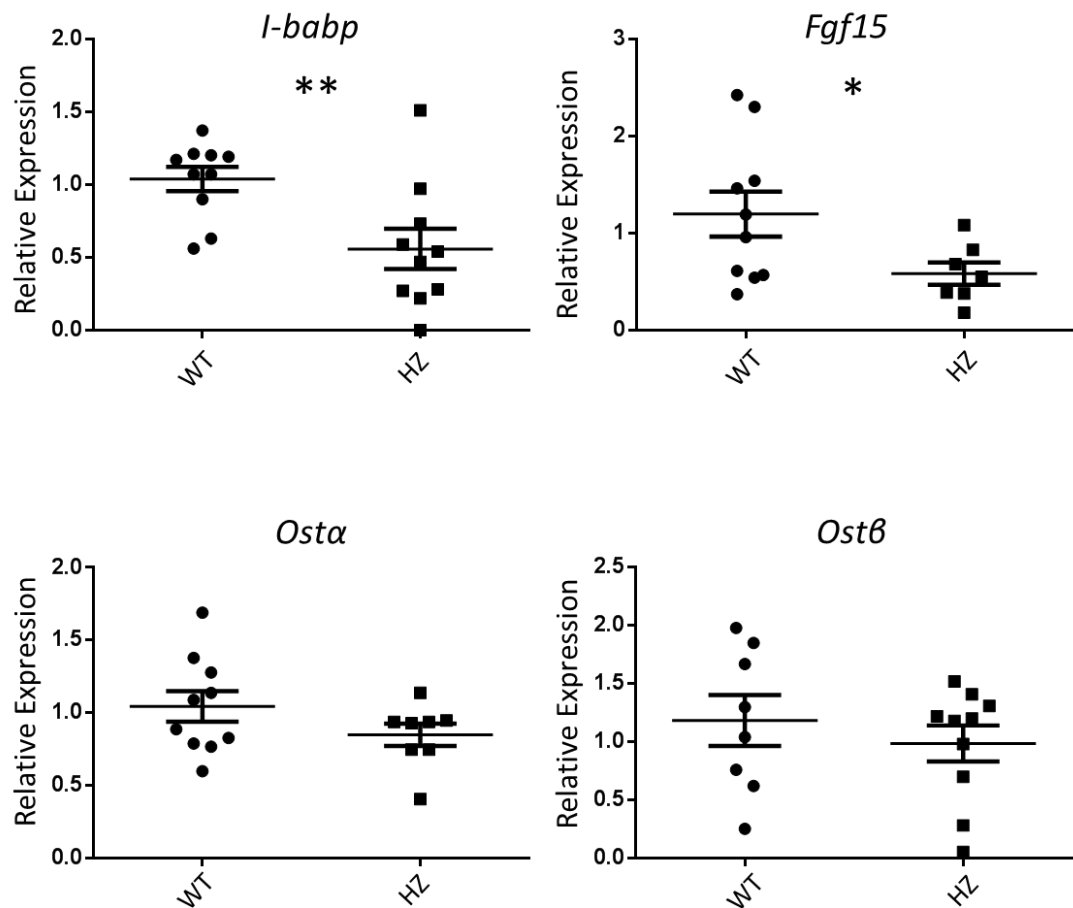


Figure 4.11: Transcription of FXR target genes is decreased in the ileum of APC^{MIN} mice at Week 14. FXR signalling has been shown to be protective against CRC. We measured the expression of four FXR target genes in the distal ileum of control and HZ mice by qPCR. Significantly lower expression of *I-babp* and *Fgf15* was detected in APC^{MIN} mice relative to WT animals, indicating reduced FXR signalling (T-test, $p < 0.05$).

The transcription of *I-babp* and *Fgf15* was significantly suppressed in our CRC model (Figure 4.11; T-tests, $p < 0.05$), indicative of reduced engagement of FXR. The transcription of the enterocyte basolateral bile acid transporters *Ost α* and *Ost β* was marginally reduced but not significantly different between groups (Figure 4.11; T-tests, $p > 0.05$).

4.2.8 Correlation analysis reveals a possible relationship between APC^{MIN} polyps and both FXR and Wnt signalling

We ran correlation analyses between polyp numbers, α -diversity indices, CAG relative abundances, faecal and plasma bile concentrations, and the relative transcription of our cytokines and FXR target genes within each group, in order to reveal any interactions between these parameters in the context of CRC. The significant correlations, after FDR correction for multiple comparisons, are presented in Figure 4.12.

The number of polyps in the middle and distal portions of the small intestine in our APC^{MIN} mice correlated negatively with FXR target genes *Ost α* (Figure 4.12; Pearson's $R^2 = -0.8$ and -0.79 , $p < 0.001$) and *Ost β* (Figure 4.12; Pearson's $R^2 = -0.9$ and -0.91 , $p < 0.001$). *Il10* also correlated positively with *Ost β* in APC^{MIN} mice (Figure 4.12; Pearson's $R^2 = 0.88$, $p < 0.05$), while the plasma concentration of two tauro-conjugated bile acids, tauro-hyodeoxycholic (T-HDCA) acid and T- $\alpha/\beta/\omega$ -MCA, correlated negatively with FXR target gene *Fgf15* (Figure 4.12; Spearman's $Rho = -1$, $p < 0.001$). T-MCAs act as antagonists to FXR but our analysis cannot distinguish between the α -, β - and ω -isomers.

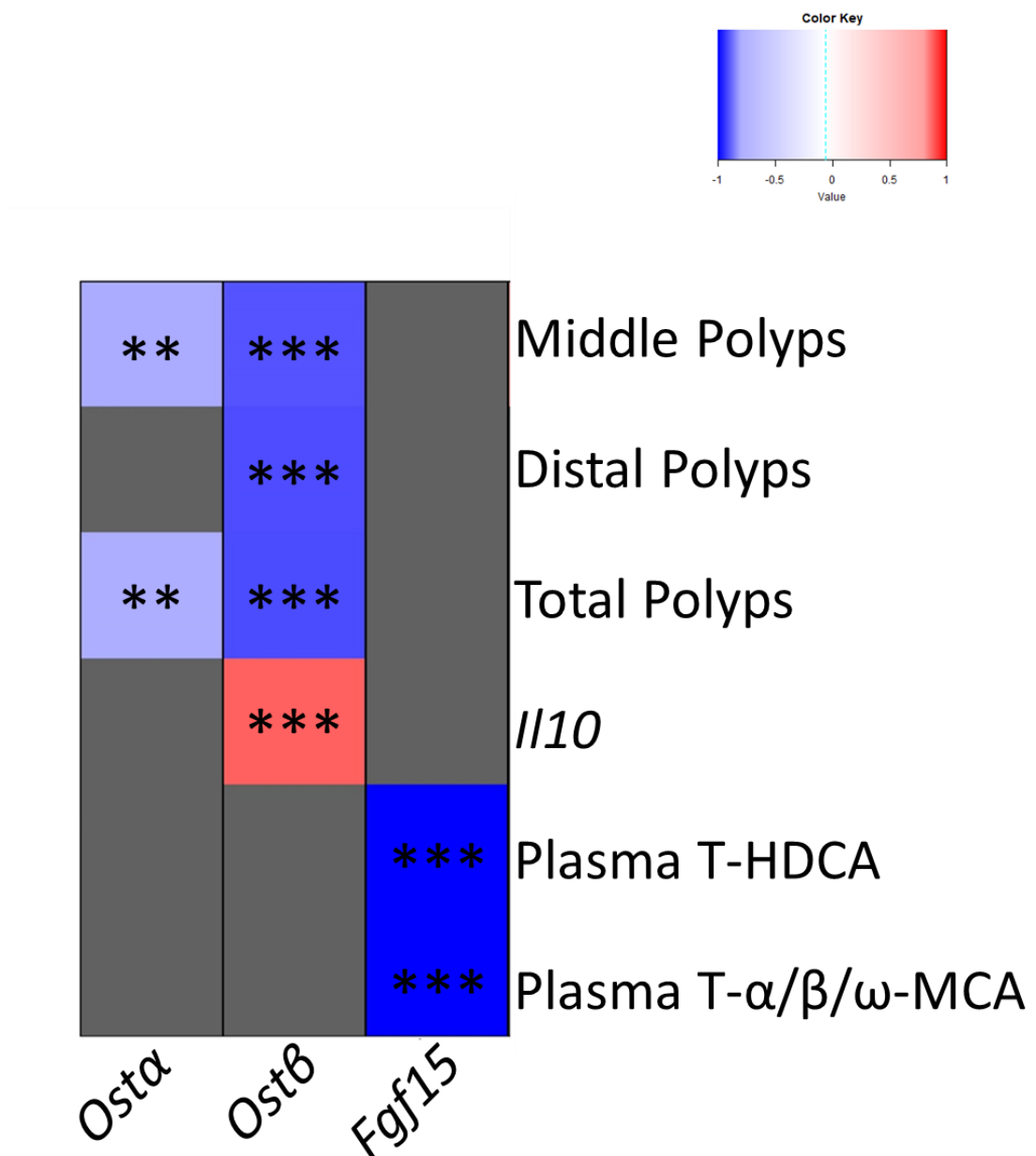


Figure 4.12: Correlation analysis reveals a relationship between polyp multiplicity and FXR target genes. The gut presents a complex milieu of interacting forces. In order to examine possible interactions between different aspects of our data in this environment, we performed a correlation analysis between several datapoints collected in our study. After correction for multiple comparisons, this revealed a strong negative correlation between our FXR target genes and polyp number. *Il10* also correlated positively with FXR target gene *Ost β* , while *Fgf15* expression correlated negatively with the plasma concentrations of T-HDCA and FXR-antagonist T-MCA (Pearson's R and Spearman's R^2 , $p < 0.05$ after FDR adjustment).

4.2.9 PICRUSt predicts a significant increase in bile acid-metabolising genes in the faecal microbiota of APC^{MIN} mice at Week 7

We investigated the faecal microbial metagenomic function content of control and APC^{MIN} animals by applying PICRUSt, a software package which predicts whole microbial gene content from 16S rRNA gene sequencing data. This revealed a significant reduction in the α -diversity of the metagenome predicted by PICRUSt in APC^{MIN} mice at Week 7 (Figure 4.13; MWU-test, $p=0.002$). The β -diversities of the predicted metagenomes were also significantly different between genotypes at Weeks 4 and 7 (Figure 4.13; PERMANOVA, $p<0.05$).

In order to assess bile acid metabolism, the relative abundances of *baiCD* (COG1902), a gene cluster involved in the 7 α -dehydroxylation of bile acids into secondary bile acids, and *bsh* (COG3049), the enzyme responsible for cleaving the amino acid conjugate from bile acids, were examined. These were significantly increased in their predicted abundance in APC^{MIN} mice at Week 7 (Figure 4.14; MWU-test, $p<0.05$), but not at Week 7, 11 or 14 (Figure 4.14; T-test, $p>0.05$).

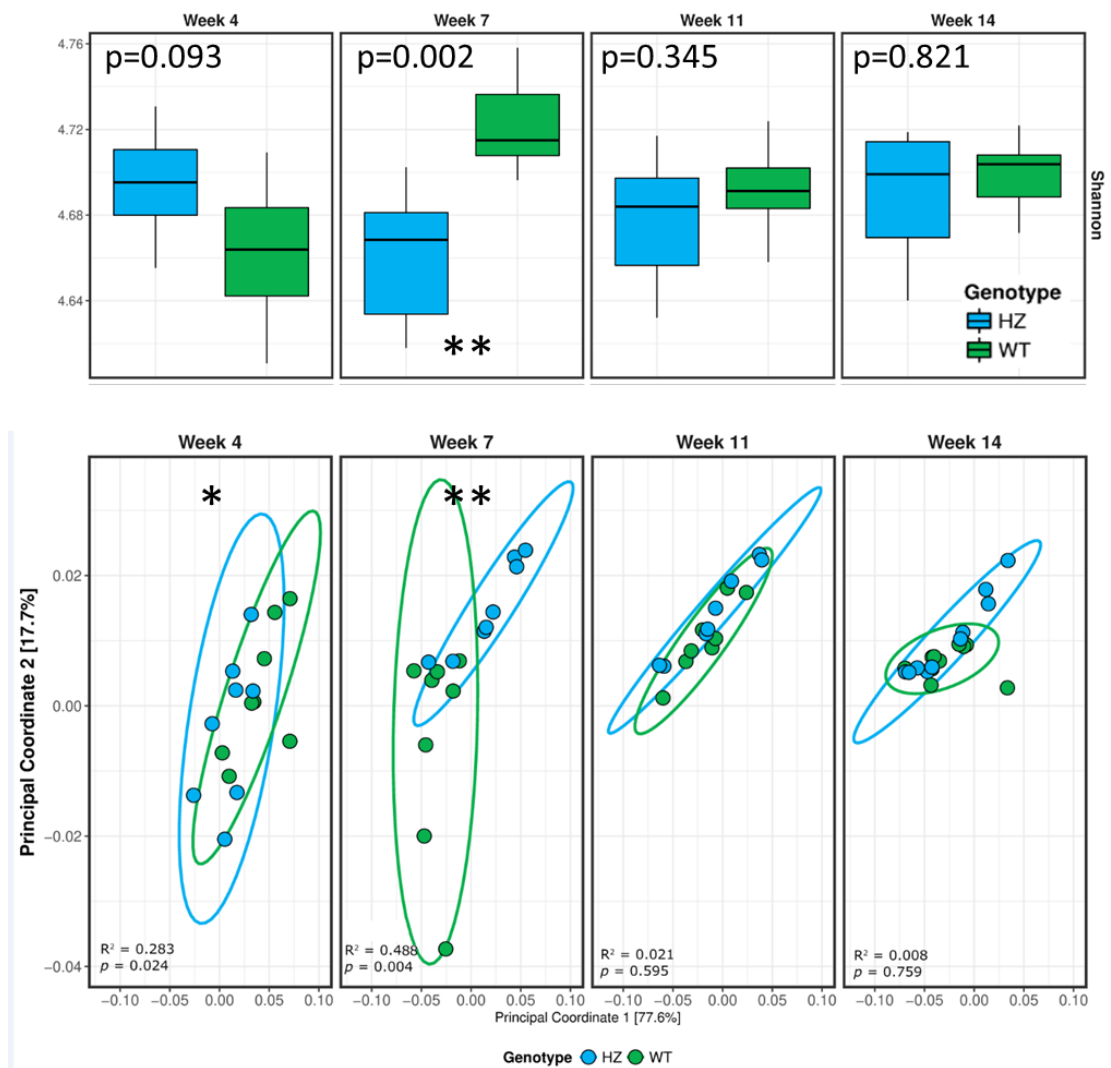


Figure 4.13: A- and β -diversity of the metagenome predicted by PICRUSt is altered in APC^{MIN} mice. We calculated the α - and β -diversity of the metagenomes predicted by PICRUSt and found that these measures are significantly different in APC^{MIN} mice. **A)** The Shannon diversity index of the metagenomes predicted by PICRUSt differed significantly between wild-type (WT) and APC^{MIN} (HZ) mice at Week 7 (MWU-test). **B)** The β -diversity of the metagenomes predicted by PICRUSt differed significantly between WT and HZ mice at Week 4 and 7 (PERMANOVA). Figure produced by Calum Walsh at Teagasc, Moorepark.

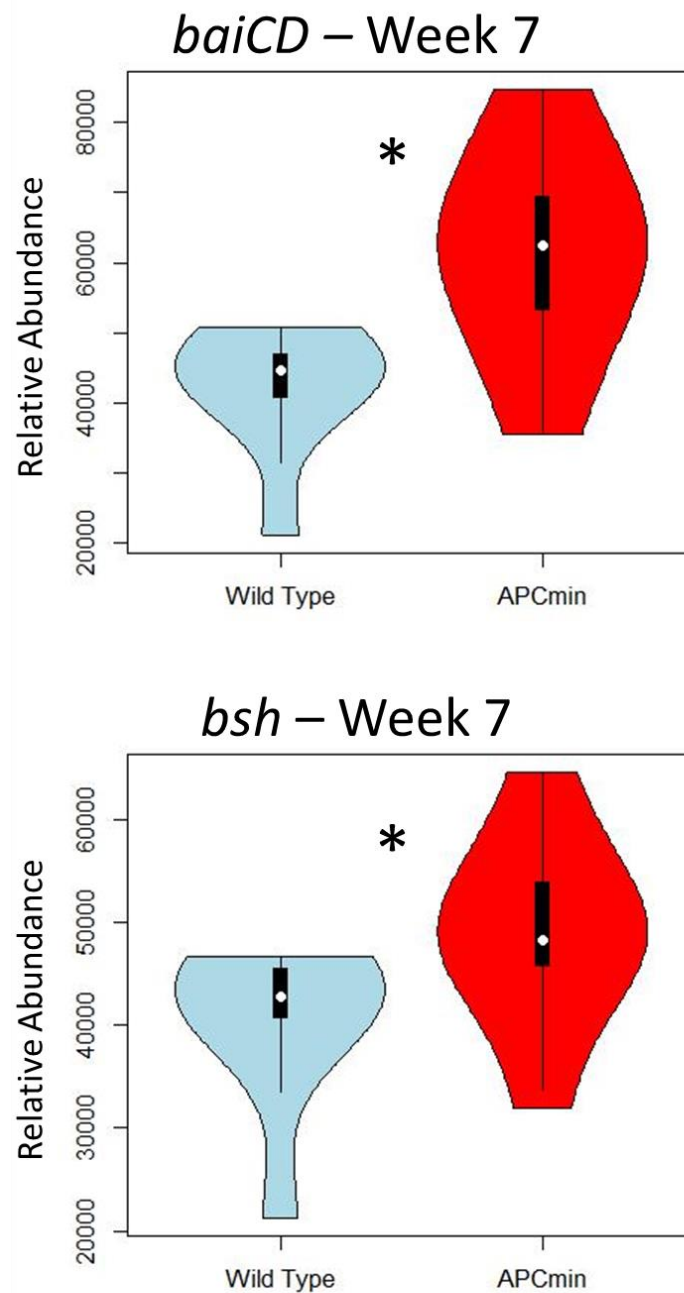


Figure 4.14: Primary and secondary bile acid metabolism gene content predicted by PICRUSt is significantly different between treatments at Week 7 only. We used PICRUSt to predict the gene content of the faecal microbiota in both our groups and compared the abundance of *baiCD* (COG1902), a gene cluster involved in the 7 α -dehydroxylation of bile acids into secondary bile acids, and *bsh* (COG3049), the enzyme responsible for cleaving the amino acid conjugate from bile acids. Their predicted abundances were increased in APC^{MIN} mice at Week 7 but not at any other time (MWU-test, $p < 0.05$). $N=8$ per group for Weeks 4-11, $n=10$ per group for Week 14.

4.3 Discussion

The microbiota is altered in human CRC, but the degree to which the host affects the microbiota and the microbiota alters the host is unclear (31). It is clear that an intricate dialogue exists between the microbiota and the host which is important in many disease states, particularly in cases of IBD, *C. difficile* and pathogen infections, and the evidence points to altered microbiota in the context of CRC. It is possible that the gut microbiota can both promote or inhibit tumour initiation, and evidence for this has been provided by animal studies (2, 3). However, it is also possible that tumorigenesis occurs entirely as a result of host processes independent of the microbiota. Similarly, after cancer initiation, components of the microbiota may suppress or promote growth, invasion and metastasis (32).

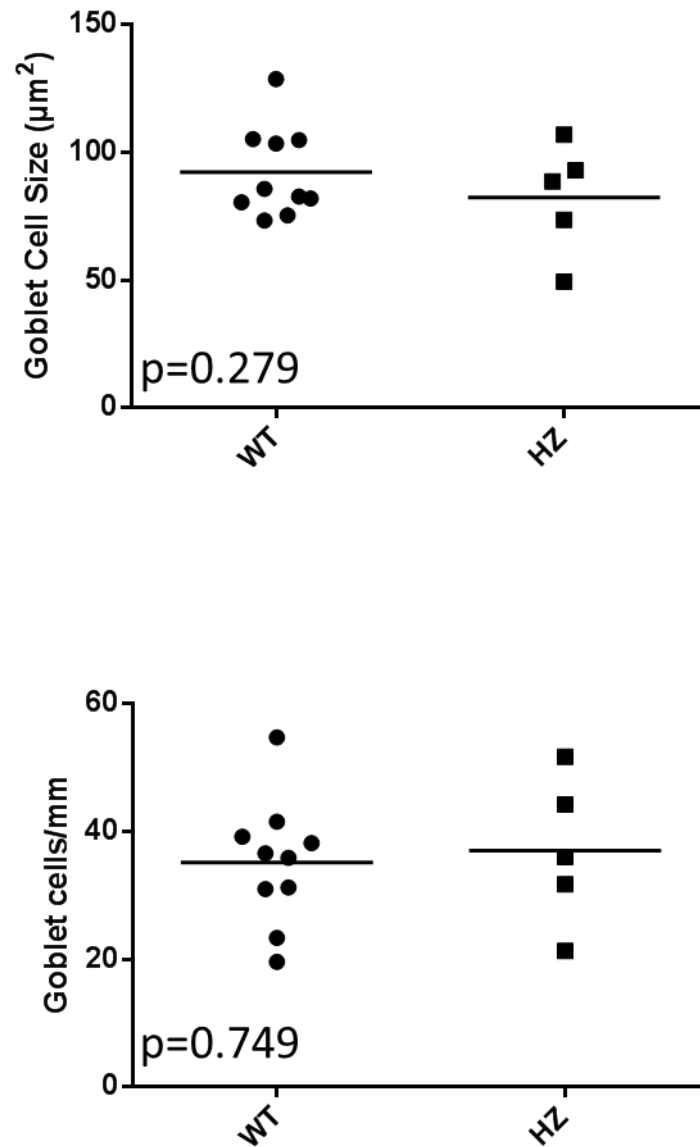
Human CRC pathogenesis is a process that takes place over decades (33). This makes it difficult to track the microbiota in a sufficiently large cohort and impossible to combine those data with the invasive colonoscopies and biopsies necessary to provide comprehensive samples before, during and after tumorigenesis. Some studies have compared the microbiota of human patients presenting with polyps and tumours but these were examined at a single time-point and so did not track the changes that occur across cancer development (28, 34). This provides the rational for using mouse models of CRC to investigate these changes. In this study, an integrated approach was applied through a time-course study of host and microbial changes in a model of familial colon cancer, the APC^{MIN} mouse. Material was collected over 10 weeks which encompassed the period before polyposis to established disease (5, 6). Assessment was made of parameters that are believed to influence the aetiology of CRC including the microbiota, metabolite adjustments by both the host and the microbiota, pro- and anti-apoptotic bile acids and inflammation.

We observed significant polyp development in the genetic CRC model in all areas of the small intestine, as anticipated, at 14 weeks of age. These were accompanied by significant

differences in the diversity and composition of the faecal microbiota in this mouse model. These differences may have initially been driven by pre-neoplastic changes in the intestine of APC^{MIN}, as we observed the first microbial alterations at Week 4, prior to the onset of polyposis reported in the literature (5, 6). These changes could be caused by mechanisms such as depletion of the intestinal mucus layer reported in antibiotic-treated APC^{MIN} mice (6), however no changes in goblet cell size or number were detected in APC^{MIN} mice in a pilot study (Supplementary Figure S4.1).

Uncertainty surrounds the degree to which the faecal microbiota represent the luminal- and mucosal-associated bacteria of the large intestine, and the composition of the microbiota at the site of polyposis in the small intestine of APC^{MIN} mice. Research in rhesus macaques found high correlations between the composition of the stool microbiota and that of the colonic lumen and mucosa, and a moderate correlation between the stool and distal small intestine (35). In mice, the microbial composition detected in the contents of the large intestine was similar to the stool but distinct from the small intestine, which clustered with the stomach contents instead (36). However, another study by Onishi et al. found that the major genera identified in the small intestine were identical to those in the large intestine (37). In addition, faecal microbial transfer (FMT) from APC^{MIN} mice treated with bile acid DCA to untreated APC^{MIN} mice increased tumour size and number in the small intestine in untreated mice, suggesting that this effect was driven by microbiota present in the faeces (10). The faecal microbiota is also commonly altered in CRC (38), which implies that it reflects some aspect of CRC that may be informative, regardless of how accurately it represents the luminal- and mucosal-associated microbiota. Nonetheless, these limitations should be considered when analysing faecal microbial content in the context of intestinal disease.

Although other studies have reported no change, or a decrease, in the α -diversity in APC^{MIN} mice (5, 39, 40) we detected an increase at 4 and 14 weeks of age, but no change at Weeks



Supplementary Figure S4.1: Goblet cell size and number was not different in APC^{MIN} mice.

A pilot study was conducted to estimate appropriate *n*-numbers. During this pilot, the number of goblet cells per millimetre of ileal villus, as well as the average size of goblet cells, was calculated in wild-type (WT) and APC^{MIN} (HZ) mice. No difference was detected in these measures (T-test, $p > 0.05$).

7 or 11. These time-points coincide with the stage immediately prior to polyposis, and just before the onset of inflammation and tumour progression as reported by others (5, 6). Increases in α -diversity during cancer onset occur due to an outgrowth of opportunistic pathogens in the disrupted epithelium, often followed by a decrease in α -diversity due to the subsequent immune response (25, 41-43). This could account for the increase in α -diversity we observed, while the instigation of the inflammatory response is expected soon after our final time-point at Week 14. However, the two-stage increase early and late in the experiment is a novel observation. This may be explained by the enrichment of opportunistic pathogens in the disrupted epithelium immediately prior to tumorigenesis, followed by the detection of tumour-associated taxa at Week 14, as has been described previously (43).

Beta (β)-diversity also differs significantly at each time-point we examined, demonstrating that even though α -diversity recovers between Week 7 and Week 11, the composition of the microbiota does not. This implies that there is a consistent baseline α -diversity value to which the faecal microbiota returns after disruption, even though the species comprising the α -diversity do not return to the previous composition. The α -diversity of the microbial metagenome predicted by PICRUSt differs significantly between APC^{MIN} and wild-type mice at Week 7, and the predicted β -diversity differs at Weeks 4 and 7, which is distinct from the changes observed in our microbial α - and β -diversity. The gut microbiota has a consistent set of base metabolic functions that are robust against disruptions to the community (44) and this is demonstrated in our data here, as the predicted metagenome does not exactly parallel the structural changes in microbiota detected in Shannon or β -diversity. The metabolic changes predicted at Week 7, which correlate with the onset of intestinal neoplasia (5, 6), highlight this as a possible key stage in the functional activity of the microbiome during the onset of intestinal neoplasia.

We also considered the individual taxa that were significantly different between groups, where we do not observe a clear pattern of pro-tumorigenic microbial changes in APC^{MIN} mice. Instead, changes include enrichment and suppression of bacteria associated with both pro- and anti-tumorigenic effects at each time-point (45-53). Potentially pro-tumorigenic changes that are observed at Week 4 include enrichment of *Blautia*, reported to be increased in CRC (28), and *Turicibacter*, which is associated with IBD and colitis-associated cancer (54, 55). In contrast, *Ruminiclostridium 5* and *Ruminococcus 1* were suppressed in APC^{MIN} mice at this time-point and are negatively associated with CRC and IBD respectively (56, 57). At Week 7, several genera were increased which are associated with CRC or colitis (58-60), while suppressed species include *Faecalibaculum*, which is protective against inflammation and CRC (61). *Delftia* and *Peptoclostridium* are enriched in APC^{MIN} mice at Week 11, both of which are associated with gastrointestinal inflammation. *Peptoclostridium* genus also includes the pathogen *C. difficile* as well as 7 α -dehydroxylating bacteria *C. hiranonis* which may increase the intraluminal concentrations of cytotoxic secondary bile acids (62-64). Taxa suppressed at this time-point include several associated with health and negatively associated with CRC and IBD, such as *Anaerostipes* and *Papillibacter* (52, 65). A similar pattern emerges at Week 14 where *Odoribacter* and the uncultivable *Candidatus Arthromitus* were enriched in APC^{MIN} mice, having previously been associated with inflammation and CRC (54, 66).

Eight taxa enriched in either APC^{MIN} or wild-type mice switched to become more abundant in the opposite experimental group during the study, perhaps reflecting the influence of the changing histopathological environment. These included an uncultured member of the *Rhodospirillaceae* family which was more abundant in APC^{MIN} mice at Week 4 and wild-type mice at Week 7, while *Lachnoclostridium* and the uncultured taxon *Lachnospiraceae* NK4A136 displayed the opposite pattern. The former family was reported to be decreased by the chemotherapy drug cyclophosphamide (67), while the latter two taxa were increased by this drug (68, 69), which reflects the changes in abundances in these taxa between Weeks

4 and 7. Cyclophosphamide modulates the immune response to cancer by a number of mechanisms including activation T_H17 cells (69) which are key to intestinal tumorigenesis in APC^{MIN} mice (70, 71) and may be partly regulated by the microbiota (28, 72). Cyclophosphamide also depletes B-cells (73), an effect that is also observed in APC^{MIN} mice (74). These data imply an interaction between the microbiota and immune response in APC^{MIN} mice which reflect those seen in cyclophosphamide chemotherapy, and encompasses a similar pattern of suppression of susceptible bacteria such as *Rhodospirillaceae* and enrichment of resistant species such as *Lachnospiraceae* (75). This could provide another mechanism by which the gut bacteria influence intestinal tumorigenesis and microbial composition early in the experiment.

The *Lachnospirillum* genus also contains the 7 α -dehydroxylating bacteria, *C. scindens* and *C. hylemonae*, which may account for part of the enrichment of the *baiCD* gene predicted by our PICRUST analysis in APC^{MIN} mice at Week 7. 7 α -dehydroxylation is responsible for the formation of secondary bile acids which are cytotoxic and have been implicated in CRC. The enrichment of this taxon at Week 7, after an initial suppression in APC^{MIN} mice, suggests an adaptation to the intestinal environment generated during the onset of polyposis, and that disruption to bile acid signalling by microbial metabolism mediated by *Lachnospirillum* could play a key role during the disease initiation. This has been previously suggested in human studies where a high fat diet, another risk factor for CRC, has been shown to enrich for bacterial species capable of 7 α -dehydroxylation, which may explain the high rate of CRC in western populations (76).

The enrichment of other taxa also alternates between groups across the experiment. These include *Allobaculum*, which is initially more abundant in WT mice but is increased in APC^{MIN} mice at Weeks 7 and 11. This taxon is enriched in models of sporadic and colitis-associated CRC (77-79). *Ruminiclostridium 9* is suppressed in APC^{MIN} mice at Week 4 and enriched at

Week 7. Members of the genus *Ruminiclostridium* are decreased in human CRC patients and APC^{MIN} mice (50, 56) and are associated with high fat diets (50). *E. ventriosum* is suppressed in APC^{MIN} mice at Weeks 4 and 6, and enriched at Weeks 11 and 14. This taxon was also decreased by CRC and increased by high fat diet (80, 81), and was negatively associated with IL6, IL8 and colitis (82, 83). While we did not detect any changes in these cytokines at Week 14, the suppression of this inflammation-intolerant microbe between Weeks 4 and 7 may indicate immune involvement at this stage. The final two taxa that alternate over the course of the experiment are both from the uncultured *Bacteroidales* S24-7 group. However, one subgroup is first enriched in APC^{MIN} before expanding in WT, while the other is increased in WT then APC^{MIN}.

A number of CAGs also differed between groups across the experiment which may describe sets of interacting taxa, as described in section 3.3. The relevance of these groups and interactions to CRC is being investigated.

In summary, our microbiota data provide us with a set of changes associated with different stages of the tumorigenic process in APC^{MIN} mice. Many of these changes have a pro-tumorigenic character but this is not universal. This may be due to limitations in our understanding of the roles of rare or uncultivable taxa *in vivo*, as well as the lack of species-level taxon identification provided by 16S rRNA gene sequencing. It is also possible that APC^{MIN} mice are not solely enriched in pro-tumorigenic species, in which case the contribution of each taxon to tumorigenesis may need to be considered individually. Species associated with CRC are not consistent across studies and the relationship between the microbiota and CRC is likely to be complex (2, 3). In this context, it will require consistent association between taxa and CRC to define key species, which should be combined with a mechanistic understanding of their interaction with the host *in vivo*. Our study does reveal novel temporal variations in the patterns of microbial changes, potentially associating some

taxa with cancer initiation early in the model and others with tumour progression later. This will inform future research and potentially, targeted therapies for specific stages of disease, and could also explain some of the inconsistent associations observed in other studies of the microbiota in CRC which sample the microbial composition at different times.

One of the primary mechanisms by which the gut microbiota may influence CRC initiation and progression is via production of bioactive microbial metabolites. The relative abundance of bile acid-metabolising taxa were altered at different time-points during the study and the predicted abundance of bile acid metabolising genes *baiCD* and *bsh* was also increased in APC^{MIN} mice at Week 7. Bile acids and microbial metabolism of bile acids have been implicated in the pathogenesis of CRC and so we measured faecal and plasma bile acids in our model. Of note, the transcription of target genes of the bile acid receptor FXR, *I-babp* and *Fgf15*, were significantly decreased in the ileum of APC^{MIN} mice which suggests that bile acid signalling was suppressed, which would limit the cancer-protective role of FXR (11, 30). This was supported by the fact that *Fgf15* correlated negatively with tauro-conjugated MCAs, which are FXR antagonists. *Fgf15* also correlated negatively with the conjugated secondary bile acid T-HDCA. HDCA can be formed by 7 α -dehydroxylation of ω -MCA, or by 7 α -dehydroxylation followed by epimerisation of the 6-carbon hydroxyl group of α - or β -MCA, a capability possessed by the microbiota (84). Another set of FXR target genes, *Ost α* and *Ost β* , also correlate negatively with the total number of polyps, and polyps in the middle and distal portions of the small intestine, of APC^{MIN} mice. The transcription of anti-inflammatory *Il10* also correlated positively with *Ost α* and this cytokine is also up-regulated by FXR (85, 86) and was significantly reduced in APC^{MIN} mice at this time-point, implicating FXR in this effect. This suggests the possibility that T-MCAs antagonise FXR, a known tumour suppressor, leading to increased polyp multiplicity in the middle and distal small intestine, potentially involving suppression of IL10. While T-MCAs were not significantly different between groups despite their correlation with *Fgf15*, the concentration of potent FXR-agonist bile acids CDCA

and DCA were significantly decreased in APC^{MIN} mice before correction for multiple comparisons. It should be noted, however, that hypermethylation of the *Fxr* promoter has been reported in the tumour-adjacent mucosa of APC^{MIN} mice, and so the relative contribution of bile acid signalling and/or promoter methylation to suppression of FXR signalling is difficult to elucidate (87). Nonetheless, we see alterations in bile acid-metabolising bacteria in APC^{MIN} mice and differences in the predicted abundance of microbial bile acid-metabolising genes, suggesting that the microbiota do play a role in bile acid homeostasis in our model. Additionally, our correlation analysis provides evidence that bile acids are involved in FXR signalling in APC^{MIN} mice.

In conclusion, our data highlight a progression of bacterial changes with the potential to contribute to disease at each time-point. These include distinct changes associated with the periods of cancer onset and progression. This dataset will inform future studies to understand the functional impact of the microbiota on tumorigenesis and to understand the implications of alterations in microbial community structure during this process. Herein we also identify a possible role for bile acid signalling in small intestine polyposis in APC^{MIN} mice mediated by FXR which supports previous studies.

4.4 References

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Chapter 5

Polyposis in APC^{MIN} mice is suppressed by bacteria expressing *bile salt hydrolase*

5.0 Abstract

Background: Bile salt hydrolase (Bsh) is an enzyme prevalent in probiotic bacteria but concerns exist regarding its ability to increase the concentration of cytotoxic secondary bile acids in the intestines. We investigated the role of Bsh in colorectal cancer (CRC) by treating APC^{MIN} mice, a model for familial colorectal cancer, with *E. coli* engineered to overexpress this enzyme.

Methods: APC^{MIN} mice were treated by gavage with PBS, *E. coli* MG1655 or *E. coli* MG1655 overexpressing Bsh at 4, 8 and 12 weeks of age before cull at 14 weeks of age. Intestinal polyps were enumerated, the faecal microbial composition was examined by 16S rRNA gene sequencing, faecal and plasma bile acid profiles were determined by UPLC-MS and ileal cytokine expression was measured by qPCR. We also examined FXR and Wnt signalling by qPCR for target genes, and performed a correlation analysis between features of our data.

Results: Gavage with *E. coli* expressing *bsh* significantly reduced small intestine polyposis in APC^{MIN} mice. This occurred alongside an enrichment of hydrophilic bile acid tauro-ursodeoxycholic acid and alterations in the composition of the microbiota. Our correlation analysis revealed a negative association between FXR target gene *I-babp* and the number of small intestinal polyps.

Conclusions: Surprisingly, *Bsh* reduced small intestine polyposis in APC^{MIN} mice by a mechanism which may involve bile acid signalling and a reduction in the hydrophobicity of the bile acid pool, as well as an induction of protective changes in the microbiota.

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5.13b. UPLC-MS and extraction from faecal and plasma samples for the detection of bile acids was performed by Peter Cronin (*Materials and Methods* section 2.7, *Ultra-Performance Liquid Chromatography – Mass Spectrometry*). Some of this work is presented in his submitted MRes thesis (April 2019). The materials and methods sections for these processes are provided by their respective executors. Gavage and mouse culling were performed by Cara Hueston.

5.1 Introduction

The aetiology of colorectal cancer (CRC) involves the complex interaction of genetic and environmental factors, and recent evidence also suggests it could be influenced by the gut microbiota (1). The gut microbiota of human CRC patients is significantly altered by disease and certain microbial patterns are associated with increased cancer risk, but definitive identification of mechanisms and specific taxa involved has proven elusive as bacterial species associated with human CRC are not consistent across studies (2, 3).

APC^{MIN} mice are used as a model of familial CRC as they harbour heterozygous mutations in the *Apc* gene, similar to human patients suffering from Familial Adenomatous Polyposis (FAP). These mice develop intestinal polyps starting at 7-8 weeks of age, with inflammation becoming apparent at approximately 12 weeks of age (4, 5). In FAP, the microbiota is enriched for pro-tumorigenic bacteria such as *Escherichia coli* expressing colibactin and enterotoxigenic *Bacteroides fragilis* (6), and a similar phenomenon is observed in APC^{MIN} mice (4). Importantly human tumour-associated bacteria has been shown to aggravate tumorigenesis in APC^{MIN} mice (7).

The gut microbiota can influence the development of CRC by production of pro-carcinogenic bacterial metabolites, for example secondary bile acids (8). This was demonstrated by dietary administration of secondary bile acids to APC^{MIN} mice which increased tumour multiplicity in a microbiota-dependent manner (9). Bile acid receptor FXR has also been shown to suppress intestinal tumorigenesis *in vivo* by mechanisms including promotion of apoptosis and differentiation (10). High fat diets also increase the faecal levels of secondary bile acids and are associated with increased cancer risk (11, 12). This suggests that bile acids and the microorganisms that influence their metabolism may represent potential aetiologic agents of high fat diet-induced colon cancer risk.

In this context, the prevalence of bile salt hydrolase (Bsh) enzymes among probiotic bacteria is surprising (13). Bsh enzymes cleave the amino acid side chain of bile salts to form free bile acids, which is a prerequisite for subsequent 7 α -dehydroxylation to secondary bile acids (14). Increasing the prevalence of Bsh in the microbiota therefore has potential to increase the luminal concentration of secondary bile acids with the potential to increase cancer risk, although the 7 α -dehydroxylation pathway has not been observed in probiotic species (8, 15). Bsh is also found in pathogenic bacteria such as *Listeria monocytogenes* where it improves its gastrointestinal persistence (16). This raises the question as to the role of Bsh *in vivo*, its interaction with the host and its desirability in probiotic bacterial strains.

We applied an engineered strain of laboratory *E. coli* MG1655 to overexpress *bsh* in order to investigate the role of this enzyme in APC^{MIN} mice and determine its effects on polyp multiplicity, microbial diversity and composition, proliferation, Wnt signalling, inflammatory transcription, as well as bile acid composition and signalling. We also performed a correlation analysis to identify any relationships between these factors. This work revealed a significant reduction of small intestine polyp number in mice administered *E. coli* MG1655 expressing *bsh* by a mechanism which may involve bile acid signalling and a reduction in the hydrophobicity of the bile acid pool, as well as an induction of protective changes in the microbiota.

5.2 Results

5.2.1 Administration of *E. coli* expressing *bsh* significantly decreased polyposis in APC^{MIN} mice

APC^{MIN} mice develop dozens of polyps in their small intestine, and we used the multiplicity of these polyps to determine the effect of bacterial gavage on tumorigenesis in this model. The total number of polyps in the small intestine of APC^{MIN} mice administered EC-BSH was significantly lower than the groups administered either PBS or EC (Figure 5.1; ANOVA and Tukey's HSD, $p < 0.05$). The number of polyps in the distal small intestine of EC-BSH mice is also significantly lower than the PBS group (Figure 5.1; ANOVA and Tukey's HSD, $p = 0.015$). There is no difference in polyp number between groups in the proximal or middle sections of the small intestine (Figure 5.1; ANOVA, $p > 0.05$) or the colon (Figure 5.1; KW test, $p = 0.4$).

5.2.2 Levels of bacterial colonisation were similar in EC and EC-BSH mice

Successful colonisation was confirmed by analysis of faecal colony forming units (CFUs) of *E. coli* and *E. coli* overexpressing *bsh* on LB agar containing streptomycin. No streptomycin-resistant bacteria were detected in mice treated with PBS. CFUs/mL only differed between EC and EC-BSH at 6 weeks of age, with higher colonisation in EC mice (Figure 5.2; Two-way ANOVA followed by T-test, $p = 0.023$).

5.2.3 Epididymal fat is reduced in mice administered *E. coli*

Cancer-associated weight loss, or cachexia, is an indicator of severe disease in human cancer that is also observed in late-stage APC^{MIN} mice. We tracked the body weight of our mice at weekly intervals throughout the study and measured the mass of their mesenteric and

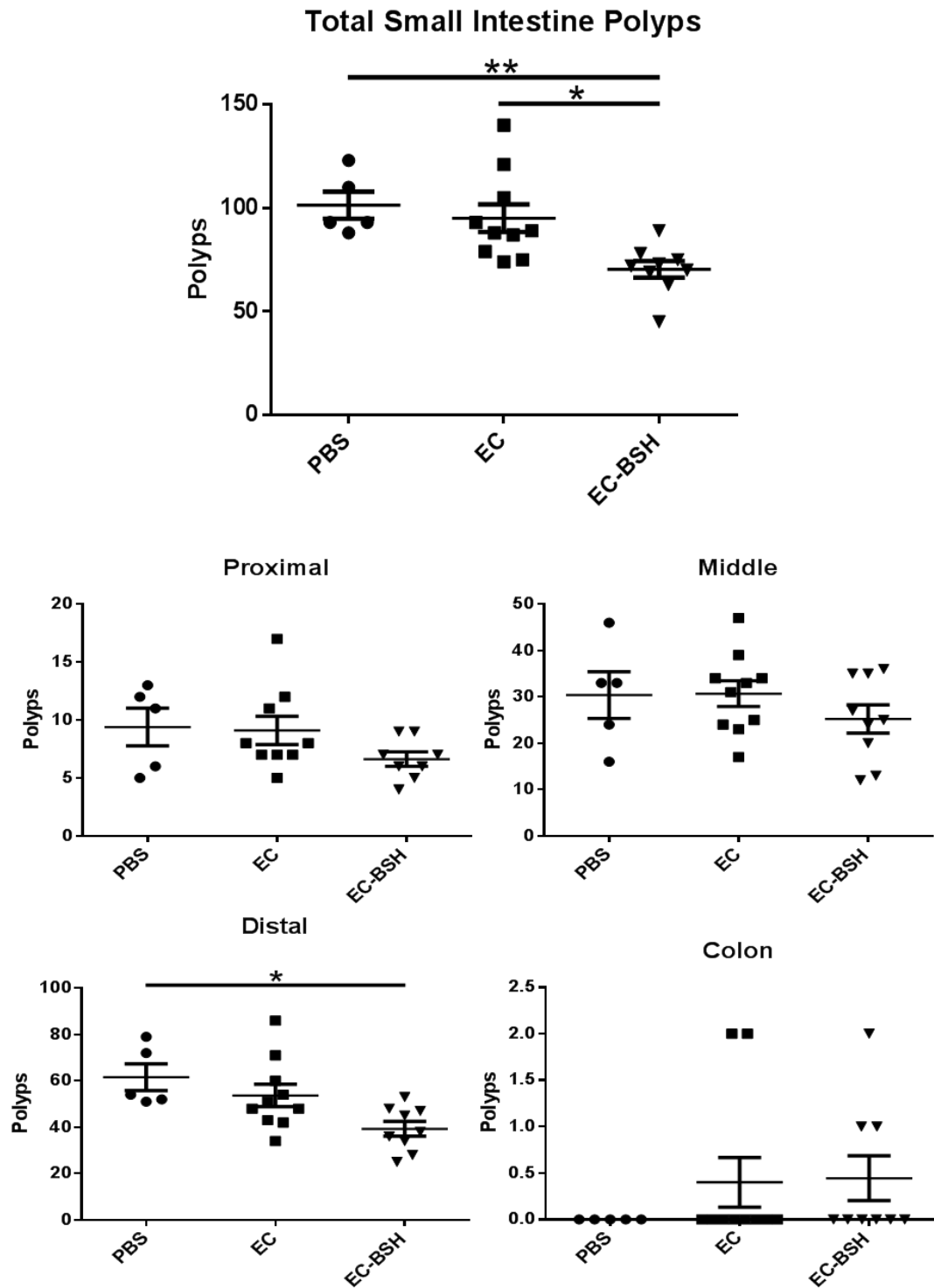


Figure 5.1: EC-BSH mice develop significantly fewer polyps in their small intestine. Mice were culled at 14 weeks of age and 2cm of their proximal and distal small and large intestine was removed for RNA analysis. The remaining small intestine was divided into three equal portions and the number of polyps in each section was enumerated under a dissecting microscope. The number of polyps in the colon was also determined. EC-BSH mice had significantly fewer total polyps in their small intestine than PBS and EC mice (ANOVA and Tukey's HSD, $P < 0.05$), and fewer polyps in their distal small intestine than PBS-treated mice (ANOVA and Tukey's HSD, $P = 0.015$).

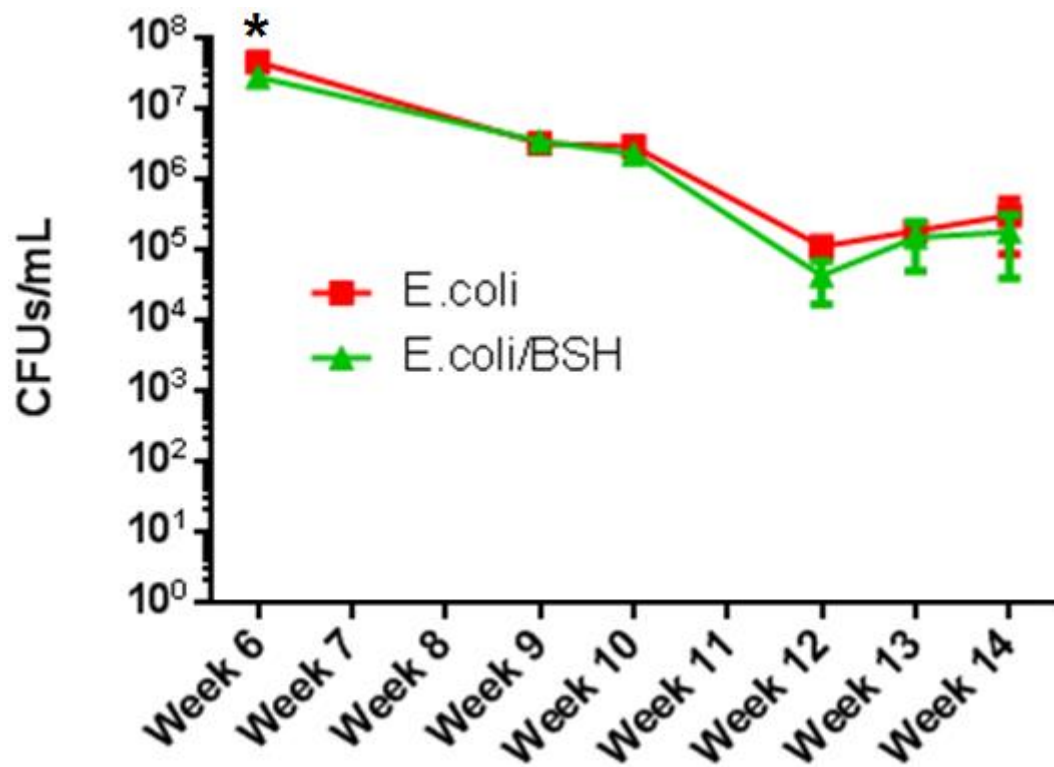


Figure 5.2: Faecal colonisation by *E. coli* and *E. coli* overexpressing *bsh*. Colonisation was determined by plating serial dilutions of faeces suspended in PBS onto LB agar containing streptomycin. CFUs/mL of *E. coli* and *E. coli* overexpressing *bsh* only differed at Week 6 (mixed ANOVA followed by T-test, $p=0.023$), while no streptomycin-resistant bacteria were detected in the PBS group.

epididymal fat after cull. Body weight did not differ between groups across the study (Figure 5.3; two-way ANOVA, $p=0.4212$). The mass of epididymal fat was reduced in the EC group (Figure 5.3; ANOVA and Tukey's HSD, $p=0.036$) and there was no difference in the mass of mesenteric fat between any of the treatments (Figure 5.3; ANOVA, $p=0.1$).

5.2.4 Ki67 staining is not increased in APC^{MIN} mice

Loss of cell cycle control in APC^{MIN} mice has been shown to precede the development of intestinal polyps (4). In order to determine how far this loss of control extends, we measured the degree of proliferation in normal villi of each group at our end-point. The expression of proliferation indicator Ki67 did not differ between groups (Figure 5.4; ANOVA, $p=0.11$).

5.2.5 Bacterial gavage affects cytokine and chemokine transcription in APC^{MIN} mice

Inflammation is a risk factor for colon cancer and the tumour microenvironment can modulate inflammatory conditions to promote tumour growth. As such, we measured the transcription of cytokines and chemokines in the distal small intestine by qPCR, as well as measuring the spleen weight and the length of the small and large intestine of our mice, which can be used as indicators of intestinal inflammation (Figure 5.5).

The relative transcription of *Cxcl1* was significantly decreased in both EC and EC-BSH mice compared to PBS (Figure 5.5; ANOVA and Tukey's HSD, $p<0.05$), while *Cxcl5*, *Tgfb* and *Jun* are decreased in EC compared to PBS (Figure 5.5; ANOVA and KW tests, followed by Tukey's HSD and MWU tests, $p<0.05$). There were no significant differences between relative transcription of *Il1b*, *Il10*, spleen weight or in the length of small intestine between groups (Figure 5.5; ANOVA, $p>0.05$).

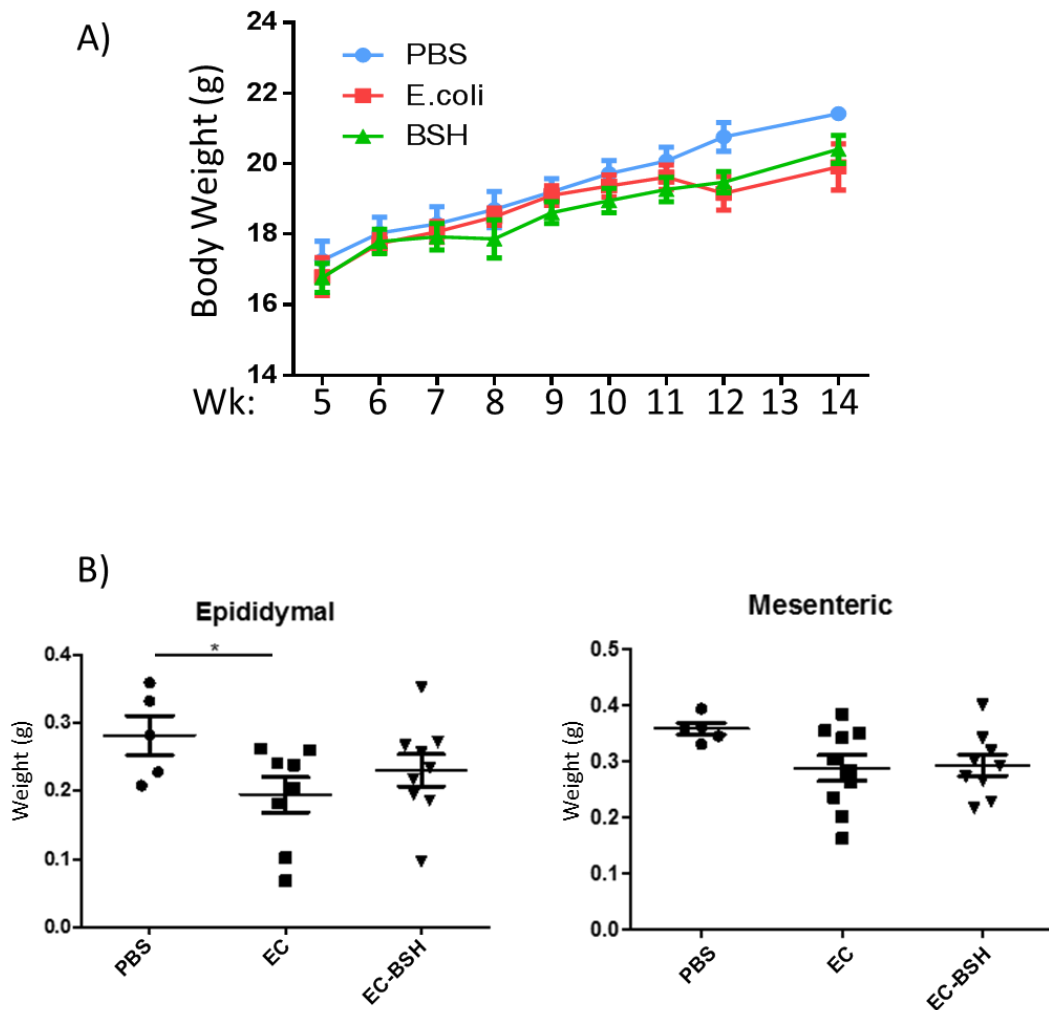


Figure 5.3: Epididymal fat was reduced in EC mice. Weight loss or inhibited weight gain is a sign of severe pathology in human cancer and mouse models of cancer. We tracked the body weight of our mice every week from 5 weeks of age (A) and recorded the mass of their mesenteric and epididymal fat after cull (B). The weight of epididymal fat was reduced in the EC group compared to PBS (ANOVA and Tukey's HSD, $p=0.036$). Body weight or mesenteric fat did not differ between groups (Two-way ANOVA and ANOVA, $P>0.05$).

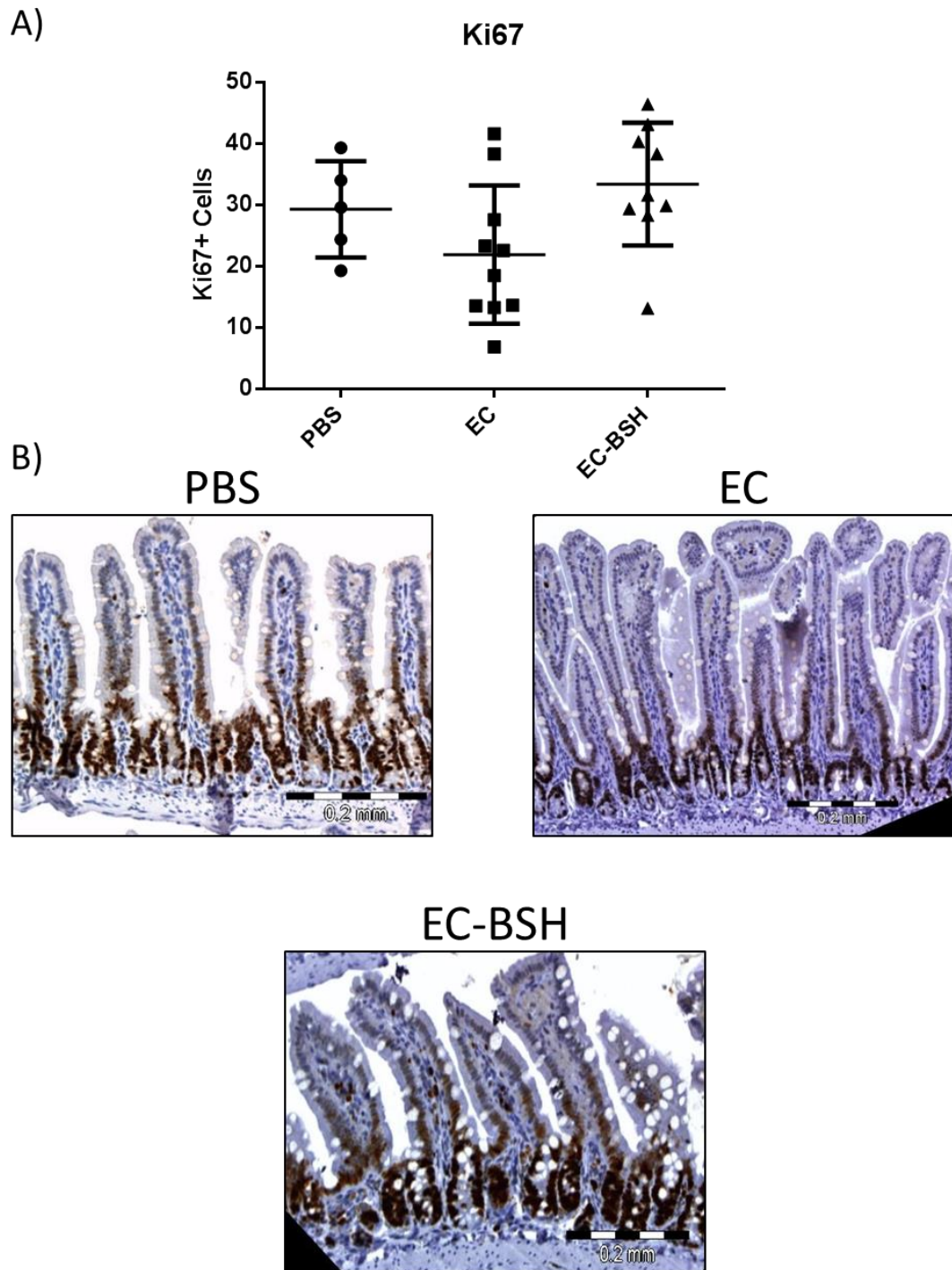


Figure 5.4: Proliferation in normal villi did not differ between groups. Mutation in the tumour suppressor *Apc* gene can lead to constitutive Wnt pathway activation and aberrant cell proliferation. In order to investigate if proliferation is up-regulated in the normal-appearing villi in the small intestine of APC^{MIN} mice prior to the development of polyps, we stained the epithelium for Ki67 and determined the percentage of Ki67-positive cells per crypt-villus unit for ten such units per mouse. **A)** There was no significant difference in Ki67 expression in the normal villi of APC^{MIN} mice, which suggests that dysregulated proliferation is not ubiquitous in the small intestine of these mice (ANOVA, $P=0.11$). **B)** Sections of the distal ileum of wild-type and APC^{MIN} stained for Ki67 on a background of haematoxylin. Magnification 200x.

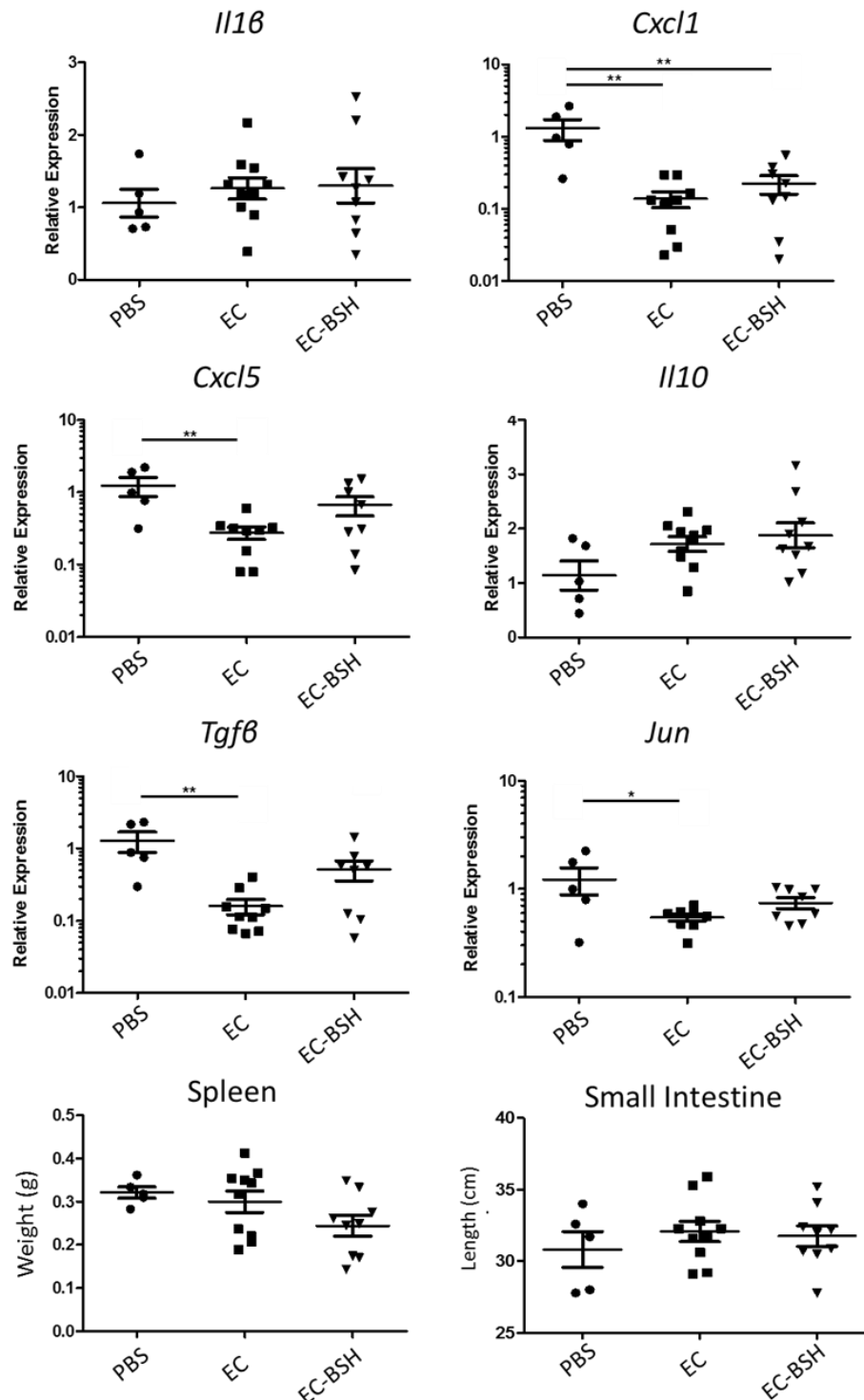


Figure 5.5: Changes in inflammatory transcription are observed in *APC^{MIN}* mice. Chronic inflammation is a risk factor for colorectal cancer and inflammation in the tumour microenvironment can drive cancer progression. In order to establish if inflammation played a role in the pathogenesis of our model, we extracted RNA from the distal small intestine, the primary site of polyposis in *APC^{MIN}* mice, and performed qPCR for several cytokines. We also measured spleen weight and the length of the small intestine. *Cxcl1* was increased in PBS mice compared to both other groups, while *Cxcl5*, *Tgfβ* and *Jun* were decreased in EC compared to PBS (ANOVA, $p < 0.05$).

5.2.6 The faecal microbiota is significantly different in APC^{MIN} mice

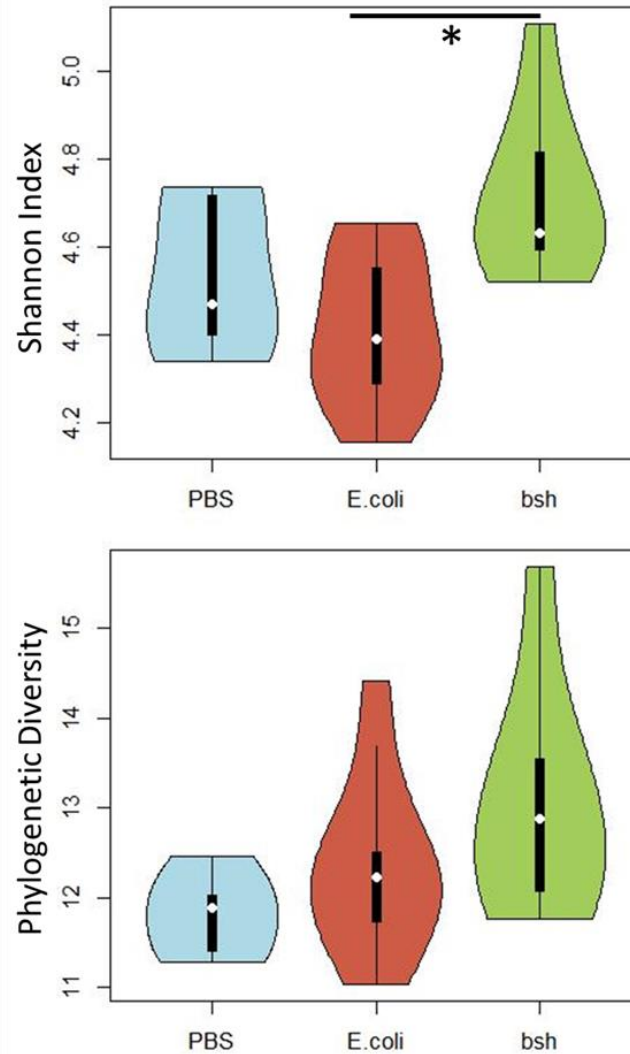
We collected faecal pellets from each mouse at the end-point of the experiment and performed 16S rRNA gene sequencing to characterise the microbial diversity and composition. We measured the α -diversity by two metrics. These were Shannon diversity, a mathematical measure of the number of species and their relative abundances, and phylogenetic diversity (PD), which measures the total length of the minimum path spanning each node of a cladogram featuring the detected species but does not consider the relative abundance of those species (Figure 5.6A).

Shannon diversity is significantly higher in the EC-BSH group compared to EC (Figure 5.6A; KW test and MWU-test, $p=0.017$). No changes were observed in PD (Figure 5.6A; KW test, $p=0.112$).

Beta (β)-diversity represents between-individual diversity in the microbiota which we measured by PERMANOVA of the unweighted Unifrac distances, visualised by PCoA. All groups were significantly different from each other, and treatment explained between 13% and 24% of the variation (Figure 5.6B; PERMANOVA, $P<0.01$).

Finally, we considered individual taxa differences between groups. The significantly different taxa are presented in Figure 5.7 (KW test with FDR adjustment, $p<0.05$). The largest changes are observed in *Lachnospiraceae UCG-001*, which is 50-fold more abundant in EC than PBS and 19-fold more abundant in EC than EC-BSH. *Ruminococcaceae UCG-010* is 19.3-fold more abundant in PBS than EC and *Faecalibacterium* is 13.9-fold more abundant in PBS than EC. *Clostridiales vadinBB60; uncultured rumen bacterium (URB)* is significantly higher in EC than EC-BSH, where it is completely absent, while this pattern is reversed for *Acetatifactor*. *Anaeroplasma* is also significantly higher in EC and EC-BSH compared to PBS, being absent in the latter group, while *Tyzzzeria* is significantly more abundant in EC-BSH than EC or PBS.

A)



B)

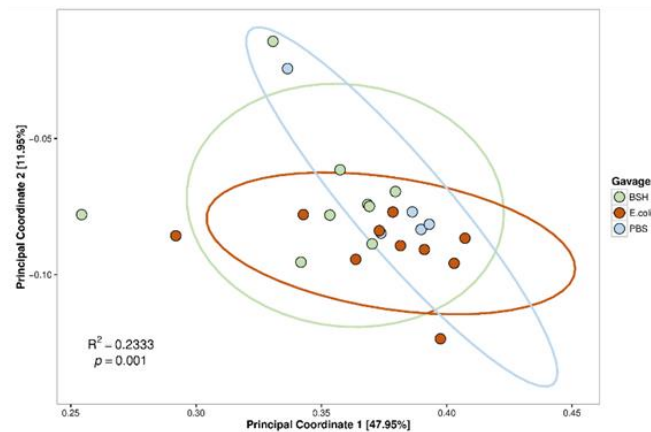


Figure 5.6: α - and β -diversity are significantly different between groups. We performed 16S rRNA gene sequencing for the V3-V4 region and examined the α - and β -diversities by Shannon and Phylogenetic Diversity (PD) metrics and unweighted UniFrac PCoA. **A)** This revealed a significant increase in Shannon diversity, but not PD, in the EC-BSH group (KW test and MWU-tests, $p < 0.05$). **B)** In addition, the β -diversity of each group was significantly different (PERMANOVA, $p < 0.01$). $N = 5, 10$ and 9 for PBS, EC and EC-BSH. Figure in **B** was provided by Calum Walsh at Teagasc, Moorepark.

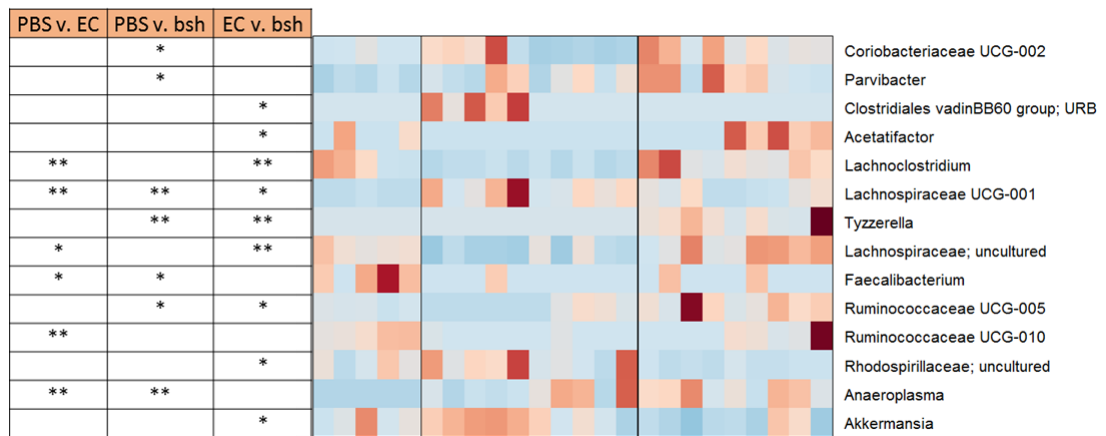


Figure 5.7: Taxa significantly different between groups. We performed 16S rRNA gene sequencing for the V3-V4 region and compared the relative abundance that each taxon comprised in each group. This revealed a number of significantly different bacteria (KW test and MWU-test with FDR adjustment, $p < 0.05$). Only the significantly different OTUs are presented here. Data are presented as Z-scores normalised to the row average with asterisks representing the degree of significance as determined by post-hoc analysis ($p < 0.05$, < 0.01 and < 0.001 for *, ** and *** respectively). $N=5$, 10 and 9 for PBS, EC and EC-BSH.

In order to investigate the alterations in the gut bacteria at a higher structural level, we defined a set of bacterial co-abundance groups (CAGs) by finding the Kendall correlation between different bacterial taxa and Ward-clustering these values based on their Pearson correlation coefficients (Figure 5.8A) (17). These correlations were converted to a distance matrix on which PERMANOVA was performed which determined that all CAGs were significantly different (Figure 5.8C, $p < 0.01$). The composition of each CAG can be found in Table 5.3.

Seven CAGs were identified, labelled CAG1-7. The relative abundance of CAG1 was higher in EC-BSH mice than EC (Figure 5.8B; KW test and MWU test, $p = 0.009$). The relative abundance of CAG3 is increased in EC-BSH mice compared to the PBS and EC groups (Figure 5.8B; KW test and MWU test, $p < 0.05$), while CAG4 is increased in PBS compared to EC and EC-BSH (Figure 5.8B; KW test and MWU test, $p < 0.05$). The relative abundance of CAG5 is higher in the EC group compared to PBS (Figure 5.9B; ANOVA and Tukey's HSD, $p = 0.029$), while the abundance of CAG7 is lower in EC-BSH compared to EC (Figure 5.9B; KW test and MWU test, $p = 0.006$).

CAG1 is dominated by an uncultured member of the *Lachnospiraceae* family and *Ruminiclostridium 9*, and comprises an average of 10.6% of the microbiota detected in our mice. CAG3 is more diverse and features large portions of *Blautia*, *Ruminococcaceae* UCG-005, *Eubacterium xylanophilum* group and *Tyzzterella* but makes up less than 1% of the total microbiome of each mouse. *Christensenellaceae*; *R7* group and *Eubacterium coprostanoligenes* group predominant in CAG4, but this CAG comprises less than 0.1% of the average total abundance of the microbiome. Taxa in CAG5 constitute 9.6% of the average abundance of the microbiome and the majority of this CAG consists of *Clostridiales vadinBB60* group; uncultured bacterium, an uncultured *Bacteroidales* bacterium and *Lachnospiraceae* UCG-008. *Akkermansia* and *Clostridiales vadinBB60* group; ambiguous taxa

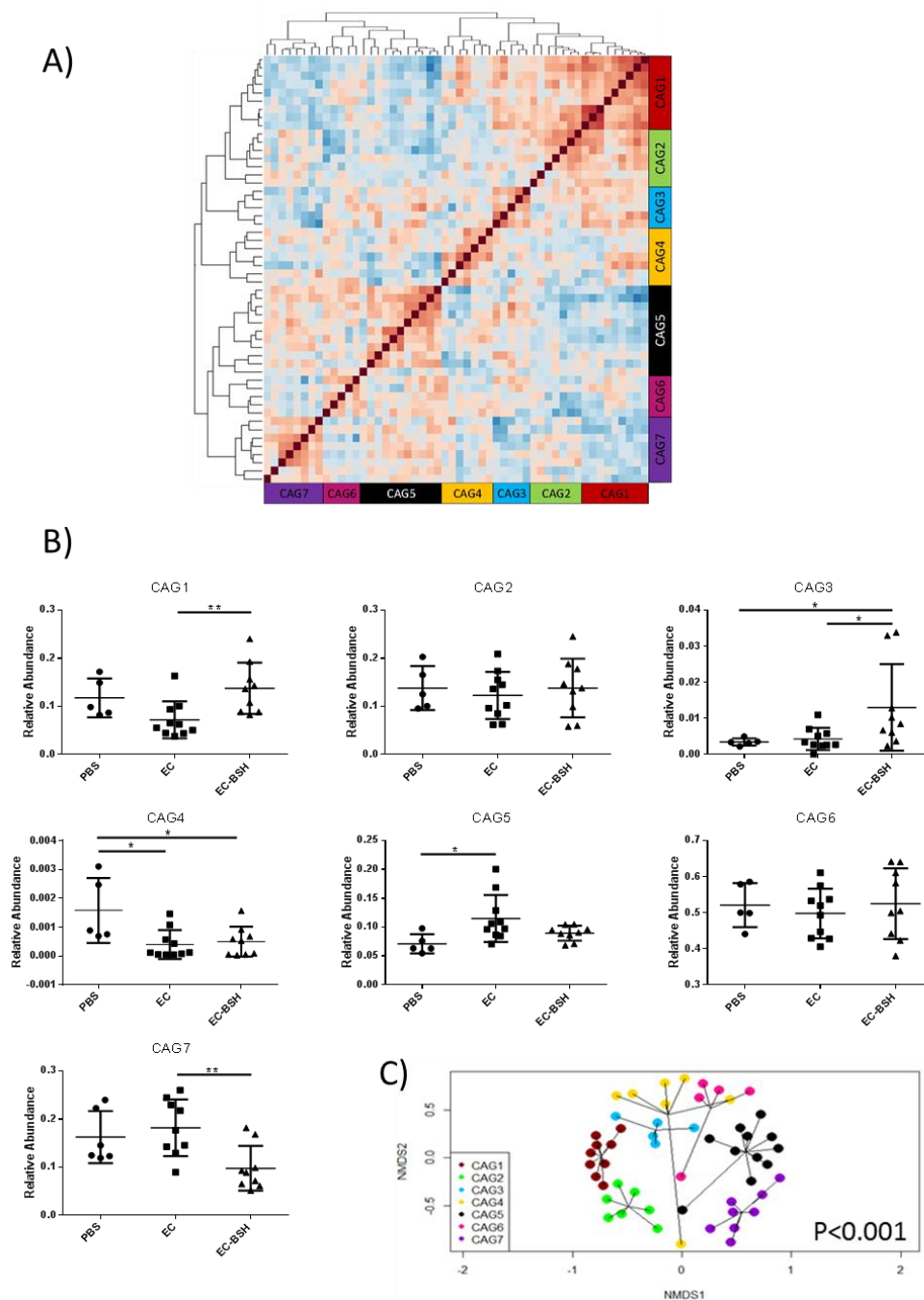


Figure 5.8: Generation of co-abundance groups. The Kendall correlations of OTUs' relative abundances were generated and clustered by Ward-linkage and Pearson correlation. This defined a set of "co-abundance groups" (CAGs) which describe higher-level structures of the microbiota by highlighting taxa for which changes in abundance correlate. These Kendall correlations were then converted to a distance metric and analysed by PERMANOVA which determined that each CAG was significantly different. **A)** A heatplot of Kendall correlation values for each pair of taxa clustered by ward linkage of their Pearson correlations. Coloured bars along the axes denote the CAGs. The composition of each CAG is presented in Table 3.1. **B)** A number of significantly different CAGs were identified (ANOVA and KW tests, with post-hoc analysis by Tukey's HSD and MWU-tests, $p < 0.05$) **C)** Non-metric multidimensional scaling (NMDS) of the distance metrics of the CAGs' correlation values. All CAGs were determined to be significantly different (PERMANOVA, $p < 0.01$).

Table 5.3: CAG Composition

CAG1	Ruminiclostridium 9
	Anaerotruncus
	Lachnospiraceae Uncultured
	Intestinimonas
	Lachnospiraceae UCG-006
	Lachnoclostridium
	Ruminococcus 1
	Ruminococcaceae UCG-003
	Ruminiclostridium 5
CAG2	Peptococcaceae Uncultured
	Lachnospiraceae NK4A136 Group
	Oscillibacter
	Ruminiclostridium
	Marvinbryantia
	Delftia
	Anaerobacillus
CAG3	[Eubacterium] xylanophilum group
	Syntrophococcus
	Blautia
	Ruminococcaceae UCG-005
	Tyzzerella
CAG4	Ruminococcaceae UCG-014
	Lactococcus
	Coriobacteriaceae Uncultured
	Ruminococcaceae UCG_010
	Christensenellaceae R7 Group
	[Eubacterium] Coprostanoligenes Group
	Faecalibacterium
CAG5	Escherichia-Shigella
	Lachnospiraceae UCG-008
	Ruminococcaceae UCG-013
	Parvibacter
	Coriobacteriaceae UCG-002
	Allobaculum
	Uncultured Bacteroidales Bacterium
	Bifidobacterium
	Anaeroplasma
	Lachnospiraceae UCG-001
	Clostridiales VadinBB60 Group; Uncultured Bacterium
CAG6	Candidatus Saccharimonas
	Ruminococcaceae Uncultured
	Christensenellaceae Uncultured
	Bacteroides
	Bacteroidales S24-7 Group; Uncultured Bacterium
CAG7	Akkermansia
	Rhodospirillaceae Uncultured
	Chloroplast; Uncultured Bacterium
	Bacteroidales S24-7 Group; Ambiguous Taxa
	[Eubacterium] Nodatum Group
	Alistipes
	Ochrobactrum
	Clostridiales VadinBB60 Group; Ambiguous Taxa

compose over 95% of CAG7, which itself forms 14.5% of the average abundance of the microbiota. Of the two CAGs that do not differ between groups, the majority of CAG2 consists of *Lachnospiraceae NK4A136 group* and this CAG encompasses 13.1% of the microbiota on average, while CAG6 is dominated by *Bacteroidales S24-7 group; uncultured bacterium* and *Bacteroides*, and this CAG comprises 51% of the average abundance of the microbiome due to the prevalence of these two taxa.

5.2.7 Tauro-ursodeoxycholic acid is enriched in EC-BSH mice

In order to investigate the potential role of microbial bile acid modification in our model, we analysed the faecal and plasma bile acid profile of each group at the experimental end-point by UPLC-MS. After correction for multiple comparisons, only the faecal concentration of tauro-conjugated ursodeoxycholic acid (T-UDCA) was increased in the EC-BSH group (Figure 5.9; KW test, $p=0.001$). The list of bile acids measured is presented in Table 5.4. Neither total faecal nor plasma bile acid concentration differed between groups (ANOVA and KW test, $P>0.05$), and there were no differences among the plasma bile acids (ANOVA and KW-test, $P>0.05$).

5.2.8 The transcription of FXR target gene *I-babp* is increased by bacterial gavage in APC^{MIN} mice

Bile acid receptor FXR regulates the transcription of a number of genes in the ileum and has also been implicated in protection from CRC (10, 18). In order to determine if bile acid signalling is affected in our model, we measured the transcription of four FXR target genes in the distal ileum (Figure 5.10).

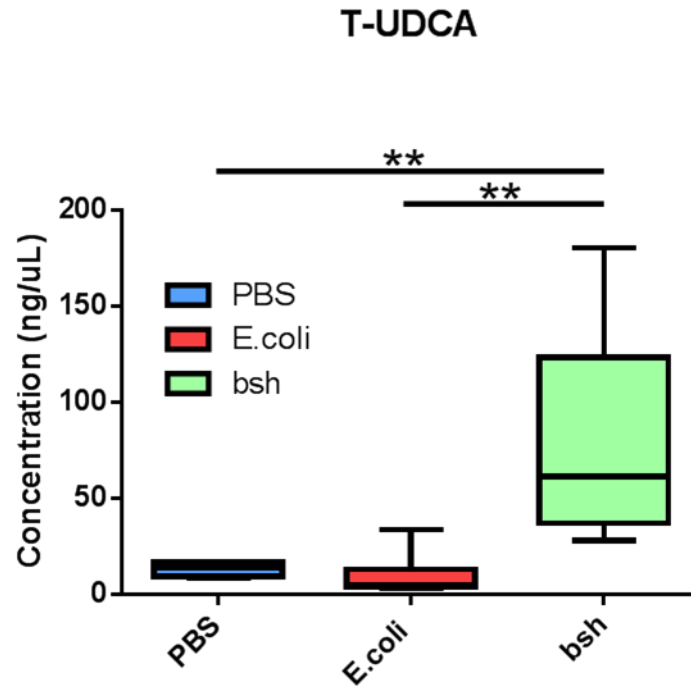


Figure 5.9: T-UDCA is enriched in EC-BSH mice. Microbial metabolism of bile acids has been implicated in the pathogenesis of colon cancer. To investigate the role bile acids play in our model, we measured the concentration of faecal and plasma bile acids by UPLC-MS. After correction for multiple comparisons, only the faecal concentration of T-UDCA was significantly different between groups (KW test and MWU-test with FDR adjustment, $p=0.001$). Each bile acid measured in presented in Table 5.4. $N=3$, 6 and 7 for plasma bile acids; 4, 8 and 8 for faecal bile acids; for PBS, EC and EC-BSH respectively.

Table 5.4: Bile acids measured

	Bile Acid	Abbreviation
Bile Acids	Dehydrocholic Acid	DHCA
	Lithocholic Acid	LCA
	Ursodeoxycholic Acid	UDCA
	Chenodeoxycholic Acid	CDCA
	Hyodeoxycholic Acid	HDCA
	Deoxycholic Acid	DCA
	Cholic Acid	CA
	Hyocholic Acid	HCA
	7-Keto-Lithocholic Acid	7-Keto-LCA
	Tauro-Cholic Acid	T-CA
	Tauro-Hyocholic Acid	T-HCA
	Tauro-Chenodeoxycholic Acid	T-CDCA
	Tauro-Ursodeoxycholic Acid	T-UDCA
	Tauro-Deoxycholic Acid	T-DCA
	Tauro-Hyodeoxycholic Acid	T-HDCA
	Tauro-Lithocholic Acid	T-LCA
	Glyco-Cholic Acid	G-CA
	Glyco-Hyocholic Acid	G-HCA
	Glyco-Chenodeoxycholic Acid	G-CDCA
	Glyco-Ursodeoxycholic Acid	G-UDCA
	Glyco-Deoxycholic Acid	G-DCA
	Glyco-Hyodeoxycholic Acid	G-HDCA
	Glyco-Lithocholic Acid	G-LCA
	Beta (β)-Muricholic Acid	B-MCA
	Alpha/gamma (α/ω)-Muricholic Acid	α/ω -MCA
	Murocholic Acid	MoCA
	Tauro α -, β -, and ω -Muricholic Acid	T- $\alpha/\beta/\omega$ -MCA
	Total Bile Acids	Total BAs

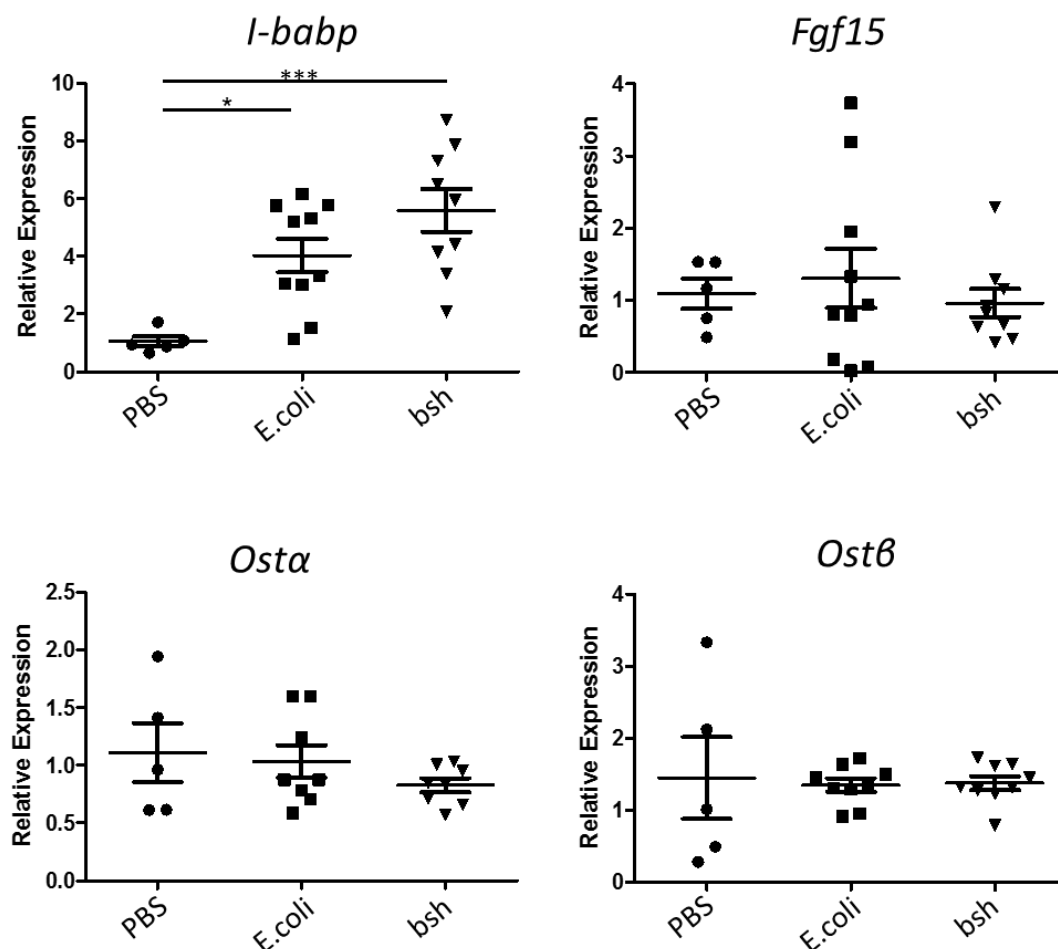


Figure 5.10: Transcription of *I-babp* is increased by bacterial gavage. One mechanism by which bile acids can affect tumour risk is by FXR signalling, which has been shown to be protective against CRC. We measured the expression of four FXR target genes in the distal ileum of our mice by qPCR. This revealed significantly increased expression of *I-babp* and EC and EC-BSH mice, which may indicate activation of FXR signalling (ANOVA and Tukey's HSD, $p < 0.05$).

The transcription of *I-babp* was significantly increased in EC and EC-BSH groups compared to PBS (Figure 5.10; ANOVA and Tukey's HSD, $p < 0.05$). The transcription of *Fgf15* and enterocyte basolateral bile acid transporters *Osta* and *Ostb* were not significantly different between groups (Figure 5.10; ANOVA, $p > 0.05$).

5.2.9 The transcription of Wnt target genes is significantly increased by bacterial gavage

In order to determine if the changes in polyposis induced by EC-BSH occur upstream or downstream of activation of Wnt signalling, we measured the transcription of Wnt target genes *Axin2*, *Myc* and *Sox9* in the distal ileum of each mouse (Figure 5.11).

The relative transcription of *Sox9* was increased in EC and EC-BSH groups compared to PBS (Figure 5.11; ANOVA and Tukey's HSD, $p < 0.01$), while the levels of *Axin2* was increased in EC-BSH compared to PBS (Figure 5.11; ANOVA and Tukey's HSD, $p < 0.05$) and *Myc* was increased in EC compared to PBS (Figure 5.11; ANOVA and Tukey's HSD, $p < 0.01$).

5.2.10 Correlation analysis reveals a relationship between polyp multiplicity, *I-babp* and T-UDCA

We ran correlation analyses between polyp numbers, α -diversity indices, CAG relative abundances, faecal and plasma bile concentrations, and the relative transcription of our cytokines, Wnt and FXR target genes. The significant correlations, after FDR correction for multiple comparisons, are presented in Figure 5.12.

Our correlation analysis revealed a negative association between the transcription of *I-babp* and the number of polyps in the distal small intestine, as well as the total number of polyps in the small intestine of each mouse (Figure 5.12A, Pearson's R , $p < 0.05$ after FDR adjustment). In contrast, there was a positive relationship between *I-babp* and both *Axin2*

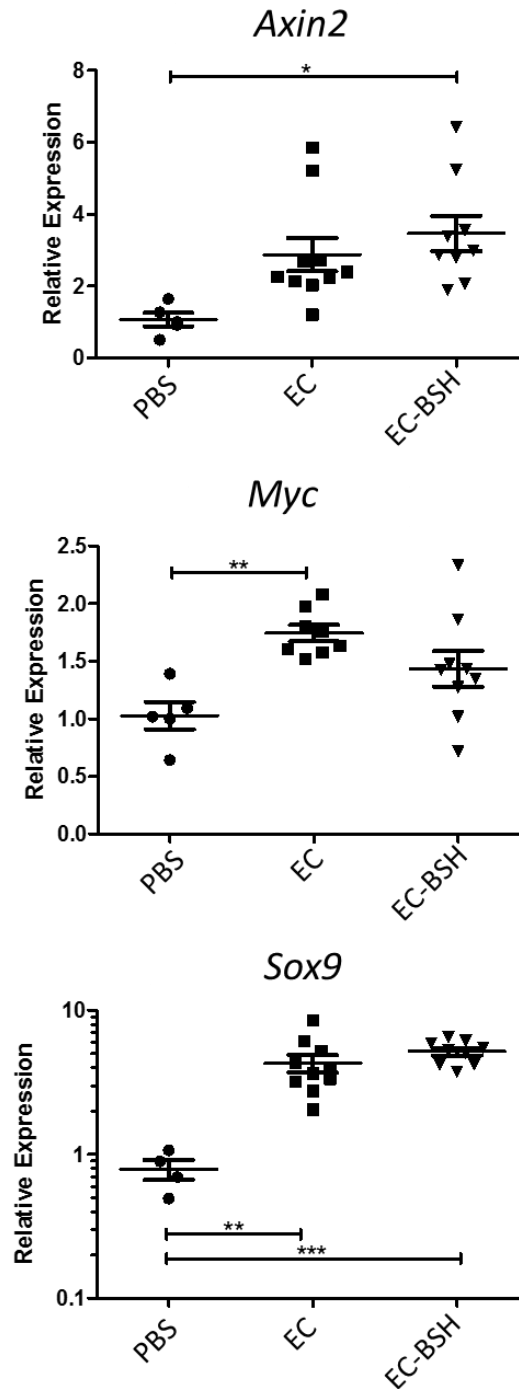


Figure 5.11: Transcription of Wnt target genes is upregulated by bacterial gavage. In order to determine if the changes in polyposis induced by EC-BSH occur upstream or downstream of activation of Wnt signalling, we measured the transcription of Wnt target genes *Axin2*, *Myc* and *Sox9* in the distal ileum of each mouse. mRNA levels of these genes were increased in EC and EC-BSH mice, indicating an activation of Wnt signalling by bacterial gavage (ANOVA and Tukey's HSD, $p < 0.05$).

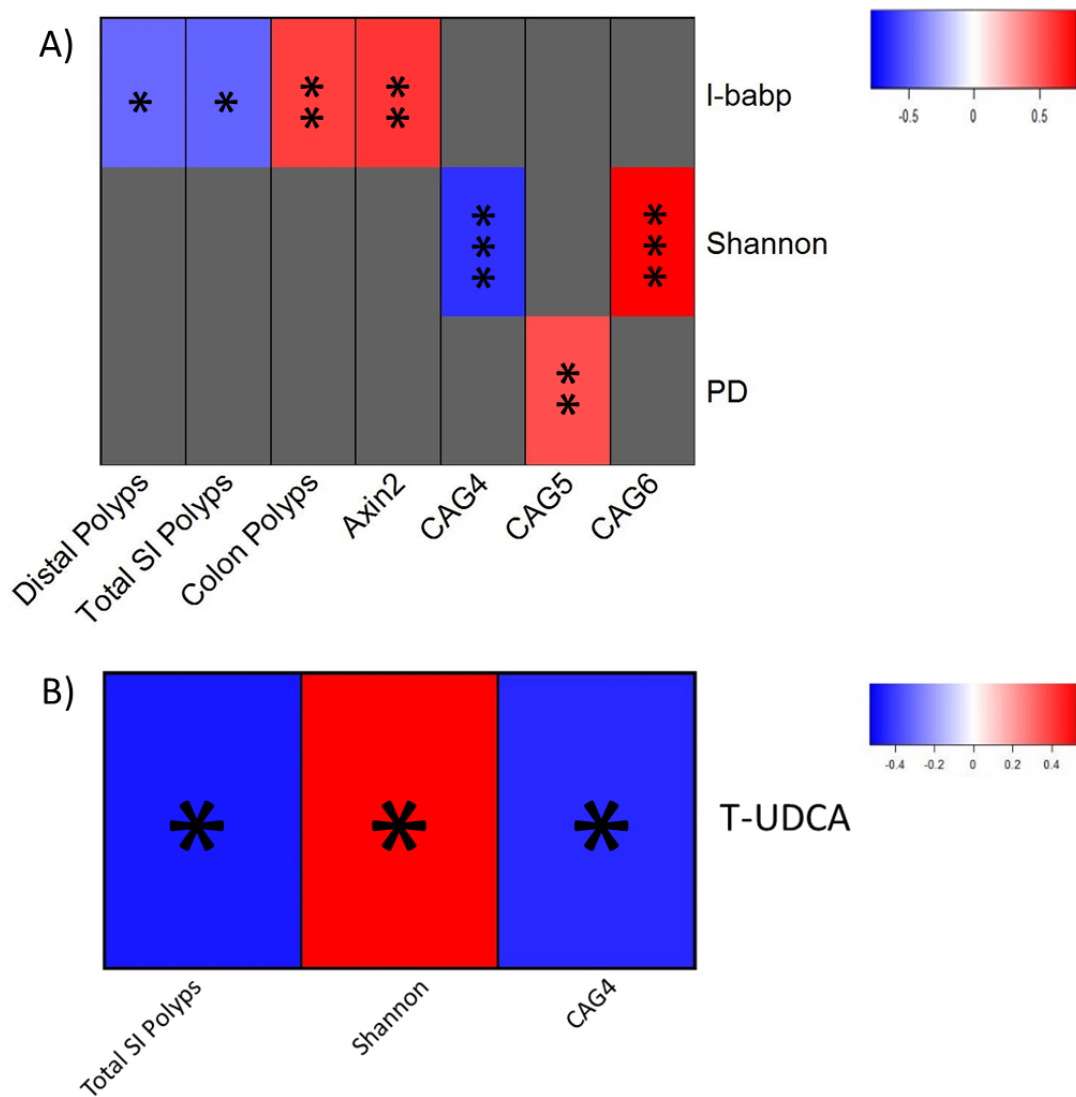


Figure 5.12: Correlation analysis reveals significant relationships between polyp multiplicity, *I-babp* and T-UDCA. The gut presents a complex milieu of interacting forces. In order to examine possible interactions between different aspects of our data in this environment, we performed a correlation analysis between several data-points collected in our study. **A)** After correction for multiple comparisons, this revealed a negative correlation between our FXR target gene *I-babp* and polyp number, and a positive correlation between *I-babp* and colonic polyps and transcription of Wnt target gene *Axin2* (Pearson's R and Spearman's R^2 , $p < 0.05$ after FDR adjustment). **B)** Given the enrichment for tauro-ursodeoxycholic acid (T-UDCA) detected by UPLC-MS, we also considered the correlations between this bile acid and other features of our data without correction for multiple comparisons. This revealed a negative correlation between T-UDCA and the total number of small intestine (SI) polyps (Spearman's $R = -0.48$, $p = 0.03$).

and the number of colonic polyps in each mouse, although the number of colonic polyps was zero-inflated (Figure 5.12A, Pearson's R and Spearman's R^2 , $p < 0.01$ after FDR adjustment).

There were also three correlations between our α -diversity matrices and the relative abundance of CAGs 4, 5 and 6 (Figure 5.12A, Pearson's R, $p < 0.01$ after FDR adjustment).

Furthermore, given the enrichment of T-UDCA detected by UPLC-MS, we also considered the correlations between this bile acid and other features of our data without correction for multiple comparisons. This revealed a negative correlation between T-UDCA and the total number of small intestine (SI) polyps (Figure 5.12B; Spearman's $R = -0.48$, $p = 0.03$).

5.2.11 Diversity of the metagenome predicted by PICRUSt differs between groups

In order to investigate any changes in the capacity of the microbiota to metabolise bile acids after bacterial gavage, we compared the predicted abundance of *baiCD* (COG1902), a gene cluster involved in the 7α -dehydroxylation of bile acids into secondary bile acids, and *bsh* (COG3049), the enzyme responsible for cleaving the amino acid conjugate from bile acids (19). These genes represent the two major pathways of microbial bile acid metabolism. No differences were detected in the abundance of these genes predicted by PICRUSt (Figure 5.13A; ANOVA, $p > 0.05$).

We also calculated the α - and β -diversity of the metagenomes predicted by PICRUSt. Alpha (α)-diversity was significantly lower in EC-BSH mice than EC or PBS (Figure 5.13B; KW test followed by MWU-test, $p < 0.05$), while β -diversity was significantly different between EC-BSH and EC groups (Figure 5.13B; KW test followed by MWU-test, $p = 0.032$) but not between any other group (Figure 5.13B; KW test followed by MWU-test, $p > 0.05$).

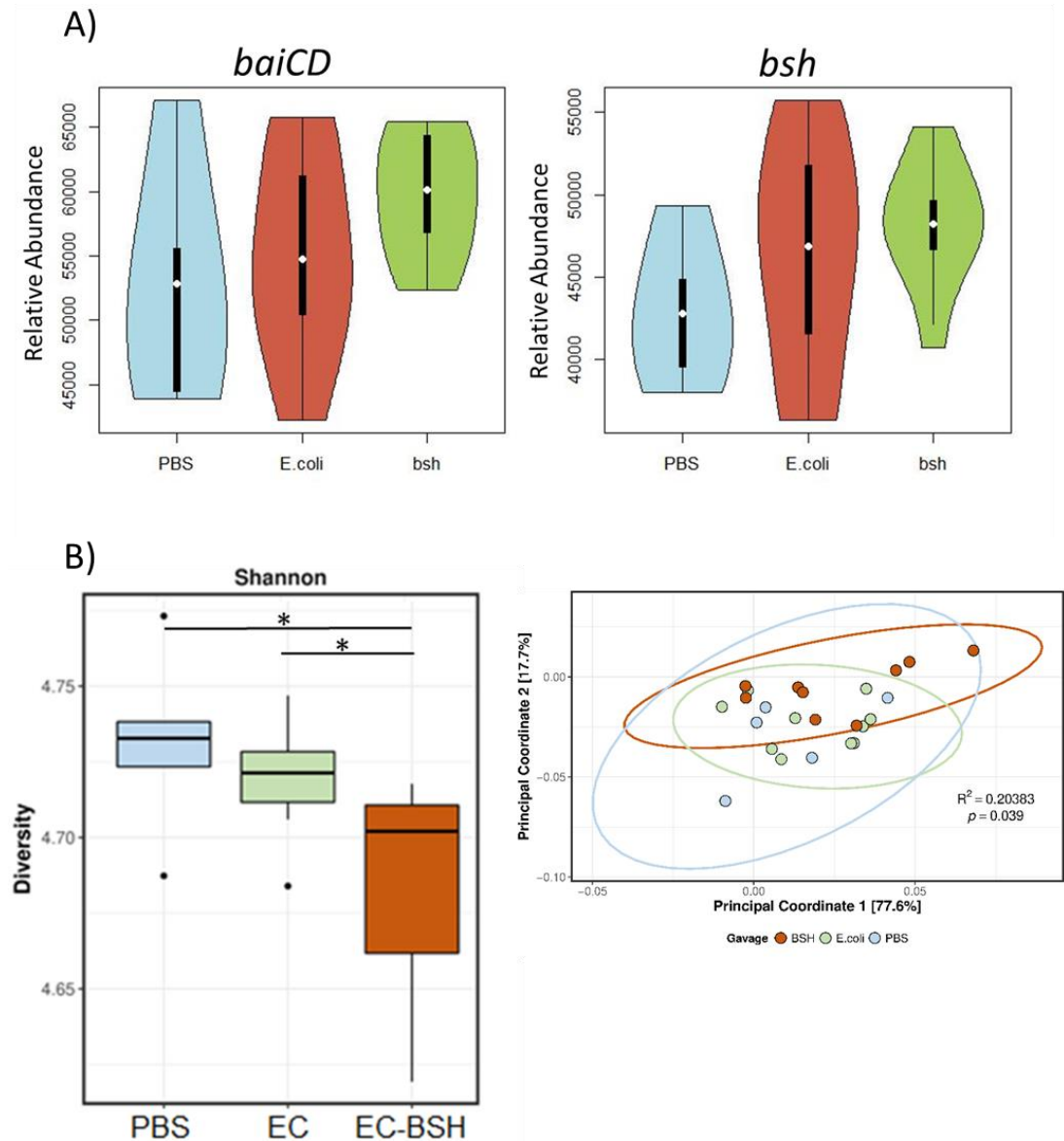


Figure 5.13: Metagenomic α - and β -diversity predicted by PICRUSt differ between groups, but the abundance of bile acid metabolising genes *bsh* and *baiCD* do not. We used PICRUSt to predict the gene content of the faecal microbiota in our groups. We compared their α - and β -diversity, and the abundance of genes *bsh* (COG3049) and *baiCD* (COG1902), a gene involved in the 7α -dehydroxylation of bile acids into secondary bile acids. A- and β -diversity differed significantly between groups, while the predicted abundance of *bsh* and *baiCD* did not (ANOVA, KW test and PERMANOVA, with MWU-test post-hoc analysis). $N=5$, 10 and 9 for PBS, EC and EC-BSH. Figures in **B** were provided by Calum Walsh at Teagasc, Moorepark.

5.3 Discussion

Probiotic supplementation is becoming a popular health-promoting regime in modern society and has been linked with a number of positive effects (20). The bile salt-hydrolysing enzyme Bsh is widespread among probiotic bacteria but there is some uncertainty concerning its effects on human health. This is foremost the case for CRC, where the potential for Bsh to increase the colonic luminal content of cytotoxic secondary bile acids could increase cancer risk. We sought to clarify the influence of Bsh by treating a mouse model of CRC with a laboratory strain of *E. coli* overexpressing Bsh. This revealed a significant decrease in polyposis in the small intestine of this model, an effect that was primarily manifested in the ileum.

We also investigated other facets of the host and microbial response in an attempt to identify the mechanism involved in this effect. Modulation of the intestinal immune response may affect carcinogenesis (21). Cytokines and chemokines are soluble immune factors that can increase cancer risk by a number of mechanisms which may also be influenced by the microbiota (22-34). However, cytokine and chemokine transcription in the EC-BSH group did not differ from EC for any of our measures genes. Transcription of *Cxcl1* was decreased in EC-BSH compared to PBS, but this was also the case for EC mice and so does not explain the decrease in polyposis in Bsh-treated mice. It also concurs with the understanding that inflammation occurs late in APC^{MIN} mice and is not required for tumour initiation in the small intestine (4, 35). Chemokines are a class of chemotactic cytokines and their control of immune cell infiltration modulates the composition of the tumour microenvironment (36). Generally, ELR⁺ chemokines such as CXCL1 are pro-angiogenic while ELR⁻ chemokines are angiostatic (37). Chemokines can also drive the expression of growth-promoting genes such as *CCND1*, and suppress apoptosis in tumour cells by up-regulating *MDM2*, inhibiting *BCL-2* expression and suppressing caspase activation (26). Chemokines' ability to induce cell

infiltration has also been co-opted by tumours to promote invasion and migration (38). There is also evidence that each of these chemokines is regulated by the microbiota, as their expression was reduced by a probiotic cocktail containing four strains of *Lactobacillus*, three strains of *Bifidobacterium* and one strain of *Streptococcus*, which also suppressed tumour formation in the colon of a mouse model of colitis-associated CRC (34).

We did observe disruption to the microbial diversity and composition as a result of Bsh treatment, including a significant increase in Shannon diversity compared to the EC group. Studies on α -diversity in CRC have presented conflicting results, reporting both increased and decreased tumour-associated α -diversity (2, 39-54). Further research is necessary to determine if the α -diversity is a useful marker in CRC or whether it simply reflects more informative changes at a compositional level.

The increased Shannon diversity of the microbiota of the EC-BSH group contrasted with the decrease in the diversity of its metagenome predicted by PICRUSt. The microbial and metagenomic β -diversities of this group were also significantly different. The richness of the microbial metagenomic content has previously been associated with positive metabolic effects in obese individuals (55). Our data suggest significant disruption to the composition and function of the microbiome in EC-BSH mice which could have significant effects on host function.

Next, we considered the specific taxa that differed between groups. The abundance of three taxa in the EC-BSH group were significantly altered compared to both other groups. *Ruminococcaceae* UCG-005, an uncultured member of the *Ruminococcaceae* family, was significantly more abundant in EC-BSH than EC or PBS groups. This taxon has been labelled a universal health biomarker and many *Ruminococcaceae* produce butyrate, a short chain fatty acid (SCFA) which is protective against CRC (56, 57). The abundance of *Tyzzarella* was also higher in EC-BSH than EC or PBS. One member of this genus, *T. piliformis* (previously

Clostridium piliformis), was identified as the causative agent of an infectious diarrhoea of laboratory mice (58). It was reduced in Chinese gastric cancer patients (59), and by probiotic treatment of dairy calves (60). *Tyzzellerella* is also a member of the *Lachnospiraceae* family which have been associated with butyrate production (57). The abundance of *Lachnospiraceae* UCG-001 is increased in EC-BSH compared to PBS but lower than EC. Members of *Lachnospiraceae* family have been associated with butyrate production and some produce Bsh (57, 61). This taxon was decreased in Type II Diabetes Mellitus (TIIDM), where it was negatively associated with TIIDM biomarkers such as cholesterol, triglycerides, IL6 and TNF α (62). It was associated with reduced systemic inflammation and improved intestinal barrier function in a model of liver injury, and with SCFA production and suppression of inflammation in a study of antimicrobial nanocomposites (63, 64). It was also decreased in hepatocellular carcinoma but enriched in obese mice (65, 66), and was suppressed by T-CA injection into the pancreatic duct (67). Of these three taxa, two have strong evidence of positive effects on host health, while the few reports available on *Tyzzellerella* are ambivalent. The abundance of *Lachnospiraceae* UCG-001 in EC-BSH as an intermediate between both other groups may also hint at two or more interacting effects contributing to the observed differences.

In order to investigate these possible separate effects, we considered the taxa that differed between EC-BSH and one of either PBS or EC. The relative abundances of six taxa were significantly different between EC-BSH and EC groups. *Akkermansia* is a symbiotic mucin degrader of the *Verrucomicrobia* phylum which was decreased in EC-BSH compared to EC (68). It does not produce butyrate but facilitates butyrate production by breaking down complex mucins for fermentation by other species (69). It improves intestinal barrier function and a reduction of *Akkermansia* later in life is associated with inflammation and reduced barrier function which can be rectified by butyrate supplementation (70-72). However, its abundance was increased in CRC patients compared to controls and it was

enriched by a fibre-free and high fat diet with the associated negative metabolic effects (73, 74). *Acetatifactor* is also enriched in EC-BSH compared to EC, where it was completely absent. This *Lachnospiraceae* produces butyrate and was increased in mice by treatment with anti-inflammatory milk (75, 76). However, it was also enriched in mice fed a high fat diet and was suppressed by administration of cancer-protective complex oligosaccharides (77, 78). It was more abundant in arthritic mice and may aggravate colitis (79, 80). It also grew on bile salt-containing media, was induced by FXR agonist fexaramine, and correlated positively with UDCA in mice fed a high-fat diet (75, 77, 81). *Clostridiales vadinBB60; uncultured rumen bacterium (URB)* was also more abundant in EC than EC-BSH, where it was completely absent. This taxon was enriched in mice treated with environmental carcinogen *N*-nitrosamine and by administration of a high fat diet (82, 83). It was increased in mice with an induced allergic response and in protozoan-infected chickens, which could be ameliorated by administration of sodium butyrate (84, 85). It was also suppressed by cancer-protective complex oligosaccharides (86). *Clostridiales vadinBB60; URB* was also completely absent from the PBS group but this was not significantly different from EC, most likely due to the smaller *n*-number in this group, and this suggests its enrichment may be a result of *E. coli* treatment or a cage effect. The uncharacterised taxon *Rhodospirillaceae; uncultured* was decreased in EC-BSH compared to EC. Data are sparse on the role of this *Proteobacterium* in CRC but it was increased in a mouse model of diet-induced obesity, and reduced by vancomycin and the chemotherapeutic drug cyclophosphamide (87, 88). The taxa *Lachnospiraceae; uncultured* and *Lachnoclostridium* were both more abundant in the EC-BSH and PBS groups compared to EC. *Lachnospiraceae* have been discussed previously, while the *Lachnoclostridium* genus has been reported to be decreased in CRC compared to healthy tissue and healthy individuals (89, 90), although the *Lachnoclostridium* species *Clostridium symbiosum* was enriched in CRC patients (91). *Lachnoclostridium* also contains major bile acid 7 α -dehydroxylating species *C. scindens* and *C. hylemonae*, responsible for the formation of secondary bile acids.

The relative abundance of four taxa were significantly different between EC-BSH and PBS groups. *Coriobacteriaceae* UCG-002 was enriched in EC-BSH compared to PBS. This *Actinobacterium* was associated with propionate and acetate production in dairy calves, but not butyrate, and was increased in mice fed a processed meat diet (92, 93). Other *Coriobacteriaceae* degrade mucin and were associated with cholesterol and triglyceride levels as well as Crohn's disease, while members of the genus *Eggerthella* can oxidise bile acids (94-97). They also correlated positively with reduced IL6 after whole-grain supplementation, and were reduced by butyrate-fortified milk (98, 99). *Coriobacteriaceae* also metabolise isoflavones, including daidzein and genistein (100). Hydrolysing phenylated compounds such as flavonoids has been suggested as a function of Bsh in the microbiota due to their homology to penicillin amidases (13). Consequently, increased luminal expression of Bsh could select for taxa which can utilise this conversion, such as *Coriobacteriaceae*, although Bsh is primarily located intracellularly. Typical isoflavone concentration in the rodent diet provided to our mice was 150 to 250 mg/kg. Consumption of these compounds is associated with reduced CRC risk, possibly mediated by alterations in the composition of the microbiota or by interaction with the oestrogen receptor, which provide additional mechanisms by which Bsh may influence CRC risk (101-104). *Parvibacter* was also increased in EC-BSH compared to PBS. *Parvibacter* is a member of the *Coriobacteriaceae* family which was isolated from a mouse model of IBD (105). It was enriched by cancer-protective green tea supplements, but associated with a reduction in SCFAs in mice fed a high fat diet (82, 106). *Anaeroplasma* is enriched in both EC-BSH and EC compared to PBS, while *Faecalibacterium* shows the opposite pattern. The opportunistic pathogen *Anaeroplasma* is a member of the *Mollicutes* class in the phylum *Tenericutes*, and was enriched in mice treated with genotoxic azoxymethane and fed a high fat diet (107). *Faecalibacterium* is a major butyrate producer associated with anti-inflammatory properties and improved

intestinal barrier function (108-110). It was reported to be reduced in CRC (111, 112), although another study found no change in the stool CRC patients (113).

Finally, one taxon differed between PBS and EC; *Ruminococcaceae* UCG-010. The *Ruminococcaceae* family has been discussed previously while the specific taxon described here was increased in the caecal microbiota of hens by probiotic treatment (114).

A number of CAGs also differed between groups across the experiment which may describe sets of interacting taxa, as described in section 3.3. The relevance of these groups and interactions to CRC is being investigated.

Bsh may modulate the microbiota by a number of mechanisms. The level of *bsh* expression in different bacteria correlates with their sensitivity to bile salts (115). A reduction in luminal bile salt concentrations could reduce this stress on bile salt-sensitive taxa leading to their enrichment. Increased bile salt deconjugation could also provide a nutritional advantage for taxa which can utilise the liberated amino acid as a source of carbon, nitrogen or energy (13). Bsh may also facilitate the incorporation of cholesterol or bile into bacterial membranes to improve membrane integrity and bacterial persistence (13). Bile acid receptor FXR has also been shown to regulate innate immunity in the intestine, and alterations in the bile acid pool precipitated by Bsh may also interfere with intestinal host-microbiota homeostasis (116, 117).

In summary, there is ample evidence of probiotic, anti-tumorigenic changes in the microbiota of mice administered in the EC-BSH group which may account for the reduction in polyps observed in these mice. This is presented most clearly by species whose relative abundance is greater in EC-BSH than both other groups, which was the case for *Ruminococcaceae* UCG-005, a taxon with strong positive associations with health. Taxa which only differed between EC-BSH and one other group only explain part of the discrepancy in polyp multiplicity but may still contribute to our understanding, such as the enrichment of *Lachnoclostridium* and

the suppression of *Clostridiales vadinBB60*; *URB* in EC-BSH compared to EC; taxa which have conspicuous positive and negative associations with health, respectively. We also observe changes counter to expectation in EC-BSH mice, such as the enrichment of opportunistic pathogen *Anaeroplasm* and suppression of beneficial *Faecalibacterium* compared to PBS. Several taxa have ambivalent or ambiguous effects on health, which may be due to the lack of species-level taxonomic identification provided by 16S rRNA gene sequencing or uncertainty regarding the metabolic capacity of rare and uncultured members of the gut microbiota. In this case, consistent association with health or disease states in studies such as this may highlight key species for deeper investigation.

We also measured the levels of faecal and plasma bile acids in our model at the end of the study. This revealed a significant increase in T-UDCA in the EC-BSH group. This equated to an average of 0.3% of the faecal bile acid content in the EC-BSH group, or 166µM, but this concentration has been shown to have a significant effect *in vitro*, including an inhibition of proliferative response in a murine mixed lymphocyte culture and enhancement of FXR activation by CDCA in Caco-2 cells (118, 119). Additionally, human subjects consuming 900mg of UDCA had average faecal water concentrations of this bile acid of 47µM, and this dose of UDCA has been reported to have biologically relevant effects (120, 121). UDCA protects against cancer and has been reported to be reduced in CRC patients (120, 122-130). UDCA has also been shown to reduce cancer risk factors such as inflammation, oxidative stress and obesity-associated metabolic dysfunction (118, 131-136), and enhance MHC-mediated tumour surveillance (137). T-UDCA may activate FXR (138) and free UDCA can potentiate activation of FXR by other bile acids by increasing their binding affinity for I-BABP (119). The authors suggest this could reduce the levels of free bile acids in colonocytes, which could decrease the risk of unintended pro-tumorigenic effects resulting from an enrichment of free and secondary bile acids by Bsh. This could operate in combination with the inherent reduction in the hydrophobicity of the bile acid pool induced by UDCA (139). FXR has been

shown to be protective against CRC, and suppression of FXR expression by promoter methylation is a feature of APC^{MIN} mice and advanced colorectal carcinomas (10, 18, 140). T-UDCA also correlated negatively with the total number of small intestine polyps in our data before correction for multiple comparisons. We also observed an increase in RNA levels of *I-babp* in our EC-BSH group compared to PBS, and the transcription of this gene correlated negatively with total small intestine polyps, suggesting enhanced activation of tumour-suppressor FXR and adding plausibility to this mechanism (10). However, the increase in transcription of *I-babp* was also observed in the EC group compared to PBS.

The origin of increased T-UDCA in the EC-BSH group is unclear. Low levels of UDCA are found in germ-free mice and higher levels are present in conventionally colonised mice, suggesting that UDCA is a primary bile acid in mice but that levels are increased by microbial bile acid metabolism (141). There have also been reports of a reduction in UDCA in response to treatment with a single antibiotic but a large increase in T-UDCA in response to combined antibiotic treatment, suggesting that the increase is not a product of intestinal bacteria (142). Our mice were exposed to streptomycin but this was the case for all three groups and so would not explain the differences between EC-BSH and the PBS and EC groups, unless there was an interaction between streptomycin and Bsh. Endogenous concentrations of murine CDCA and UDCA are low as these bile acids are converted to α - and β -MCA respectively, by hydroxylation at the 6-carbon position by the *Cyp2c* gene cluster (143). *Cyp2c*-null mice also have high concentrations of UDCA and T-UDCA, and reduced levels of FXR-antagonist MCAs, due to lack of conversion from CDCA and UDCA (143). We did not observe changes in the faecal or plasma concentrations of MCAs, however, which suggests that the increase in T-UDCA is not due to alterations in hepatic bile acid synthesis. The enrichment of T-UDCA in faecal samples, but not plasma, further implies that the changes are as a result of microbial action in the large intestine. UDCA can be formed from CDCA by 7 α -hydroxysteroid dehydrogenase enzymes present in the microbiota. These microbial enzymes account for the

greater abundance of β -MCA in mice, despite the greater affinity for the formation of α -MCA in liver extracts, by epimerising the hydroxyl group located in the 7-carbon position of α -MCA. This is the same modification that converts CDCA to UDCA (144). This enzyme is also produced by strains of *E. coli* K12 and so epimerisation may be enriched in our model (145, 146). However, levels of endogenous murine CDCA are low, as described previously, and so may not explain the enrichment of UDCA in our data. Other potential routes for UDCA generation include dehydroxylation of the 3-carbon hydroxyl group of CA or ursocholic acid, or the 6-carbon hydroxyl group of MCAs, but enzymes capable of these reactions have not been identified.

Finally, we considered the colonisation levels detected in our gavaged mice. Colony forming units (CFUs)/mL of *E. coli* and *E. coli* overexpressing *bsh* were similar in the EC and EC-BSH groups across the experiment, while no bacteria were detected in the PBS group. This suggests that the effects observed in the EC-BSH group are a consequence of the expression of *bsh* rather than colonisation potential, despite the ability of *bsh* to improve bacterial persistence *in vivo* (147).

In conclusion, we have provided evidence for the protective role of Bsh in polyposis in APC^{MIN} mice. Mechanisms may involve alterations to the composition and function of the microbiota, including in microbial bile acid metabolism resulting in enrichment for T-UDCA. This may reduce polyp multiplicity via FXR signalling, and/or a reduction in bile acid pool hydrophobicity and the intracellular levels of free and secondary bile acids.

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6.0 Final Discussion

The aim of my thesis was to investigate whether alterations in the gut microbiota are a cause or consequence of intestinal neoplasia. Benign and pre-malignant changes in the host can alter the gut microenvironment in such a way as to impact the microbiota. The interactions which occur during the transition from normal to abnormal tissue are less well understood, however, and may reveal a role for the microbiota in cancer initiation. There is also evidence that taxa in the tumour microenvironment influence tumour progression (1), but questions remain regarding the breadth of taxa involved and the mechanisms by which they influence disease. Therefore, we examined host and microbial alterations before, during, and after cancer onset, and set them to a timeline (**Chapters 3 and 4**) and investigated a microbial intervention in CRC (**Chapter 5**). Our time-course studies identified distinct sets of taxa associated with cancer onset and progression which were differentially associated pro- or anti-tumorigenic properties. We also investigated host bile acid homeostasis and revealed its potential role in CRC development and interaction with the gut bacteria. An overview of the effects we saw in each group is presented in Table 6.1.

In **Chapter 3**, we observed pro-tumorigenic changes in the microbial composition of mice administered AOM prior to detection of host neoplastic transformation, which suggests that the microbiota may act as an instigating factor in the development of sporadic CRC and that prophylactic modulation of the gut microbiota could potentially reduce cancer risk. This longitudinal study also provided us with the opportunity to track changes in the microbiota during cancer progression and this identified a set of taxa distinct from those associated with tumour initiation. The bacterial changes at our earliest time-point in mice administered AOM primarily reflected a suppression of anti-inflammatory bacterial species such as those which produce butyrate, which was followed by pro-inflammatory cytokine transcription in these mice. Later, we observed an enrichment of pathogenic taxa which may contribute to cancer

Table 6.1: Comparison of major study effects

Time-point 1		Time-point 2		Time-point 3		Time-point 4			
AOM	APCmin	AOM	APCmin	AOM	APCmin	AOM	APCmin	APC/bsh	
ns	ns	ns	ns	ns	ns	ns	ns	ns	Body Weight
↑	↑	↓	ns	ns	ns	ns	↑↑	↑	Microbial α-diversity
**	**	*	**	*	**	**	**	**	Microbial β-diversity
↓↓	ns	↓↓	ns	ns	ns	ns	ns	ns	Total Bile Acids
ns	ns	ns	↓↓	ns	ns	ns	ns	↓	PICRUSt α-diversity
ns	ns	ns	**	ns	ns	ns	ns	*	PICRUSt β-diversity
ns	ns	ns	↑	ns	ns	ns	ns	ns	baiCD/bsh
↓	-	↑↑	-	↑↑	-	↑	↑	ns	Inflammation
ns	-	ns	-	↑	-	↑↑	↑↑↑	↓	Neoplasia
ns	-	ns	-	ns	-	ns	ns	ns	Proliferation
-	-	-	-	-	-	-	↓	ns	FXR

Note that time-points 1-4 correspond to Weeks 8, 12, 24 and 48 in the AOM study and Weeks 4, 7, 11 and 14 in the APC^{MIN} longitudinal study, as defined in the respective chapters. The data from our intervention study is presented from mice treated with *E. coli* overexpressing *bsh* compared to wild-type *E. coli* at time-point 4 (Week 14) only. Direction of arrows signify the direction of change compared to control, with the number of arrows representing significance. Cells marked *ns* were not significantly different. Cells marked with a dash (-) were not measured. Asterisks represent degree of significance for statistics without directionality (<0.05, <0.01 and <0.001 for *, ** and *** respectively).

progression. We also measured the α -diversity of the microbiota in this model. The literature is inconsistent with respect to the effects of CRC on α -diversity, with reports of increased and decreased diversity in the microbiota of CRC patients (2-6). Our results may explain part of this discrepancy as we saw a sequential increase and decrease in α -diversity in our AOM-induced mice over time. The reduction in α -diversity occurred concurrently with the initiation of the inflammatory response, suggesting that the inconsistencies observed in the published literature may be explained, in part, by the inflammatory state in the host and that the relationship between the host and microbiota is dynamic over the course of tumorigenesis.

One thread common to each of our studies was the indication that bile acid alterations accompanied changes in the gut microbiota and host in both the AOM and APC^{MIN} mouse models and informed our microbial intervention strategy in Chapter 5. FXR is a nuclear receptor for bile acids and is particularly noteworthy as it regulates bile acid homeostasis and has recently been reported to play an important role in the suppression of CRC by inducing apoptosis of genetically altered cells and inhibiting proliferation by the Wnt signalling pathway (7). In mice which were administered AOM, the reduction in faecal bile acids and concomitant increase in plasma bile acids prior to CRC development might suggest an increase in ileal bile acid uptake by ASBT which is under negative regulation by FXR. This may have been mediated by enrichment for FXR antagonist bile acids such as tauro-conjugated β -muricholic acid (T- β MCA), which we detected in the plasma. Bile acids also correlated with features of the microbiota in this model such as α -diversity and co-abundance groups (CAGs), suggesting a role for the gut bacteria in bile acid dysregulation. One interesting observation from the longitudinal studies in both mouse models was the emergence of key time-points for changes in the microbiota. In AOM-treated mice, we observed a reduction in α -diversity and a number of significantly difference taxa at Week 12, which occurred concurrently with the onset of inflammatory cytokine transcription. This also

signified a switch from a suppression of anti-inflammatory species to the enrichment of opportunistic pathogens in these mice. Separately, correlation analysis suggested major interactions between the microbiota, bile acids and inflammation at Week 8, possibly feeding into these phenomena at Week 12.

Our APC^{MIN} longitudinal study in **Chapter 4** also presented a view of the temporal microbial landscape during tumour development in a model of familial CRC. This separately identified species associated with cancer initiation and progression, as well as taxa consistently altered throughout the experiment and taxa that remained in flux. This highlights an advantage of research which samples the cancer-associated microbiota at several time-points, as a single snapshot may not fully represent the temporal variation of luminal microbial environment. The microbial composition in APC^{MIN} mice did not have distinct pro-inflammatory associations, as observed in **Chapter 3**, but was enriched for other species associated with CRC. In **Chapter 4**, we observed a significant down-regulation of FXR target gene transcription and a negative correlation between these genes and polyp multiplicity. A key time-point in our APC^{MIN} longitudinal study occurred around Week 7, when there was a significant disruption to the predicted metagenome of these mice, including the abundance of bile acid metabolising genes *bsh* and *baiCD*. This coincided with the onset of polyposis reported in the APC^{MIN} model (8, 9). The timing of these key events suggests their relevance to the role of the microbiota in CRC initiation and invites deeper examination in the future.

This association between the microbial patterns, bile acid signalling and CRC lead us to consider the role of Bsh in CRC development. In our intervention study in **Chapter 5**, mice administered a strain of *E. coli* overexpressing *bsh* developed significantly fewer intestinal polyps compared to *E. coli*-treated or PBS-treated controls. This intervention has previously been shown to have positive effects on lipid metabolism, weight gain and cholesterol levels in the host (10). Our analysis of the bile acid pool in these mice revealed an enrichment of

tauro-conjugated ursodeoxycholic acid (T-UDCA). The enrichment of a conjugated bile acid in mice administered a bile acid-deconjugating enzyme is surprising, and may hint at wider alterations in bile acid regulation than simple deconjugation. This enrichment may account for the decrease in intestinal polyposis we observed, however, as T-UDCA and its unconjugated form UDCA, which has been shown to have similar effects *in vivo*, protect against cancer development and have been reported to be reduced in CRC patients (11-20). T-UDCA also correlated negatively with the total number of small intestinal polyps in our study, before correction for multiple comparisons, reinforcing the possibility that it is responsible for the reduction in polyposis in these mice. Moreover, the FXR target gene *I-babp* was up-regulated in mice exposed to *bile salt hydrolase* (Bsh) compared to PBS-treated, but not *E. coli*-treated mice, and a negative correlation was observed between *I-babp* and small intestinal polyps. Each of these results support the hypothesis that cancer risk is increased by suppression of FXR signalling, possibly mediated by microbial-induced bile acid dysregulation.

6.1 Future Perspectives

The initial changes observed in mice administered AOM was a suppression of species negatively associated with cancer risk, including a number of butyrate producers. Supplementation of these mice with butyrate-producing bacteria before Week 8 may prevent the subsequent inflammatory activation and enrichment for opportunistic pathogens, and would confirm the significance of these changes in CRC initiation. In contrast, the contribution of the microbiota to CRC in APC^{MIN} mice is less obvious. However, our *bsh* intervention study also highlighted a possibly key role of T-UDCA which invites further investigation into the role of this bile acid *in vivo*, and into the effect of *bsh* supplementation on wider bile acid regulation. The effect of microbial metabolism of bile acids such as

conjugated muricholic acids (MCAs) was also highlighted in our research as potentially relevant to CRC pathogenesis. MCAs do not feature in the human bile acid pool but the degree of FXR activation and/or antagonism as a result of microbial action should be considered.

Here, we have established a temporal progression of bacterial changes during the process of tumorigenesis in two mouse models of CRC. We identified patterns in the gut bacteria associated with disease initiation and progression. However, the association of any one species with health or disease is difficult, and considering their effects in isolation, for example in mono-associated animals, is perhaps not reflective of the diverse communities which reside in the gut. In this regard, there are parallels between study of the gut microbiome and genomic research since the turn of the millennium. The microbiome has been described as the second genome and the presence or absence of risk-associated taxa may reflect the role of alleles for complex genetic traits, in that they may contribute to overall disease risk, but the scale of that contribution is influenced by cis- and trans-acting elements. Like research into the human genome, elucidation of these interactions may require large-scale meta-analyses such as the Human Microbiome Project; phase II of which considered microbial configurations observed in irritable bowel disease but not CRC (21). Investment into microbiome research has increased considerably in recent years (22), and this research will continue into the foreseeable future. Our identification of species associated with different stages of the tumorigenic process, as well as the temporal variation apparent in our data, may inform this analysis. We furthermore attempted to address this problem by considering co-abundance groups (CAGs) as structural units of the microbiota. The abundances of these CAGs also appeared to vary during disease progression. The relevance of these variations to CRC, and utility of CAGs as a tool to describe the microbiota, requires further investigation.

We also interrogated our 16S rRNA gene sequencing analysis and produced a significant amount of PICRUSt data to generate a picture of the wider metabolic functioning of the microbiota. PICRUSt predicts the metagenomic content of the microbiome by associating 16S marker gene data with closely related whole genome sequences. We detected a large number of significantly different Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathways in each of our models, such as *linoleic acid metabolism* and *bacterial toxins*, which are presented in the appendix. Picking apart these pathways to identify the specific bacterial genes and functions involved may identify novel metabolic processes which influence the role of the microbiota in CRC.

In conclusion, our research has provided a timeline of host and microbial interactions during the onset and progression of intestinal neoplasia, and has provided new evidence that pro-tumorigenic alterations in the microbiota are an instigating factor in sporadic CRC and a contributing factor in familial CRC. It also highlights a number of mechanisms by which this might occur and suggests possible therapeutic interventions, one of which we validated in a mouse model of familial CRC.

6.2 References

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7.0 Appendix

Table A1: Significantly different pathways predicted by PICRUSt in mice administered AOM

Time	Function	p
Week 8	G protein-coupled receptors	0.001
Week 8	C5-Branched dibasic acid metabolism	0.016
Week 8	Betalain biosynthesis	0.027
Week 8	Indole alkaloid biosynthesis	0.027
Week 12	Vibrio cholerae infection	0.015
Week 12	Ethylbenzene degradation	0.049
Week 12	Ion channels	0.049
Week 12	Linoleic acid metabolism	0.049
Week 24	Amino sugar and nucleotide sugar metabolism	0.008
Week 24	Bacterial toxins	0.011
Week 24	alpha-Linolenic acid metabolism	0.015
Week 24	Bacterial invasion of epithelial cells	0.015
Week 24	Cyanoamino acid metabolism	0.015
Week 24	Phenylpropanoid biosynthesis	0.015
Week 24	Calcium signaling pathway	0.017
Week 24	Lysine degradation	0.021
Week 24	Starch and sucrose metabolism	0.021
Week 24	Carbohydrate digestion and absorption	0.028
Week 24	Glycosphingolipid biosynthesis - lacto and neolacto series	0.028
Week 24	Inorganic ion transport and metabolism	0.028
Week 24	RIG-I-like receptor signaling pathway	0.028
Week 24	D-Alanine metabolism	0.037
Week 24	Insulin signaling pathway	0.049
Week 24	Sphingolipid metabolism	0.049
Week 24	Stilbenoid, diarylheptanoid and gingerol biosynthesis	0.049
Week 24	Transcription machinery	0.049
Week 48	1,1,1-Trichloro-2,2-bis(4-chlorophenyl)ethane (DDT) degradation	0.003
Week 48	Pyruvate metabolism	0.006
Week 48	C5-Branched dibasic acid metabolism	0.009
Week 48	G protein-coupled receptors	0.009
Week 48	Selenocompound metabolism	0.012
Week 48	Valine, leucine and isoleucine biosynthesis	0.012
Week 48	General function prediction only	0.021
Week 48	RIG-I-like receptor signaling pathway	0.021
Week 48	Sphingolipid metabolism	0.021
Week 48	Function unknown	0.036
Week 48	Stilbenoid, diarylheptanoid and gingerol biosynthesis	0.036
Week 48	Apoptosis	0.046

Week 48	Caffeine metabolism	0.046
Week 48	Circadian rhythm (plant)	0.046
Week 48	Fatty acid elongation in mitochondria	0.046
Week 48	Fluorobenzoate degradation	0.046
Week 48	Steroid biosynthesis	0.046
Week 48	Systemic lupus erythematosus	0.046
Week 48	Transcription machinery	0.046

Note: Significance determined by Mann-Whitney U-test. No correction for multiple comparisons. 252 comparisons made.

Table A2: Significantly different pathways predicted by PICRUSt in APC^{MIN} mice at Week 4

Time	Function	p
Week 4	Bisphenol degradation	1.55E-41
Week 4	Carbohydrate digestion and absorption	1.55E-41
Week 4	Other transporters	1.55E-41
Week 4	Insulin signaling pathway	3.11E-41
Week 4	Amino acid metabolism	6.22E-41
Week 4	Ether lipid metabolism	6.22E-41
Week 4	G protein coupled receptors	6.22E-41
Week 4	Pentose phosphate pathway	6.22E-41
Week 4	Aminoacyl tRNA biosynthesis	0.001
Week 4	Fructose and mannose metabolism	0.001
Week 4	Glycosphingolipid biosynthesis lacto and neolacto series	0.001
Week 4	Ascorbate and aldarate metabolism	0.002
Week 4	Carbohydrate metabolism	0.002
Week 4	Pentose and glucuronate interconversions	0.002
Week 4	RIG I like receptor signaling pathway	0.002
Week 4	Colorectal cancer	0.003
Week 4	Huntingtons disease	0.003
Week 4	Influenza A	0.003
Week 4	Small cell lung cancer	0.003
Week 4	Toxoplasmosis	0.003
Week 4	Tryptophan metabolism	0.003
Week 4	Viral myocarditis	0.003
Week 4	p53 signaling pathway	0.003
Week 4	Antigen processing and presentation	0.005
Week 4	Apoptosis	0.005
Week 4	Cell division	0.005
Week 4	Glycine serine and threonine metabolism	0.005
Week 4	Linoleic acid metabolism	0.005
Week 4	NOD like receptor signaling pathway	0.005
Week 4	Others	0.005
Week 4	Progesterone mediated oocyte maturation	0.005
Week 4	Proteasome	0.005
Week 4	Taurine and hypotaurine metabolism	0.005
Week 4	Valine leucine and isoleucine biosynthesis	0.005
Week 4	beta Alanine metabolism	0.005
Week 4	Nucleotide excision repair	0.007
Week 4	Nucleotide metabolism	0.007
Week 4	Other ion coupled transporters	0.007
Week 4	Parkinsons disease	0.007
Week 4	Starch and sucrose metabolism	0.007
Week 4	Amino acid related enzymes	0.010

Week 4	Biosynthesis of ansamycins	0.010
Week 4	Cardiac muscle contraction	0.010
Week 4	Cell cycle Caulobacter	0.010
Week 4	Cellular antigens	0.010
Week 4	DNA repair and recombination proteins	0.010
Week 4	DNA replication	0.010
Week 4	Homologous recombination	0.010
Week 4	N Glycan biosynthesis	0.010
Week 4	Pantothenate and CoA biosynthesis	0.010
Week 4	Ribosome	0.010
Week 4	Translation proteins	0.010
Week 4	Folate biosynthesis	0.015
Week 4	One carbon pool by folate	0.015
Week 4	Propanoate metabolism	0.015
Week 4	Protein export	0.015
Week 4	Purine metabolism	0.015
Week 4	Ribosome Biogenesis	0.015
Week 4	Ribosome biogenesis in eukaryotes	0.015
Week 4	Translation factors	0.015
Week 4	Alzheimers disease	0.021
Week 4	D Glutamine and D glutamate metabolism	0.021
Week 4	Glycan biosynthesis and metabolism	0.021
Week 4	MAPK signaling pathway yeast	0.021
Week 4	Mismatch repair	0.021
Week 4	Pathways in cancer	0.021
Week 4	Peroxisome	0.021
Week 4	Phosphotransferase system	0.021
Week 4	Photosynthesis proteins	0.021
Week 4	Transcription factors	0.021
Week 4	Type I diabetes mellitus	0.021
Week 4	Amino sugar and nucleotide sugar metabolism	0.028
Week 4	Bacterial secretion system	0.028
Week 4	Base excision repair	0.028
Week 4	Biosynthesis of siderophore group nonribosomal peptides	0.028
Week 4	Biosynthesis of vancomycin group antibiotics	0.028
Week 4	DNA replication proteins	0.028
Week 4	Energy metabolism	0.028
Week 4	Epithelial cell signaling in Helicobacter pylori infection	0.028
Week 4	Galactose metabolism	0.028
Week 4	Lipoic acid metabolism	0.028
Week 4	Lipopolysaccharide biosynthesis	0.028
Week 4	Lipopolysaccharide biosynthesis proteins	0.028
Week 4	Penicillin and cephalosporin biosynthesis	0.028
Week 4	Phosphatidylinositol signaling system	0.028
Week 4	Photosynthesis	0.028

Week 4	Benzoate degradation	0.038
Week 4	Biotin metabolism	0.038
Week 4	Glycerophospholipid metabolism	0.038
Week 4	Histidine metabolism	0.038
Week 4	Oxidative phosphorylation	0.038
Week 4	Prenyltransferases	0.038
Week 4	Protein processing in endoplasmic reticulum	0.038
Week 4	RNA degradation	0.038
Week 4	RNA polymerase	0.038
Week 4	Riboflavin metabolism	0.038
Week 4	Sporulation	0.038
Week 4	Terpenoid backbone biosynthesis	0.038
Week 4	Thiamine metabolism	0.038
Week 4	Ubiquinone and other terpenoid quinone biosynthesis	0.038

Note: Significance determined by Mann-Whitney U-test. No correction for multiple comparisons. 252 comparisons made.

Table A3: Significantly different pathways predicted by PICRUST in APC^{MIN} mice at Week 7

Time	Function	p
Week 7	Arachidonic acid metabolism	1.55E-41
Week 7	Glycerophospholipid metabolism	1.55E-41
Week 7	Parkinsons disease	1.55E-41
Week 7	Phenylalanine metabolism	1.55E-41
Week 7	Protein processing in endoplasmic reticulum	1.55E-41
Week 7	Tryptophan metabolism	1.55E-41
Week 7	Type II diabetes mellitus	1.55E-41
Week 7	Ubiquinone and other terpenoid quinone biosynthesis	1.55E-41
Week 7	Ubiquitin system	1.55E-41
Week 7	PPAR signaling pathway	3.11E-41
Week 7	Transporters	3.11E-41
Week 7	Amyotrophic lateral sclerosis	6.22E-41
Week 7	Biosynthesis of vancomycin group antibiotics	6.22E-41
Week 7	Cell division	6.22E-41
Week 7	Circadian rhythm plant	6.22E-41
Week 7	N Glycan biosynthesis	6.22E-41
Week 7	Phosphonate and phosphinate metabolism	6.22E-41
Week 7	Prenyltransferases	6.22E-41
Week 7	Prion diseases	6.22E-41
Week 7	Ribosome biogenesis in eukaryotes	6.22E-41
Week 7	Thiamine metabolism	6.22E-41
Week 7	Tropane piperidine and pyridine alkaloid biosynthesis	6.22E-41
Week 7	Valine leucine and isoleucine biosynthesis	6.22E-41
Week 7	Carbon fixation in photosynthetic organisms	0.0011
Week 7	DNA replication proteins	0.0011
Week 7	Lipoic acid metabolism	0.0011
Week 7	Meiosis yeast	0.0011
Week 7	Mismatch repair	0.0011
Week 7	Non homologous end joining	0.0011
Week 7	Prostate cancer	0.0011
Week 7	RNA polymerase	0.0011
Week 7	Retinol metabolism	0.0011
Week 7	alpha Linolenic acid metabolism	0.0011
Week 7	Alanine aspartate and glutamate metabolism	0.0021
Week 7	Bacterial motility proteins	0.0021
Week 7	Biosynthesis of unsaturated fatty acids	0.0021
Week 7	D Alanine metabolism	0.0021
Week 7	Drug metabolism other enzymes	0.0021
Week 7	Fatty acid metabolism	0.0021
Week 7	Flagellar assembly	0.0021
Week 7	Fluorobenzoate degradation	0.0021
Week 7	Homologous recombination	0.0021

Week 7	Lipopolysaccharide biosynthesis	0.0021
Week 7	Others	0.0021
Week 7	Pentose and glucuronate interconversions	0.0021
Week 7	Photosynthesis proteins	0.0021
Week 7	Primary immunodeficiency	0.0021
Week 7	Protein kinases	0.0021
Week 7	Translation proteins	0.0021
Week 7	Tyrosine metabolism	0.0021
Week 7	Vitamin B6 metabolism	0.0021
Week 7	Zeatin biosynthesis	0.0021
Week 7	Atrazine degradation	0.0031
Week 7	Bacterial invasion of epithelial cells	0.0031
Week 7	Butanoate metabolism	0.0031
Week 7	Carbon fixation pathways in prokaryotes	0.0031
Week 7	Cell cycle Caulobacter	0.0031
Week 7	Citrate cycle TCA cycle	0.0031
Week 7	Cyanoamino acid metabolism	0.0031
Week 7	Cysteine and methionine metabolism	0.0031
Week 7	Cytoskeleton proteins	0.0031
Week 7	General function prediction only	0.0031
Week 7	Glycolysis Gluconeogenesis	0.0031
Week 7	Huntingtons disease	0.0031
Week 7	Lysine degradation	0.0031
Week 7	Other ion coupled transporters	0.0031
Week 7	Pantothenate and CoA biosynthesis	0.0031
Week 7	Pertussis	0.0031
Week 7	Plant pathogen interaction	0.0031
Week 7	Porphyrin and chlorophyll metabolism	0.0031
Week 7	Signal transduction mechanisms	0.0031
Week 7	Sphingolipid metabolism	0.0031
Week 7	Taurine and hypotaurine metabolism	0.0031
Week 7	Toluene degradation	0.0031
Week 7	Vibrio cholerae pathogenic cycle	0.0031
Week 7	beta Lactam resistance	0.0031
Week 7	African trypanosomiasis	0.0051
Week 7	Basal transcription factors	0.0051
Week 7	Base excision repair	0.0051
Week 7	Biosynthesis and biodegradation of secondary metabolites	0.0051
Week 7	Biotin metabolism	0.0051
Week 7	Chagas disease American trypanosomiasis	0.0051
Week 7	Glycan biosynthesis and metabolism	0.0051
Week 7	Lipid metabolism	0.0051
Week 7	Lipopolysaccharide biosynthesis proteins	0.0051
Week 7	RIG I like receptor signaling pathway	0.0051
Week 7	Toxoplasmosis	0.0051

Week 7	DDT degradation	0.0071
Week 7	C5 Branched dibasic acid metabolism	0.0071
Week 7	Carbohydrate digestion and absorption	0.0071
Week 7	Chromosome	0.0071
Week 7	D Arginine and D ornithine metabolism	0.0071
Week 7	Fatty acid biosynthesis	0.0071
Week 7	Flavonoid biosynthesis	0.0071
Week 7	Geraniol degradation	0.0071
Week 7	Germination	0.0071
Week 7	Glycosaminoglycan degradation	0.0071
Week 7	Glyoxylate and dicarboxylate metabolism	0.0071
Week 7	Ion channels	0.0071
Week 7	Isoquinoline alkaloid biosynthesis	0.0071
Week 7	Novobiocin biosynthesis	0.0071
Week 7	Starch and sucrose metabolism	0.0071
Week 7	Synthesis and degradation of ketone bodies	0.0071
Week 7	Viral myocarditis	0.0071
Week 7	Cell motility and secretion	0.0101
Week 7	Folate biosynthesis	0.0101
Week 7	Lysosome	0.0101
Week 7	Oxidative phosphorylation	0.0101
Week 7	Peptidoglycan biosynthesis	0.0101
Week 7	Phenylpropanoid biosynthesis	0.0101
Week 7	Polyketide sugar unit biosynthesis	0.0101
Week 7	Pores ion channels	0.0101
Week 7	Sulfur relay system	0.0101
Week 7	Two component system	0.0101
Week 7	Vibrio cholerae infection	0.0101
Week 7	Glutathione metabolism	0.0151
Week 7	Glycosphingolipid biosynthesis ganglio series	0.0151
Week 7	Glycosphingolipid biosynthesis lacto and neolacto series	0.0151
Week 7	Linoleic acid metabolism	0.0151
Week 7	MAPK signaling pathway yeast	0.0151
Week 7	Nucleotide metabolism	0.0151
Week 7	Other transporters	0.0151
Week 7	Peroxisome	0.0151
Week 7	Proteasome	0.0151
Week 7	Protein digestion and absorption	0.0151
Week 7	ABC transporters	0.0211
Week 7	Amino acid metabolism	0.0211
Week 7	Bladder cancer	0.0211
Week 7	Carotenoid biosynthesis	0.0211
Week 7	Cellular antigens	0.0211
Week 7	D Glutamine and D glutamate metabolism	0.0211
Week 7	Electron transfer carriers	0.0211

Week 7	Glutamatergic synapse	0.0211
Week 7	Glycerolipid metabolism	0.0211
Week 7	Methane metabolism	0.0211
Week 7	Nitrogen metabolism	0.0211
Week 7	Pyruvate metabolism	0.0211
Week 7	RNA degradation	0.0211
Week 7	Ribosome	0.0211
Week 7	Ribosome Biogenesis	0.0211
Week 7	Staphylococcus aureus infection	0.0211
Week 7	Tetracycline biosynthesis	0.0211
Week 7	Transcription related proteins	0.0211
Week 7	Translation factors	0.0211
Week 7	Xylene degradation	0.0211
Week 7	Amino sugar and nucleotide sugar metabolism	0.0281
Week 7	Energy metabolism	0.0281
Week 7	Lipid biosynthesis proteins	0.0281
Week 7	Nicotinate and nicotinamide metabolism	0.0281
Week 7	One carbon pool by folate	0.0281
Week 7	Pentose phosphate pathway	0.0281
Week 7	Proximal tubule bicarbonate reclamation	0.0281
Week 7	Pyrimidine metabolism	0.0281
Week 7	Riboflavin metabolism	0.0281
Week 7	Stilbenoid diarylheptanoid and gingerol biosynthesis	0.0281
Week 7	Systemic lupus erythematosus	0.0281
Week 7	Tuberculosis	0.0281
Week 7	beta Alanine metabolism	0.0281
Week 7	DNA repair and recombination proteins	0.0381
Week 7	Limonene and pinene degradation	0.0381
Week 7	Pathways in cancer	0.0381
Week 7	Penicillin and cephalosporin biosynthesis	0.0381
Week 7	Polycyclic aromatic hydrocarbon degradation	0.0381
Week 7	Protein folding and associated processing	0.0381

Note: Significance determined by Mann-Whitney U-test. No correction for multiple comparisons. 252 comparisons made.

Table A4: Significantly different pathways predicted by PICRUSt in APC^{MIN} mice at Week 11

Time	Function	p
Week 11	Cytoskeleton proteins	0.0021
Week 11	Small cell lung cancer	0.0101
Week 11	Chromosome	0.0151
Week 11	Fatty acid elongation in mitochondria	0.0211
Week 11	Lysine degradation	0.0211
Week 11	Starch and sucrose metabolism	0.0211
Week 11	C5 Branched dibasic acid metabolism	0.0281
Week 11	Fatty acid biosynthesis	0.0281
Week 11	Influenza A	0.0281
Week 11	One carbon pool by folate	0.0281
Week 11	Synthesis and degradation of ketone bodies	0.0281
Week 11	G protein coupled receptors	0.0381
Week 11	Vibrio cholerae infection	0.0381

Note: Significance determined by Mann-Whitney U-test. No correction for multiple comparisons. 252 comparisons made.

Table A4: Significantly different pathways predicted by PICRUSt in APC^{MIN} mice at Week 14

Time	Function	p
Week 14	Inorganic ion transport and metabolism	0.0071
Week 14	Meiosis - yeast	0.0091
Week 14	Bacterial toxins	0.0231
Week 14	D Alanine metabolism	0.0231
Week 14	Sphingolipid metabolism	0.0291
Week 14	Vibrio cholerae pathogenic cycle	0.0291
Week 14	alpha Linolenic acid metabolism	0.0291
Week 14	Transcription machinery	0.0351

Note: Significance determined by Mann-Whitney U-test. No correction for multiple comparisons. 252 comparisons made.

Table A5: Significantly different pathways predicted by PICRUSt between APC^{MIN} mice administered PBS, *E. coli* MG1655 or *E. coli* MG1655 expressing *bsh*

Time	Function	p
Week 14	Pathways in cancer	0.001
Week 14	RIG I like receptor signaling pathway	0.001
Week 14	Pentose and glucuronate interconversions	0.002
Week 14	Mineral absorption	0.003
Week 14	Alzheimers disease	0.004
Week 14	Primary immunodeficiency	0.004
Week 14	Transcription machinery	0.004
Week 14	Fatty acid biosynthesis	0.005
Week 14	Lipid biosynthesis proteins	0.005
Week 14	Other ion coupled transporters	0.005
Week 14	Renal cell carcinoma	0.006
Week 14	Tuberculosis	0.006
Week 14	Arachidonic acid metabolism	0.007
Week 14	Fructose and mannose metabolism	0.007
Week 14	Methane metabolism	0.007
Week 14	Starch and sucrose metabolism	0.007
Week 14	Amyotrophic lateral sclerosis	0.008
Week 14	Ether lipid metabolism	0.008
Week 14	Linoleic acid metabolism	0.008
Week 14	Phenylalanine metabolism	0.008
Week 14	Valine leucine and isoleucine degradation	0.008
Week 14	Drug metabolism other enzymes	0.009
Week 14	Huntingtons disease	0.009
Week 14	Selenocompound metabolism	0.009
Week 14	RNA transport	0.01
Week 14	Lysine degradation	0.011
Week 14	Vibrio cholerae pathogenic cycle	0.011
Week 14	African trypanosomiasis	0.012
Week 14	Biosynthesis of ansamycins	0.012
Week 14	Chagas disease American trypanosomiasis	0.012
Week 14	Cyanoamino acid metabolism	0.012
Week 14	Galactose metabolism	0.012
Week 14	Steroid hormone biosynthesis	0.012
Week 14	Sulfur metabolism	0.012
Week 14	Sulfur relay system	0.012
Week 14	Apoptosis	0.013
Week 14	Cardiac muscle contraction	0.013
Week 14	Colorectal cancer	0.013
Week 14	Fatty acid metabolism	0.013
Week 14	Influenza A	0.013
Week 14	Parkinsons disease	0.013

Week 14	Phosphatidylinositol signaling system	0.013
Week 14	Pyruvate metabolism	0.013
Week 14	Small cell lung cancer	0.013
Week 14	Toxoplasmosis	0.013
Week 14	Viral myocarditis	0.013
Week 14	p53 signaling pathway	0.013
Week 14	Cell motility and secretion	0.014
Week 14	Metabolism of cofactors and vitamins	0.014
Week 14	Ubiquitin system	0.014
Week 14	Bacterial secretion system	0.015
Week 14	Drug metabolism cytochrome P450	0.015
Week 14	Caffeine metabolism	0.017
Week 14	Carotenoid biosynthesis	0.017
Week 14	Circadian rhythm plant	0.017
Week 14	Fatty acid elongation in mitochondria	0.017
Week 14	Fluorobenzoate degradation	0.017
Week 14	Glycosyltransferases	0.017
Week 14	Ion channels	0.017
Week 14	Meiosis yeast	0.017
Week 14	Non homologous end joining	0.017
Week 14	Phenylpropanoid biosynthesis	0.017
Week 14	Steroid biosynthesis	0.017
Week 14	Systemic lupus erythematosus	0.017
Week 14	Prion diseases	0.018
Week 14	Tryptophan metabolism	0.018
Week 14	Tyrosine metabolism	0.018
Week 14	Toluene degradation	0.019
Week 14	Bacterial invasion of epithelial cells	0.02
Week 14	Geraniol degradation	0.02
Week 14	Proximal tubule bicarbonate reclamation	0.02
Week 14	Secretion system	0.02
Week 14	Glutathione metabolism	0.021
Week 14	Prenyltransferases	0.021
Week 14	Isoquinoline alkaloid biosynthesis	0.022
Week 14	Biosynthesis of unsaturated fatty acids	0.023
Week 14	Riboflavin metabolism	0.023
Week 14	Lipopolysaccharide biosynthesis	0.024
Week 14	Glycerophospholipid metabolism	0.025
Week 14	Replication recombination and repair proteins	0.027
Week 14	Base excision repair	0.028
Week 14	Caprolactam degradation	0.028
Week 14	N Glycan biosynthesis	0.028
Week 14	Propanoate metabolism	0.029
Week 14	Ubiquinone and other terpenoid quinone biosynthesis	0.029
Week 14	Valine leucine and isoleucine biosynthesis	0.029

Week 14	Penicillin and cephalosporin biosynthesis	0.03
Week 14	Lipopolysaccharide biosynthesis proteins	0.031
Week 14	alpha Linolenic acid metabolism	0.032
Week 14	Citrate cycle TCA cycle	0.037
Week 14	Metabolism of xenobiotics by cytochrome P450	0.038
Week 14	Inorganic ion transport and metabolism	0.04
Week 14	Amoebiasis	0.041
Week 14	Electron transfer carriers	0.042
Week 14	Protein processing in endoplasmic reticulum	0.042
Week 14	Limonene and pinene degradation	0.043
Week 14	Bladder cancer	0.044
Week 14	Primary bile acid biosynthesis	0.044
Week 14	Secondary bile acid biosynthesis	0.044
Week 14	Phosphotransferase system	0.045
Week 14	Amino acid metabolism	0.046

Note: Significance determined by Kruskal-Wallis test. No correction for multiple comparisons. 252 comparisons made.