The Impact of Antibiotic-Induced Gut Dysbiosis on Cholestatic Liver Disease: The Role of Age and the Effect of Bacterial Reconstitution.

Jack Cameron Hales

100316219

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Abstract

The gut and liver have a well-defined, reciprocal metabolic and signalling relationship, the gut-liver axis, which is dependent upon functional cooperation. Dysfunction in either the gut or liver can result in concurrent issues in both organs. Antibiotics can constitute a significant disruption to the gut microbiota and whilst the immediate effects of antibiotics upon the gut-liver axis are under increasing scrutiny, lingering changes to microbial population and metabolomics remain poorly defined. The effects on the gut-liver axis are also mostly unidentified.

Our study investigates long-term, antibiotic induced, perturbation to the gut- liver axis and how this may subsequently affect liver metabolism, immunity and cholestatic disease progression. To do this, we use a longitudinal murine model of antibiotic depletion, microbial recovery and surgically induced cholestatic injury. Liver, intestinal, faecal and serum samples were collected, and various analyses were conducted. Liver tissue was analysed by flow cytometry, qPCR, histochemistry and immunohistochemistry. Serum was assessed for liver damage markers by biochemical analysis, the intestinal microbiota by shotgun metagenomics and liver and intestinal metabolite content by LC-MS. Additionally, macrophages were differentiated from bone marrow haematopoietic stem cells to investigate their behaviour.

Our results suggest that, following antibiotic depletion, the recovered gut bacteriome has a modified population composition which produces an altered faecal pool of both bile acids and short chain fatty acids. These changes appear to prime the liver and general innate immune response of young mice for pro-fibrotic responses to cholestatic injury. These effects were seen to be alleviated by reconstitution of the antibiotic depleted bacterial species *Bacteroides thetaiotaomicron*. Aged mice show no antibiotic induced changes to cholestatic disease severity.

These results have significant implications, not only for the administration of antibiotics in cases of chronic disease, but also, in highlighting the role of dysbiosis and disrupted microbial metabolism in cholestatic disease more generally.

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Abbreviations

Abbreviations are listed below in order of text appearance.

- Bt- *Bacteroides thetaiotaomicron*
- GI- Gastrointestinal
- ARG- Antimicrobial resistance gene
- AMR- Antimicrobial resistance
- FXR- Farnesoid X Receptor
- TGR5- Takeda-G-protein coupled receptor
- HSC- Hepatic stellate cell
- IEC- Intestinal epithelial cell
- HSC- Hepatic stellate cell
- CDCA- Chenodeoxycholic acid
- LCA- Lithocholic acid
- DCA- Deoxycholic acid
- CA- Cholic acid
- Tα/βMCA- Tauro-alpha/beta-muricolic acid
- UDCA- Ursodeoxycholic acid
- S1PR2- Sphingosine-1-phosphate receptor-2
- PXR- Pregane-x-receptor
- CAR- Constitutive androstane receptor
- VDR- Vitamin D receptor
- RORγT- Retinoic acid related orphan receptor
- SCFA- Short chain fatty acid
- NAFLD- Non-alcoholic fatty liver disease
- GF- Germ free
- FMT- Faecal microbial transfer
- PRR- Pattern recognition receptor
- PAMP- Pathogen associated molecular pattern
- TLR- Toll-like receptor
- NLR- NOD-like receptor
- LPS- lipopolysaccharide
- NFκB- Nuclear factor κ B

- TREG- T-regulatory (Cell)
- IL- Interleukin (referring to cytokines)
- MyD88- Myeloid differentiation primary response 88
- TNFα- Tumour necrosis factor α
- NKT- Natural killer T-(cell)
- SIBO-NASH- cooccurring small intestinal bacterial overgrowth-Non-alcoholic steatohepatitis
- IBD- Inflammatory bowel disease
- DAMP- Damage associated molecular pattern
- AIMD- Antibiotic induced microbial depletion
- FGF- Fibroblast growth factor
- CYP- Cytochrome P450
- IFNγ- Interferon γ
- PSC- Primary sclerosing cholangitis
- PBC- Primary biliary cirrhosis
- HSC- Hepatic stellate cell
- αSMA- αSmooth muscle actin
- BDL- Bile duct ligation
- MDR2- Multi drug resistance gene 2
- AWERB- Animal welfare and ethical review body
- ASPA- Animal scientific procedure act 1986
- Con- Control
- AB- Antibiotics
- ABBDL- Antibiotics recovered/pretreated Bile duct ligation
- VNM(A)- Vancomycin, neomycin, metronidazole, (Ampicillin)
- (See materials and methods 2.3.1 for unlisted bile acid abbreviations)
- ALT- alanine transaminase
- AST- Aspartate transaminase
- ALP- Alkaline phosphatase
- TBil- Total bilirubin
- FITC- Fluorescein isothiocyanate
- BMDM- Bone marrow derived macrophage
- TBP- TATA box binding protein
- BSA- Bovine serum albumin

- CXCL- Chemokine (C-X-C motif) ligand
- CCL- Chemokine (C-C motif) ligand
- MMP- matrix metalloproteinase
- CD- Cluster of differentiation
- PFA- Paraformaldehyde
- Ara-C- cytosine arabinoside
- CFU- colony forming units
- SEM- Standard error of the mean
- BSH- Bile salt hydrolase
- DDC- 3,5-diethoxycarbonyl-1,4-dihydrocholine
- LEfSe- Linear discriminant analysis of effect size
- MASLD- Metabolic associated steatohepatic liver disease (new nomenclature for NAFLD like diseases)
- OMV- Outer-membrane vesicle
- CCL4- Carbon tetrachloride
- HCC- Hepatocellular carcinoma
- ABx6Mo- Antibiotic recovered (6Mo recovery time)
- ALD- Alcoholic liver disease

Figures and tables

Chapter One.
Introduction

1.1 The Microbiome: a metabolically active and stably dynamic population.

A microbiome is a vast population of bacteria, fungi, viruses, and unicellular eukaryotes and their genomic material resident within a defined environmental niche. Microbiomes are resident throughout nature, including within and upon animals, including humans, where they have many important roles in wider organism function [1]. Of particular interest in this thesis are gut-resident microbial populations, as they appear to play the largest role in whole organism functioning and are generally what is referred to by the unqualified term 'microbiome'. A healthy microbiome is highly heterogenous, metabolically active, functionally diverse and relatively stable over time, following an initial developmental period [2,3]. Although composition can vary substantially between individuals [4,5]. Such healthy gut microbial compositions arise given a set of prerequisites; a stable gastrointestinal (GI) environment, nutrient rich and diverse, low in harmful xenobiotics and antimicrobials.

The relationship between host and gut microbiome in homeostatic conditions is mostly symbiotic, the host providing both nutrition and an inhabitable niche, whilst the microbiome provides multiple benefits [6]. These benefits include: increasing resistance to infection [7]; indigestible foodstuff fermentation (which provides essential nutrients, volatile organic compounds- VOCs, and increases the bioavailability of dietary products e.g. glucose [8–10]) immune education and development; and otherwise supplementing many host metabolic processes [6,11,12].

The gut microbiome and its metabolites function and signal not only locally at the level of the GI tract but, via the hepatic portal venous, then systemic circulation, to organs and cells distant from the gut. Additionally, various signalling pathways stimulated locally in the GI tract by resident microbes or their derivatives, can have effects at distant sites, such as the liver, brain and circulatory system [1,13,14]. The products of microbial metabolism can be either beneficial or detrimental in the resolution or development of non-infectious diseases and chronic dysfunction, an effect linked heavily to population composition [12,13,15–18].

Immediately following birth infants are rapidly colonised by a novel population of bacteria viruses and fungi derived from the mother and their immediate environment [19]. The infant gut microbial population composition is highly dynamic, with stabilisation

only achieved post-infancy [20]. The community architecture and diversity of the adult microbiome, in the absence of perturbing factors, is relatively static, as is its metabolome [2,3]. Compositional diversity appears to be the greatest determinant of long-term population stability. In the presence of perturbing factors, microbiomes of greater diversity are most resilient to alteration [2]. It is also remarkable that the presence, absence or abundance of relatively few, indicative, key species is correlated to this overall population-wide diversity [21].

However, despite this relative long-term stability in adults, persistent population changes can be detected, following even brief periods of strong disruption, such as that of a short-course of antibiotics [22–25]. This is especially true in individuals treated in youth, an investigation following the recovery of the microbiome and its effects throughout life in mice having showed distinct recovered microbiome compositions. Each of these compositions had diverse effects upon health and longevity, especially regarding insulin sensitivity. These mice subsequently retained these altered populations throughout life, which stabilised following an initially unstable period of biomass recovery [26].

Microbial population changes and dysfunction arising from antibiotic use.

Dysbiosis is the alteration of the resident microbial population from an initially stable and 'healthy' composition, prompting disruption in the host-microbial network, caused by factors such as antibiotic use, inflammation, dietary change etc. and is associated with the pathogenesis of various diseases [1,20,27,28]. This constitutes an interruption in the commensal nature of host-microbe interaction, driving and driven by, the expansion of opportunistic populations and depletion of beneficial symbionts [25,29–31]. Antibiotic treatment, whilst highly beneficial in the context of infection, frequently generates dysbiosis as a secondary consequence which has been shown to significantly affect bacterial metabolomic activity and diversity down to the species and even strain level [20]. Human adult data demonstrates that in homeostatic conditions the populational composition of the microbiome is still somewhat variable over extended periods of time, however, these changes are comparatively miniscule to those of antibiotic treatment [24]. A single treatment of ciprofloxacin - a broad spectrum fluoroquinolone – for example, leads to significant decreases in microbial diversity and richness, yet these alterations are highly individualised and depend upon a multitude of factors prior to, during and following antibiotic treatment [24,32] (Fig. 1.1).

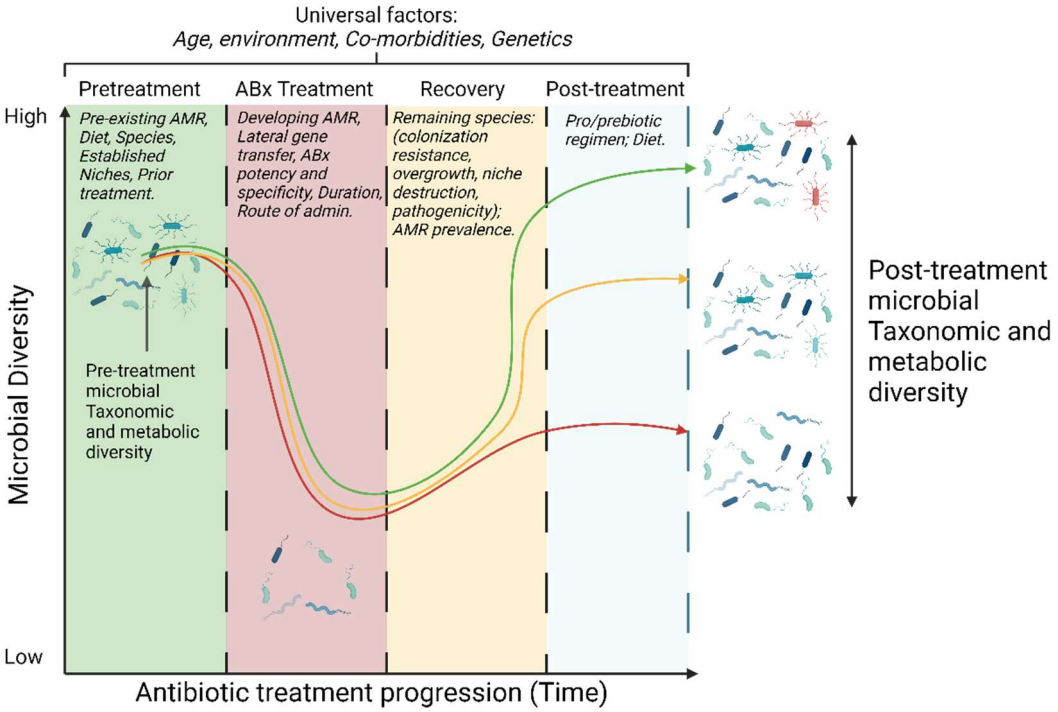

Figure 1.1. The factors influencing antibiotic treatment effects and recovery within the microbiome. *Green arrow: the optimal response and recovery of the microbiome in response to antibiotic treatment- gain of function obtained through reconstitution of the microbiome with beneficial (metabolically active) bacteria e.g. by faecal microbial transplantation. Orange: A net neutral response of the MB to antibiotics, where the recovered state resembles pre-treatment diversity and function- obtained by optimal recovery of existing species within the pre-treatment m. Red: Net-detrimental response to antibiotics where there is a loss of microbial species diversity and function.*

Adapted from: Schwartz DJ, Langdon AE, Dantas G. Understanding the impact of antibiotic perturbation on the human microbiome. Genome Med. 2020 Sep 28;12(1):82. doi: 10.1186/s13073-020-00782-x.

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Antibiotic spectrum and mode of action plays a primary role in determining the microbiota population response. In silico modelling of short-term antibiotic impact shows that, ciprofloxacin produces greater displacement than amoxicillin- as a narrower spectrum β-lactam [33,34]. Additionally, a β-lactam- cefprozil - was shown to alter population composition down to the strain level, altering the dominant strains and intraspecies composition, with limited recovery [33,34]. Differences in antimicrobial resistance gene (ARG) presence and copy number between strains are suggested to be

responsible for the described intra-species disparities. These differences arise during treatment and given the potential for metabolic divergence at the strain level, they could have unforeseen consequences for the microbial metabolome [33,34]. A single treatment of vancomycin or ampicillin, on the other hand, has been shown in mice to generate significant alterations in the microbiome and microbial metabolome, but only at defined loci in the gut, providing a far more precise picture of the effects of antibiotic treatment to the host. The treatment, whilst leaving some loci relatively unchanged, in others caused widespread disruption by reducing metabolic and population diversity and biomass, though these effects were mostly resolved within 5 days of recovery [35]. These results suggest that there may be significant local effects of antibiotic treatment in addition to the GI-wide effects, though the extent of systemic effects caused by localised population alterations are not elucidated.

These changes in population are mostly noted during or immediately following treatment, the retention of said changes into the longer-term is coming under heavy scrutiny, though this remains much less well-defined. As mentioned previously, the infant microbiome is in a dynamic state, it is perhaps unsurprising therefore, that post-natal multi-antibiotic treatment, results in significant microbiome alteration, highly enriched for antimicrobial resistant (AMR) and pathogenic organisms [36]. However, it has been subsequently shown that the healthy infant microbiome, whilst subject to acute disruption, is more prone to recovery to a developmentally consistent microbiome composition [37]. Yet, specific species and ARGs were enriched up to the end of observation (2 years) following treatment, which were virtually non-existent in the healthy control population [37].

It has recently been illustrated in a long-term study that, for adults, microbiome recovery from antibiotic treatment is more limited, yet there is seemingly no significant population bloom that is retained post-recovery. Broad spectrum antibiotic treatment results in short lived blooms of both *Enterococcus spp.* and *E.coli* with a return to normalcy within 8 days, yet after 6 months, whilst total population count was largely restored, species number was significantly reduced in comparison to control. [22]. However, this lack of significant population blooming is challenged by a more recent study by Hildebrand *et al*, which shows that monodominance of low abundance species may be an immediate consequence of antibiotic treatment. They demonstrated population increases of certain species from a near-zero proportion of the overall microbial population, which are retained into the long-term. This does, however,

constitute a relatively small whole-population change. Some species were also wholly depleted, with no recovery possible without intervention [25].

Collectively, evidence suggests that the microbiome develops into a stable composition throughout the developmental period, with the early microbiome being less immediately resilient to perturbation but more 'meta-stable', with only a few AMR and opportunistic organisms retained. Whilst the adult microbiome appears more immediately resilient – given a set of determining factors, especially initial diversity- but less meta-stable, retaining certain species and decreased diversity into the longer term. The accumulation of ARGs is also significant, suggesting that early antibiotic treatment may be more damaging in the long term. It appears that the response of the microbiome and ultimately the host to antibiotic perturbation is governed by three factors: Initial state of the microbiome and its network with the host, strength and duration of perturbation and its recovered state. [20] (Fig. 1.1).

Overall, the microbiome appears both stable and dynamic in equal measure. Its composition is gradually established in early life, during which time its composition is both highly susceptible to perturbation, yet equally prone to recovery to its initial composition. Once the composition is established in this developmental phase it becomes stable, with limited change occurring due to changes in diet, behaviour etc. As a consequence of aging, the microbiome also appears to transition to different stable compositions at different developmental periods with relatively well-defined changes occurring as a function of aging. These factors combined, result in the microbiome being 'stably dynamic'. That is to say, the composition of the gut microbiome, following the developmental period and in homeostatic conditions, is broadly stable at any given point in time, whilst undergoing gradual and somewhat predictable change. Even in cases of severe perturbation, composition shifts to a new, altered, though still stable composition as outlined by the meta-stability hypothesis. The 'metastability' hypothesis suggests that, following severe but temporary perturbation in adults using antibiotics, a recovered microbiome may transition to an alternative yet still stable composition, with similar species number and diversity [20].

The network existing between the host and resident bacteria can be detrimentally affected by microbial perturbation, with such detrimental effects being involved in the pathology of various diseases [9,29–31,38]. However, the reverse is also true, in that host disruption can drive dysfunction, notably these effects are extensive in the gut-liver axis

(discussed below) [9,39–43]. Perhaps the strongest link is with inflammation of the bowel and its associated chronic diseases [39]. However, the lack of a causative link in many non-communicable diseases involving microbiome change is stark, including in liver diseases [44], though recently a study on cholestatic liver disease, suggests an initiating role of the microbiome in the pathology of the disease [45] (discussed below).

1.2 The gut-liver axis: Metabolic, signalling and immune reciprocity

The gut-liver axis describes the system by which the gut- particularly the microbiome- and the liver functionally interact (Fig 1.2). The function and communication axis between these two organs is as a result of the reciprocal requirement for the production of essential metabolites by the other, particularly that of bile acids [17,44,46]. 70% of the

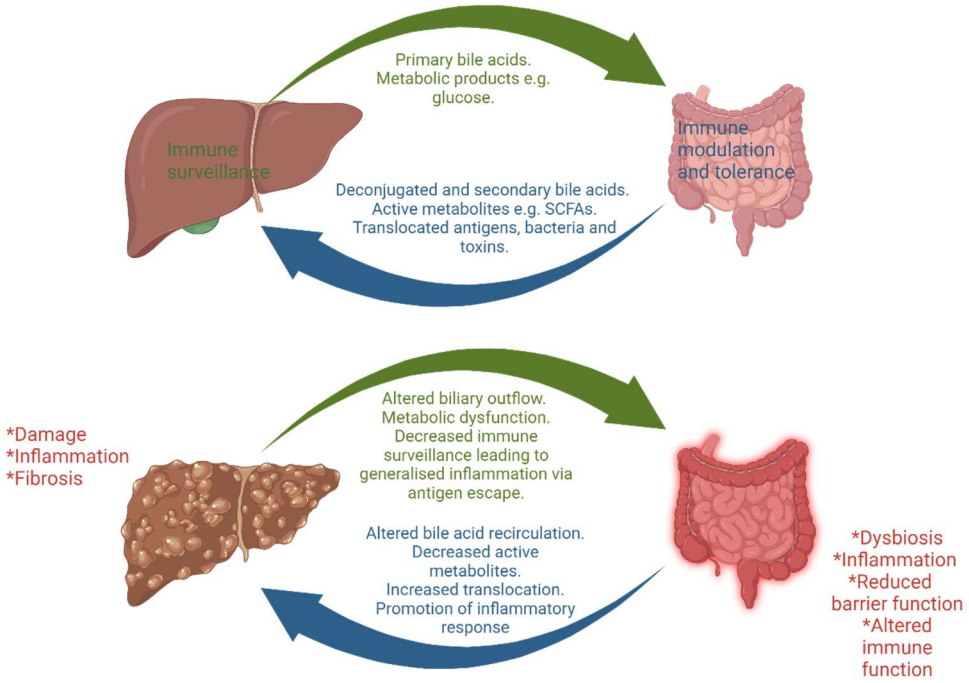

Figure 1.2. The gut-liver axis in homeostatic conditions and during dysfunction. *Top: the gut-liver axis in homeostasis; the liver synthesises conjugated bile acids de novo and provides the products of its metabolic activity and immune surveillance. The gut deconjugates the bile acids and generates secondary bile acids, provides beneficial, active compounds through fermentation of dietary residue and enhances immune tolerance. There is also some translocation of luminal material to the liver. | Bottom: The dysfunctional gut liver axis; triggers of dysfunction (red) cause the alteration of almost every component of the gut liver axis which, in turn, often exacerbate the original cause of dysfunction, generating a feedback loop of worsening disruption.*

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hepatic blood supply is derived from the gut via the hepatic portal vein, which is the direct, intestinal, venous outflow. Impaired gut barrier function, therefore, exposes the liver to gut derived toxic factors and often whole bacteria via this pathway, which can be damaging. Likewise, impaired liver physiology may prompt microbiome and gut dysfunction due to lack of, or change in, metabolites and immune function, thus generating a strong feedback loop [47] (Fig. 1.2).

The liver is a structurally and functionally heterogenous organ, comprised of a varied cellular population- the predominant types being hepatocytes, and immune cells. Its physiological location at the crossroad of portal venous outflow and the peripheral organs, indicate its function as gatekeeper and processor of antigens and digestion products arriving from the gut. The primary barrier for gut luminal material is at the intestinal interface formed of the epithelium and mucosa (including the lamina propria), where access to the circulation for bacterium and other exogenous antigens in the intestinal lumen is regulated. There is an inevitable degree of permeability to xenobiotic material, as a result of innate leakiness. This leakiness occurs as a result of the importing of essential microbiome-generated metabolites and inflammation caused by opportunistic pathogens and necessitates the constrained, but protective, immune response of the liver to infiltrating material [9].

Bile acids, synthesised in the liver by hepatocytes, are amphipathic, hydroxylated, steroid acids synthesised in hepatocyte peroxisomes from the sterol lipid precursor cholesterol [48]. These primary bile acids are then conjugated with the amino acids taurine or glycine to form the conjugated primary bile acids, with taurine being most common in murine metabolism and glycine in humans [17]. They are then circulated to the gut where they emulsify hydrophobic dietary material. 95% of these bile acids are recirculated via the enterohepatic circulation, regulating their own synthesis and cholesterol biosynthesis. [49]. The remaining 5% reach the colon where much of their metabolism into secondary bile acids occurs via deconjugation, oxidation, dehydroxylation and esterification- processes which are central to this pathway. Deconjugation is performed by most gut bacteria through the activity of bile salt hydrolases, though primarily by members of the phyla; *Firmicutes, Bacteroidetes* and *Actinobacteria* [50]. This process generates deconjugated bile acids and is often followed by subsequent biochemical processes to generate secondary bile acids. On the other hand, the ability to dehydroxylate bile acids is rare in the gut microbiome, with bile acid dehydroxylase enzymes appearing to be limited to few species of the genus *Clostridium* [51].

Dehydroxylation is one of the processes which generates secondary bile acids and results in the production of DCA and LCA- the most abundant secondary bile acids in humans [50]. Oxidation is often followed by subsequent epimerisation. These processes can co-occur in a single bacterial species or occur as a collaborative process between two distinct species. The bacterial genera responsible include *Bacteroides, Clostridium, Escherichia, Eggerthella, Eubacterium, Peptostreptococcus,* and *Ruminococcus* [50–52]. Finally, bile acid esterification activity has been identified in the genera *Bacteroides, Eubacterium* and *Lactobacillus*. Esterification plays a complex role, with some suggesting that it reduces bile acid hydrophilicity and reabsorption, which as a result increases bioavailability to the microbiome and reduces toxicity [50].

The signalling of primary and secondary bile acids occurs via the ligation of membrane-bound and cytoplasmic receptors- the two most prominent of which are the farnesoid-X receptor (FXR) and Takeda G-protein coupled receptor 5 (TGR5). These receptors are key sensors in the autoregulation of bile acid synthesis and the enterohepatic circulation and are both heavily involved in a variety of other metabolic pathways e.g. lipid metabolism and glucose homeostasis [17,53–55]. Microbial metabolism then can significantly alter these signalling properties through the processes described above and is essential for their biological function [17] (Fig. 1.3).

FXR is expressed primarily in enterohepatic cells, in particular hepatocytes, hepatic stellate cells (HSCs) and intestinal epithelial cells (IECs), however it is also expressed to a considerably lesser extent in monocytes and lymphocytes [56]. It has somewhat of a hierarchy of ligation with its most potent agonists in descending order being; Chenodeoxycholic acid (CDCA), Lithocholic acid (LCA), Deoxycholic acid (DCA) and Cholic acid (CA), whilst its main antagonist is tauromuricholic acid, both alpha and beta isoforms (Tα/Tβ-MCA) [56]. TGR5, on the other hand, is primarily expressed in nonparenchymal liver cells and innate immune cells, it, likewise, has a hierarchy of agonists:- LCA, DCA, CDCA, CA and Ursodeoxycholic acid (UDCA) (in descending order of potency) [57]. Its main functions appear to be anti-inflammatory and regulatory to glucose and energy homeostasis [58]. These are the two receptors most responsive to bile acids, activated at homeostatic concentrations and central to the enterohepatic circulation. However, there are other, less studied, receptors responsive to bile acids at higher concentrations, particularly those found in liver pathologies such as cholestasis, these being the Sphingosine-1-phosphate receptor-2 (S1PR2), Pregnane X receptor (PXR), Constitutive androstane receptor (CAR), Vitamin D receptor (VDR) and retinoic acid-

related orphan receptor γt (RORγt). Each of which, in response to bile acids at increased concentration, have primarily anti-inflammatory, proliferative and bile acid metabolising effects in the liver and gut, where these concentrations can be reached in disease [56].

The role of bacterial metabolism in the gut-liver axis.

The fermentation of indigestible dietary residue in the intestinal tract, by the gut microbiome (particularly, though not exclusively, by Firmicutes and Bacteroidetes species [59]) generates an array of metabolically active compounds which enter the portal circulation. The first direct metabolic link drawn between the microbiome and liver was through observation of prebiotic treatment, which decreased hepatic lipogenesis and plasma tri-acyl-glycerol levels [60]. The fermentation of indigestible carbohydrates, such as these prebiotics, generates short chain fatty acids (SCFAs) which are active compounds influencing cell differentiation, proliferation, ion absorption and vitamin production- especially in the liver [61]. A study has shown that, through a hepatoprotective prebiotic regimen consisting of indigestible carbohydrates, acetate and propionate concentration can be doubled. Propionate directly contributed to reducing hepatic steatosis (lipid deposition), an effect which appeared to be driven by a large increase in the proportion of *Bacteriodetes* [10]. This protective effect is also demonstrated in other alcoholic and non-alcoholic liver injuries [9].

On the other hand, reduction of short chain fatty acid production can cause significant immune dysregulation. In particular, the short chain fatty acid butyrate plays a significant role in inflammation, especially in the behaviour of macrophages. Antibiotic depletion of the microbiome, especially in early life, and the associated reduction in butyrate production has been shown to cause a significant pro-inflammatory effect. A causal link has been drawn between butyrate depletion and allergic asthma and other allergic responses [16,62]. Cait et al 2018 showed that this reduction in Butyrate was driven by reductions in the *Clostridiaceae, Lachnospiraceae, and Ruminococcaceae* families, particularly in the class *clostridia*, the primary butyrate producers of the gut microbiome [16]. Increased butyrate production or direct supplementation has been shown to ameliorate many chronic and acute inflammatory conditions particularly in the liver, an effect which appears to be organism-wide and centres on macrophages [18,63,64]. Butyrate appears to imprint an anti-inflammatory, pro-phagocytotic, antimicrobial and pro-apoptotic effect on macrophages, a state which could be described as between M1 and M2 and has been described as an M0 state [18]. This effect is mediated by direct

transcriptional regulation via histone deacetylation and appears to ameliorate inflammatory liver conditions [18,64].

In addition to SCFA production, protein metabolism via proteolytic fermentation produces a wide range of beneficial compounds which are, broadly speaking, anti-inflammatory, anti-ageing, and anti-oxidative polyphenols and SCFAs. Putrefaction on the other hand generates toxic amines, phenols, thiols, indoles and ethanol- many of which are hepatoxic and pro-inflammatory [61]. Species of the genera; *Bifidobacterium, Clostridium, Lactobacillus, Escherichia,* and *Klebsiella* have been identified as protein degraders and producers of such compounds in the gut microbiota[65]. Ammonia, ethanol and acetaldehyde are detoxified and metabolised by the liver and significantly influence resident macrophage (Kupffer cell) activity and cytokine production [66]. These metabolites are implicated in both non-alcoholic fatty liver disease (NAFLD) and Cirrhosis [44,47]. Protein metabolism by the gut microbiota is primarily conducted by

Microbial metabolism is also central to the activity and recirculation of bile acids. The secondary bile acids generated by, mostly bacterial, metabolism exhibit strong antimicrobial and cytotoxic activity for the purpose of interspecies competition. Furthermore, the metabolism of bile acids by the microbiome significantly alters their physiochemical properties and hence their role in metabolic and signalling pathways. For example, the deconjugation of bile acids by bile salt hydrolases (BSHs) (abundant enzymes, ubiquitous in all major gut microbiome phyla) is key in bile acid reabsorption, promotes intestinal colonization and the deconjugated glycine and taurine serve as an essential nitrogen and carbon source [67]. The majority of BSH encoding genes in the gut microbiome belong to bacteria of the genera; *Bacteroides, Blautia, Eubacterium, Clostridium, and Roseburia,* with *Bacteroides spp.* possessing the most BSH genes of these genera [68]. Hence, the pools of primary and secondary bile acids incoming from the liver and outgoing from the gut, significantly alter microbiome composition and host physiology both directly and indirectly [51] (Fig 1.3.).

The role of the immune system in the gut-liver axis.

The gut mucosa forms the largest interface between the external environment and the immune system. This border comprises a mucous layer laced with epithelial cell-derived antimicrobials, the physical barrier of the epithelium, and the lamina propria with a

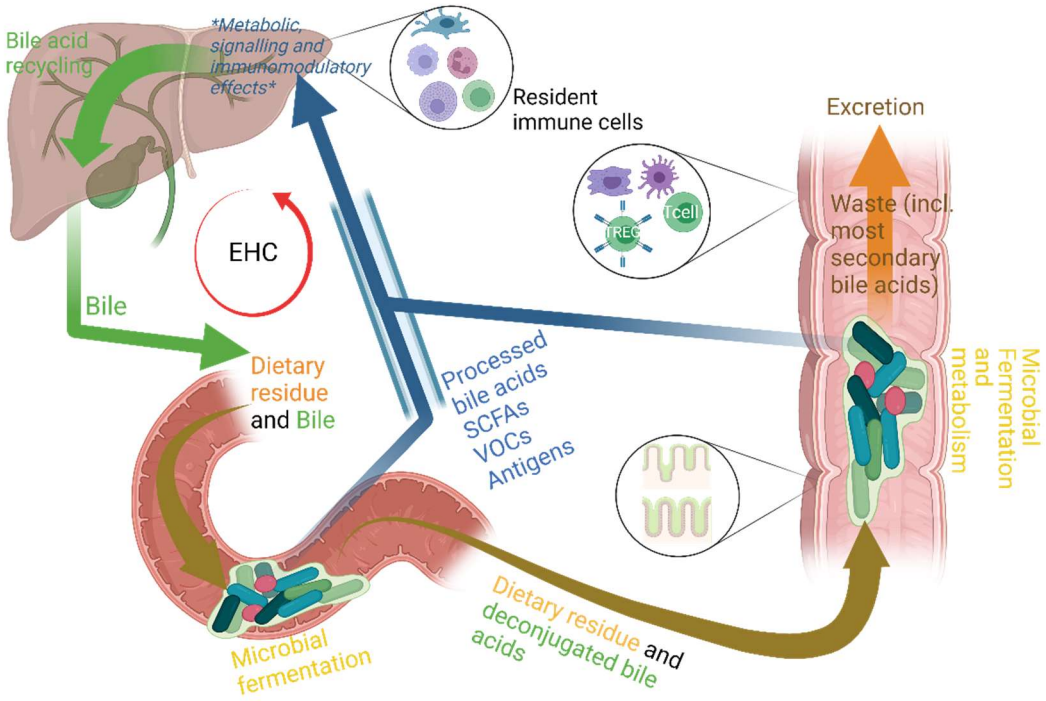

Figure 1.3. A schematic diagram of the circulation of bile acids and their signalling effect- the enterohepatic circulation (EHC)- the role of the microbiome in this pathway and the consequences of microbial metabolism. *Primary bile acids are synthesised de novo and conjugated in the liver. They are then transported to the gut via the biliary tree and much of the pool is deconjugated by the microbiome, enhancing their reuptake. 95% are recirculated to the liver via the hepatic portal vein along with SCFAs, VOCs and some luminal antigens where they have direct chemical and signalling effects, processed bile acids act upon hepatocytes, autoregulating their synthesis. The remaining 5% of bile acids escape to the lower bowel, where they are metabolised into secondary bile acids, significantly altering their physiochemical properties. There is limited passive reuptake, but most of the secondary pool is excreted in the faeces.*

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large resident population of resident adaptive and innate immune cells. The network of interaction between the gut resident bacterial population and the host plays a key role in the education and development of a tolerant yet effective immune system [27,69–72]. It is also critical for the development of the gut, and its defence mechanisms; Germ free (GF) mice (mice lacking a resident microbiome) develop with gut morphological, functional and immune aberration [14]. Reconstitution of the microbiome in these mice, via faecal microbial transfer (FMT), is sufficient to restore healthy immune physiology [71].

The microbiome constitutively elicits low-level innate immune responses through stimulation of pattern recognition receptors (PRRs) by pathogen associated molecular

patterns (PAMPs), the most prominent PRRs, in bacterial recognition, being toll-like receptors (TLRs) and nod-like receptors (NLRs). Activation of TLRs, e.g. TLR 4, by PAMPs, e.g. lipopolysaccharide (LPS), leads to nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) signalling- eliciting pro-inflammatory mediator production [73].

GALT- gut associated lymphoid tissue, is the largest immunological organ of the body comprising 4 compartments: Peyer's patches, lamina propria lymphocytes, intra-epithelial lymphocytes, and mesenteric lymph nodes. Activation of the adaptive arm of the gut immune response results from sustained and unresolved innate immune stimulation, followed by dendritic cell antigen presentation at the mesenteric lymph nodes and Peyer's patches. It leads to mucosal and serum antibody responses, pro-inflammatory and killer T-cell activation and local immune suppression by T-regulatory cells (TREGs) [47]. TREG mediated suppression is essential for preventing extensive autoimmunity, bystander injury and chronic inflammation. In mice with TREGs lacking Il2Ra (Interleukin 2 receptor a) colitis and cholangitis are developed spontaneously as a direct consequence of immune hyperactivation by commensals and other innocuous antigens [74,75]. This tolerance mechanism is driven primarily by interaction with the microbiome and its products, though it is an open question to what extent tolerance arises as a result of immune recognition of commensal microbes, or tolerogenic and immunomodulatory effects originated by the commensals. Likely, both are responsible and the particular tolerogeneic mechanism is specific to each commensal phyla.

For example, SCFAs and bile acids in the circulation and liver can tune immune cells, an effect which has been demonstrated in mice. By increasing dietary fibre, increased SCFA content in the circulation was observed, which modulated the response of dendritic cells to allergens in the lung and shaped the cell population derived from haematopoiesis in the bone marrow [62]. The significance of butyrate, in particular, to the alteration of monocyte and macrophage homeostasis in the context of allergic asthma, was also mentioned previously [18]. On the other hand, at the gut epithelium, the interaction of adaptive and innate immune cells with commensals drives extra-thymic education of lymphoid cells, induction of tolerance pathways and shapes functional diversity. This occurs primarily through antigen presentation to and response regulation by dendritic cells of naïve lymphocytes [38].

Equally essential to the immune component of the gut-liver axis is the role of the liver in the surveillance of translocated gut luminal material arriving from the hepatic portal

vein, especially bacteria and bacterial antigens and toxins, preventing infiltration into the broader circulation [76]. In the liver there are four cell types responsive to bacterial antigens; Cholangiocytes, Kupffer cells, hepatic sinusoidal endothelial cells and hepatocytes are all receptive to PAMPS via PRRs, facilitating the responses of each to gut derived antigens. These translocated bacteria and their products initially and primarily induce the activation of Kupffer cells (liver resident macrophages). As a result, signalling via the NF-kB pathway, mediated by the adaptor Myeloid differentiation primary response 88 (My-D88), results in the release of pro-inflammatory cytokines such as tumour necrosis factor alpha (TNF-a) [47].

Direct sensing of whole bacteria can overwhelm the initial innate response in the liver and generate a T-cell mediated inflammatory response- gamma-delta T-cells in particular are central to this response in the gut-liver axis given their enrichment in the liver [77]. Their inducible production of IL-17A, a highly pro-inflammatory cytokine, is negatively correlated to commensal load [78].

Both FXR and TGR-5 are expressed on innate immune cells including macrophages, Kupffer cells, dendritic cells and natural killer (NKT) cells; all of which are highly present in the lamina propria, highlighting the role of bile acids in regulating the gut epithelial, liver and general innate immune response [79]. Bile acids in addition to SCFAs also play a key role in driving the polarization of macrophages between the M1- pro-inflammatory and M2- immunomodulatory states, both in the gut and the liver. [16,18,80].

Thus, bile acids have been shown to play a significant role in the modulation of the liver immune response and in the integrity of the gut barrier [79,81–84]. FXR ligation initiates repressive pathways of pro-inflammatory cytokine production via direct NF-kB repression and regulation of TLR-4 expression and activity [46,79,81,85]. FXR ligation, hence, restores gut barrier integrity by limiting inflammation related reductions in tight-junction protein expression [86]. Furthermore, colon inflammatory phenotypes in Crohn's are associated with decreases in FXR expression [84].

Bile acids also exert these anti-inflammatory effects at the liver with bile acid accumulation (e.g. via cholestasis) demonstrated to affect macrophage function [83]. Bile acids are suppressive to the pro-inflammatory response to LPS via TGR-5 signalling [53], whilst also promoting the release of IL-10 and reducing phagocytic activity [87]. This IL-10 release can further drive the differentiation of both naïve T-cells and Th-17 cells into TREGs [80], which has been demonstrated in the gut, but likely also occurs elsewhere,

affecting the T-cell population which migrates to the liver in homeostasis and during dysfunction.

1.3 Disruption to the gut-liver axis: Swings and roundabouts.

It has been extensively shown that a co-morbidity of hepatic dysfunction and disease is GI dysbiosis and vice versa, heavily suggesting that the two are causally linked [9,41,88]. Changes in gut microbiome composition have been demonstrated in almost all majour liver dysfunctions, including both alcoholic and non-alcoholic disease [1,9,40,41,88–90].

Translocation of luminal antigens to the liver occurs both in homeostasis and disease, however, this effect is greatly exacerbated in diseases of both the liver and gut. For example, cooccurring small intestinal bacterial over growth-Non-alcoholic steatohepatitis (SIBO-NASH) results in considerably increased serum endotoxin, with TLR-4 expression increasing in response [72,91] and worsened hepatic steatosis in comparison to NASH alone. Both of which effects are driven by a functionally compromised gut epithelial barrier [74]. In addition, patients with liver cirrhosis have significantly higher bacterial translocation to the liver and corresponding circulating bacterial antigens and DNA [9]. The hepatoprotective nature of a correctly functioning gut barrier is neatly shown by the work of Schneider *et al* on CX3CR1 macrophages, which respond to bacterial detection by inducing release of IL-22 from innate lymphoid cells, promoting epithelial integrity and repair [74]. CX3CR1 deletion increases translocation, predisposition to inflammatory bowel diseases (IBDs), liver dysfunction and steatohepatitis [92]. Broadly the notion of increased translocation producing liver dysfunction and vice-versa is known as the 'leaky gut hypothesis'.

The suggested role of translocated LPS in gut-liver dysfunction is known as metabolic endotoxemia and it resembles the mechanism of the leaky gut hypothesis. The suggested effect being that, through activation of TLR-4 signalling in the circulation and liver, a variety of metabolic defects such as: insulin resistance, chronic low-grade inflammation, and diabetes are promoted. [84,93–95]. LPS alone is highly hepatoxic and endotoxemia is, hence, also related to a wide variety of liver diseases [96–100]. Exposure of the liver to increased levels of LPS is associated with morphological and functional change, altering the acute inflammatory response by causing early and sustained recruitment of neutrophils. This then results in extensive bystander injury, PAMP and DAMP (damage associated molecular pattern) release, associated amplification of the

immune response and worsened liver damage [98,99]. Though neutrophil accumulation is required for tissue remodelling, their appropriate recruitment and reverse migration is necessary to prevent cirrhosis [99,101]. Additionally, prior stimulation with LPS appears to be required for the cytotoxicity of bile acids to hepatocytes at high concentrations, such as those seen in cholestatic liver disease [45]. This gives credence both to the role of endotoxemia in the initiation and promotion of liver disease and to the role of gut barrier inflammation in cholestasis as discussed below.

Antibiotics and gut-liver dysfunction.

As has been discussed previously, antibiotics constitute a significant perturbing factor to the homeostatic conditions of the gut microbiome, this is further demonstrated by their effect upon the bidirectional metabolism of bile acids and the fermentation of dietary residue.

In a recent investigation using antibiotic induced microbial depletion (AIMD) in mice, Zarrinpar *et al* demonstrated that AIMD drives significant metabolic change in the liver, predominantly resulting from the loss of butyrate synthesis from the microbiome and alterations in bile acid metabolism. The described lack of butyrate removes the preferred energy source of the colon which drives immediate insulin sensitivity and hepatocyte gluconeogenesis, thereby causing long-term increases in serum glucose, and otherwise affecting host-wide glucose homeostasis. These changes appear to arise from signalling pathways involving bile acids [63].

Additionally, AIMD affected major changes in other gut luminal secondary metabolites, most prominently other SCFAs (reducing their production at almost every level, due to an almost total bacterial depletion) [63]. The increase in glucose sensitivity described by Zarrinpar *et al* was replicated in a study by Rodrigues *et al*, however, they discovered direct links between key gut resident bacterial species, serum glucose levels and liver function mediated via FXR. These links further strengthen the implication of bile acids [85]. More heavily implicated however, was the role of increased endotoxemia. This would be logical given the protective effect of FXR ligation in the anti-inflammatory phenotype of the gut epithelium. Moreover, FXR agonist treatment is hepatoprotective in cholestatic disease [102], the response to said treatment being driven by reduction and alteration of the bile acid pool and gut microbiome population change [81]. In addition to which, fibroblast growth factor (FGF)-19 analogue treatment is inhibitory to inflammation at

the gut epithelium (FGF-19 being a downstream, secreted product of FXR ligation, modulatory to bile acid production in the liver).

A very recent publication investigating the immediate effects of AIMD upon cholestatic injury found that the disruption in enterohepatic circulation, and more specifically decreased FXR agonists and increased antagonists in the gut, reducing FGF15 expression and caused by microbial population change, leads to significantly worsened hepatic injury and disease outcome [103]. The lack of FXR and FGF15 signalling caused something of a positive feedback loop whereby this lack promoted increased cholestasis through increased synthesis of bile acids. Moreover, antibiotics also promoted leakage of bile acids from the bile canaliculi and uptake of these bile acids by hepatocytes, promoting hepatocyte cell death [103]. These results also lend authority to the hypothesis of the role of dysbiosis in the initiation of cholestasis (discussed below).

A significant role has also been attributed to population change of the bacterium *Akkermansia muciniphilla* [39] heavily implicated in the mechanisms of metabolic endotoxemia initiation, serum glucose levels and FXR agonism [85,95].

Treatment with antibiotics and the resulting dysbiosis immediately generates a pro-inflammatory phenotype directly at the gut epithelial barrier, exacerbating gut-liver translocation and subsequent disruption to liver homeostasis [12,30,104]. A key role in the dysbiosis-inflammation pathway has recently been attributed to the hyperactivation of macrophages, driving inflammation and a decrease in tight junction protein expression, with macrophage depletion leading to the resolution of the inflammation at the epithelium and reducing translocation to the portal circulation [45]. Dysbiosis can also drive the differentiation of anti-inflammatory ROR-gamma TREGs into pro-inflammatory Th-17 cells- large scale producers of pro-inflammatory IL-17 present at the gut epithelium [105]. This is highly relevant to liver function as autoimmune liver diseases and immune cell driven liver damage are driven by increased Th-17 responses to pathogens as mentioned previously. This effect has also recently been directly linked to changes in the product of bacterial bile acid metabolism [52].

Germ free and antibiotic treated mice also have reduced bile acid excretion via the faeces, though the bile acid pool is increased and their reabsorption is enhanced, leading to systemic, metabolic alteration [63,106]. AIMD is shown to cause this selective bile acid reabsorption upregulation and as a result, body-wide serum bile acid levels increase and FXR antagonism is increased. Yet in the short-term, gut reuptake disproportionately

affects tauro-beta-muricholic acid (TbMCA)- a prominent FXR antagonist. Consequently, FXR activation is reduced in hepatocytes and de novo bile acid synthesis is increased via cytochrome P450 (CYP)7a1, whilst the higher concentration of FXR agonists in the gut promotes the restoration of homeostatic conditions [63]. The significant increase in bile acid synthesis at the hepatocyte level dramatically increases the total bile acid pool, which subsequently exposes the gut microbiome to amplified pressure from their antimicrobial effect, including pathogenic and opportunistic organisms. This induces metabolic and physiologic change, significantly increasing the resistance of gut resident pathobionts to bile acid antimicrobial properties, ultimately increasing their virulence and pathogenicity in the long run [17].

Furthermore, it has recently been shown that antibiotic treatment targeting gram negative bacteria causes disruption, and eventual stabilisation at a new equilibrium, of the primary: secondary bile acid ratio. This results in increased CXCL16 expression in sinusoidal endothelial cells, promoting NKT cell accumulation in the liver [107]. Another investigation in this area showed that following ampicillin treatment in mice NKT cells accumulate in the liver following partial hepatectomy (surgical removal of up to 2/3 of the liver). Moreover, the recruited NKT cells are functionally altered, upregulated in production of pro-inflammatory interferon gamma (IFNγ). Neutralisation of the increased IFNγ load restored homeostatic regenerative capacity. This effect appeared to be initiated by increased signalling and activation of Kupffer cells, which in turn produced higher loads of Il12, both of these effects being strongly correlated to commensal load [108]. These results suggest that antibiotics deplete key commensals in the gut, causing upset in the homeostasis of bile acids, SCFAs and other bacterial metabolites, which then causes inflammation, increased gut-liver translocation of gut luminal contents, and altered populations of functionally perturbed immune cells in the liver and gut. Combined, these effects result in altered immune and metabolic, gut-liver and organism-wide homeostasis.

1.4 Cholestasis: A disease of the gut-liver axis

Cholestatic liver disease is a rare pathology of the liver characterised by biliary atresia and subsequent accumulation of bile in the liver. There are two common aetiologies of the disease: primary sclerosing cholangitis (PSC) and primary biliary cirrhosis (PBC). PBC is thought to be predominantly autoimmune in nature, with strong genetic determinants and a catalogue of autoantibodies identified [109]. On the other hand, PSC has a much less

clearly defined causal pathway, though a relationship been proposed with inflammatory bowel diseases and intestinal/biliary dysbiosis [9,41,110,111]. 70% of PSC sufferers have IBD as a co-morbidity and up to 10% of colitis patients develop PSC, a significantly higher incidence than in the general population [112].

The role of the microbiome in both PSC and PBC appears to be highly complex with the gut microbiome being significantly altered in both diseases [41,109–113]. Given the central role of bile acids in the metabolism and population dynamics of the microbiome, this is perhaps unsurprising, however, there appear to be multiple pathways by which alteration in microbiome composition is contributing to the progression and initiation of disease [111]. Whilst it has been suggested that the initiating factor of cholestatic pathology is intestinal/biliary dysbiosis increasing intestinal permeability and microbial colonisation of the bile ducts, increasing localised inflammation in the liver, cholestasis can be initiated independent of this, by direct injury for example. The progression of the disease following obstruction is then well defined, at first causing extensive cell death via accumulation of toxic concentrations of bile acids. This results in accumulation of mobile innate immune cells- initially neutrophils followed by monocytes, macrophages and Kupffer cells. If these are insufficient to resolve the injury, adaptive immune components can be recruited. If the damage is not subsequently resolved, tissue injury can become self-promoting causing chronic inflammation and damage. This then progresses to extensive fibrosis, via activation of hepatic stellate cells by pro-inflammatory cytokines. Initially this is concentrated at the biliary tree, driving bile duct destruction, but eventually will lead to decompensated cirrhosis if untreated [109,112].

Alterations to the microbiome arising from cholestatic liver disease.

Cholestatic liver disease has a significant impact upon the microbiome, the restriction of biliary outflow from the liver profoundly alters the intestinal environment. As mentioned previously, bile acids are essential metabolites for many microbes and function as compounds for interspecies competition when converted to secondary bile acids and excreted by various microbial genera [51]. Hence, the loss of bile acids from the intestinal environment leads to a lack of key metabolites and tools for modulation of the microbial population composition, which leads to microbial population change. Generally speaking, a reduction in the intestinal bile acid content results in an expansion of gram-negative bacteria and by extension LPS abundance, which impacts upon epithelial inflammation, gut permeability and thereby can induce endotoxemia [114].

The microbiome changes induced by cholestasis depends heavily on the aetiology involved [114]. In PBC- which, as mentioned previously, is primarily an autoimmune cholestatic disease- it has been found by Tang *et al* that *Bacteroides*, *Faecalibacterium*, *Sutterella* and *Oscillospira* were significantly depleted in human PBC patients in comparison to healthy controls and *Haemophilus*, *Veillonella*, *Clostridium*, *Lactobacillus*, *Streptococcus*, *Pseudomonas* and *Klebsiella* significantly enriched [115]. Furthermore, they identified that microbial genes associated with epithelial cell invasion and antibiotic resistance were enriched, while amino acid and α linoleic acid synthesis genes were depleted [115]. This suggests that the content of pathobionts is enriched in PBC and that the population of bacteria present is more able to bypass the gut epithelium and potentially translocate to the liver, alligning with the previously suggested mechanism of bacterial driven autoimmune activation at the biliary tree[112]. Additionally, the depletion of beneficial genera, especially *Bacteroides* and *Faecalibacterium*, is particularly noteworthy.

In PSC patients on the other hand Sabino *et al* have demonstrated that few bacteria are depleted in comparison to healthy controls, with the exception of *Anaerostipes spp*. However, there is a significant enrichment in *Enterococcus*, *Fusobacterium*, *Lactobacillus*, *Morganella* and *Streptococcus*. They also noted a general decrease in microbial diversity in PSC patients. *Enterococcus*, *Fusobacterium* and *Lactobacillus* were shown to be a reliable signature of PSC, however, increased content of these bacteria was shown regardless of disease severity [41], suggesting that enrichment of these bacteria, whilst characteristic of PSC is not a determinant for worsened disease.

It has been demonstrated in various other studies however, that bacterial population can modulate PSC severity and various species have been studied. Juanola *et al*. have demonstrated that the gut microbiota is key for orchestrating an appropriate restorative response to cholestatic injury and that absence of the microbiota drives worsened biliary cholestasis post-BDL. Their study showed that germ-free mice experience greater levels of necrotic damage and inflammation in response to BDL, whilst microbially colonised mice show an enhanced ductular reaction, cellular proliferation, autophagy and collagen deposition, i.e. they undergo standard cholestatic disease progression [116]. However, other studies dispute this, a 2020 study by Isaacs-Ten *et al*. demonstrated that conventionalised germ-free mice experience significantly worsened cholestatic disease with worsened necrosis, ductular reaction and fibrosis in response to both diet and chemically induced cholestatic disease [45].

Jiang *et al* have demonstrated in a diet induced model of PSC that *Prevotella copri* is depleted and that reconstitution significantly ameliorated cholestasis and fibrosis. This alleviation in disease pathophysiology was shown to be dependent upon alterations to biliary metabolism and signalling, with limited effects upon hepatic inflammation [117].

The role of the immune response in driving cholestatic disease.

T-cells and macrophages are the cells thought to be most at fault for driving progression of cholestatic disease. Mobile T-cells in GALT are characterised by their expression of gut-tropism molecules, which respond to gut specific adhesion molecules and chemokines [74,118]. These gut adhesion molecules can be expressed in the liver in cases of gut or liver inflammation, causing erroneous recruitment of gut-localised lymphocytes which drive liver inflammation, particularly at the biliary epithelium [119]. The Biliary and GI dysbiosis caused in PSC enriches for primary amine generating bacterium[119]. Γδ T-cells are also hyperactivated in PSC by detection of the bacterium *L.gasseri,* driving increased inflammation and fibrosis [120].

Macrophages have a complex role in cholestatic disease, appearing to play both a resolving and contributory role in progression [121] and as mentioned previously, their activity is heavily modulated by bile acid accumulation [53,80,83,87]. They can also drive decreased barrier function at the gut epithelium, promoting increased translocation via LPS and pathogen stimulation driving a pro-inflammatory phenotype and reduction in tight junction expression [45]. Additionally, decreasing LPS stimulation threshold in Kupffer cells, e.g. through mutation of genes associated with detection, may permit response to commensals. In the case of PSC, fucosyltransferase variants contribute to toxic bile acid accumulation and liver injury via this mechanism [119].

The progression of fibrosis in cholestatic disease

Liver fibrosis is the excessive deposition of extracellular matrix that arises from chronic damage and is characteristic of the advanced development of most chronic liver diseases [122]. In the liver, the primary fibrogenic tissue comprises liver specific myofibroblasts- hepatic stellate cells and portal fibroblasts [123]. Hepatic stellate cells are the main contributors to fibrosis in most chronic liver diseases, however portal fibroblasts play a significant role in cholestatic liver disease, contributing meaningfully to bile duct obstruction [124]. In response to chronic damage both HSCs and portal fibroblasts are activated, proliferating and upregulating the expression of collagen 1, α-smooth

muscle actin (αSMA), TGF (transforming growth factor) β, Il6 and 13 which promotes further myofibroblast activation [125]. Whilst in homeostatic conditions portal fibroblasts constitute a small proportion of the myofibroblast population in the liver, under cholestatic conditions the population expands, either through direct sensing of damage, or by auto-activation by local portal fibroblast signalling [126]. Portal fibroblast activation is extensively stimulated in the bile duct ligation (BDL) and Multidrug resistance receptor 2 (MDR2) knockout models of cholestasis (Discussed below) [124].

As has been mentioned previously, cholestatic liver disease progresses from an initial or progressive development of biliary atresia, this obstruction then impairs bile synthesis and excretion. Accumulation of the bile to a cytotoxic concentration induces hepatic damage, reflux of bile into the periductular space and eventually the parenchyma which as a result provokes an inflammatory response resulting in fibrosis [127]. This fibrosis is initiated in distinct manners when considering HSCs or portal fibroblasts [127]. In the context of HSCs, activation from a quiescent state is largely dependent upon their physiological role as the main storage compartment of vitamin A in the liver [127]. In response to damage, HSCs downregulate vitamin A, PPARγ and GFAP expression and become activated, in which state they are analogous to collagen type 1 expressing myofibroblasts [127]. Following stimulation with profibrogenic cytokines and signalling molecules, such as TGFβ, IL6 and LPS, derived from local immune cells, hepatocellular damage or infiltrating microbes, HSCs upregulate the expression of α smooth muscle actin and other cytoskeletal components, migrating to the site of insult and depositing excess extracellular matrix, inducing localised scar formation.

Macrophages are heavily implicated in the induction of HSC fibrogenesis. Infiltrating macrophages appear to be the main source of TGFβ, the most potent HSC activating cytokine [127,128]. Deletion of macrophage TGFβ has been shown to attenuate, and its overexpression exacerbate, liver fibrosis. Furthermore, lack of TNFα, IL1β or IL6 signalling- whose activity is synergistic with that of TGFβ- also attenuates liver fibrosis, Il1β being demonstrated in a diet induced murine steatohepatitis model of fibrosis and TNFα and IL6 in the carbon tetrachloride mouse model of chronic fibrosis [129,130]. On the other hand, macrophages also play a role in the resolution of hepatic fibrosis During fibrosis regression macrophages switch to an MMP9 and MMP13 expressing phenotype [131]- each of which are ECM degrading enzymes [132]. Hence, in a model of CCL4 induced, reversible, liver fibrosis, depletion of macrophages during fibrosis progression alleviated

fibrosis, however, during regression, depletion led to a failure of fibrosis regression and persistent scarring [133].

The activation of portal fibroblasts is more poorly understood, though like HSCs, exposure to TGFβ is essential for the induction of Collagen type 1 expression in these cells [127,134]. Furthermore, portal fibroblasts possess the capacity to induce HSC activation via IL13 signalling, HSCs activated in this manner (Via cholestatic injury- BDL) possess similarities in genetic expression with portal fibroblasts and somewhat explains the differential behaviour of HSCs in cholestasis and hepatoxic injury [127,135].

1.5 Summary and thesis prospect

This chapter has highlighted the existing literature showing that the intestinal microbiota is an essential component within the host organism and is key to proper functioning. Though an organism can survive when the gut microbiota is depleted or absent, homeostasis is perturbed and metabolic and immune pathways are impacted, affecting the progression of many diseases. Additionally, we have demonstrated how the gut microbiota is altered by many factors, not least by ageing. It has explored how antibiotics possess the capacity for perturbation of the microbiota and the impacts upon said pathways. Furthermore, it has placed these observations in the context of the gut-liver axis and explored cholestasis, a disease of the gut-liver axis. It has explored the pathophysiology of the disease and enumerated the ways in which the microbiome is affected by and may be involved in the progression of the disease. The following chapters will illustrate my research into the impact of antibiotic induced microbial population change upon cholestatic liver disease progression, how this may be impacted by informed probiotic intervention and the role of ageing on the effects of antibiotic induced microbial population change in cholestatic liver disease. The rationale of my work is outlined below, along with the overall aims of this research.

1.5.1 Thesis rationale

Premise one- The gut-liver axis is a well-established bidirectional and co-dependent relationship between the gut and its microbial inhabitants and the liver [44].

Premise two- The composition of the gut bacteriome has been shown to influence host homeostasis, immunity, metabolism and by extension the susceptibility to various chronic diseases [1,136–138].

Premise three- The composition and compositional stability of the gut bacterial community alters throughout life, which impacts the metabolite output and immunogenic status of the gut microbiota in addition to the susceptibility to chronic disease [19,20,136,139].

Premise four- Antibiotics significantly impact the gut microbiota both in terms of total biomass immediately and compositionally both immediately and into the long-term, which is influenced by many factors prior to administration, not least of which being age [20,22,143,144,23,25,26,31,37,140–142].

Premise five- Cholestatic liver disease has been described as a disease of the gut-liver axis, in patients the disease has been hypothesised to arise from gut microbial stimulus arriving in the bile ducts due to dysbiosis which promotes autoimmunity against the biliary tree [112]. Furthermore, PSC as a particular manifestation of human cholestatic disease, is often accompanied by inflammatory bowel disease, termed PSC-IBD with a distinct disease aetiology [112], suggesting a heavy gut microbial component to disease manifestation. Finally recent studies have shown that in germ free mice, or mice immediately post antibiotic induced microbial depletion, cholestatic disease progression is significantly worsened, mediated by changes to, or absence of, the gut-liver axis and microbial communities [116,145].

Hypothesis- Antibiotics will induce microbial population change following bacterial biomass recovery, which will significantly impact the severity of cholestatic liver disease, mediated by microbial metabolite output in addition to specific changes to gut bacterial communities. Furthermore, intervention to restore key microbial communities may alleviate the impact upon cholestatic liver disease. Finally, age will play a role in determining the impact of antibiotics upon cholestatic liver disease progression.

1.5.2 Thesis aims

1. To determine the changes to gut microbial population elicited following the recovery of bacterial biomass after antibiotic treatment (antibiotic induced microbial population change).
2. To analyse the impact of antibiotic induced microbial population change upon the pool of bile acids and short chain fatty acids in both the gut and liver.
3. To uncover the long-term effect of antibiotics upon cholestatic liver disease pathophysiology following the recovery of bacterial biomass.

4. To elucidate how these changes to cholestatic liver disease pathophysiology may be mediated by changes to immune cell function.
5. To examine whether the effects of antibiotic induced microbial population change may be reversed by replacement of beneficial, depleted, species.
6. To investigate the impact of age upon antibiotic induced microbial population change and its effects upon SCFA and bile acid production and cholestatic liver disease pathophysiology.

Chapter Two.
Materials and Methods

Experimental procedures and reagents

All experimental procedures were conducted by the PhD candidate unless otherwise stated, reagents were obtained from Sigma-Aldrich (Merck) unless otherwise stated.

2.1 Animals and animal procedures

2.1.1 Legal and ethical approval and animals

Experimental procedures were performed on 8 week to 18-month-old wild type males at the Disease Modelling Unit (University of East Anglia, UK). These mice were housed in individually ventilated cages, 1-5 animals per cage and maintained on a 12-hour light/dark cycle at standard laboratory conditions, with free access to standard chow diet (SDS/SAFE diets) and water. standard chow is a grain or cereal based pellet feed supplemented with animal byproducts and essential micronutrients for optimum nutrient balance in rodents). These procedures were approved by the Animal Welfare and Ethical Review Body (AWERB, University of East Anglia, UK), performed according to the guidelines of the National Academy of Sciences (National Institutes of Health publication 86-23, revised 1985) and were conducted within the provisions of the Animal Scientific Procedures Act 1986 (ASPA). Surgery was conducted according to the LASA Guiding principles for Preparing for and Undertaking Aseptic Surgery 2010. All procedures were performed with UK Home Office approval (70/8929) under project licence PP9417531 assigned to Dr Naiara Beraza.

2.1.2 Antibiotic preparation and administration.

Mice of greater than 8 weeks of age were randomly assorted into treatment groups, generally these groups were: Untreated or control (Con), Antibiotic recovered (3w) (AB), Bile duct ligation (BDL) and antibiotic pretreated or recovered BDL (ABBDL) unless otherwise stated. Antibiotics were administered by gavage in a mixture diluted in PBS, consisting of vancomycin at 50mg/kg, Neomycin at 100 mg/kg and metronidazole at 100 mg/kg (VNM). Ampicillin was administered in the drinking water, at 1g/L, ad libitum, the ampicillin drinking water was changed every 2 days (VNMA). This procedure was conducted for one week after which the ampicillin was withdrawn, and standard drinking water provided.

2.1.3 Bile duct ligation and sample collection.

After a defined microbiota recovery period of between three weeks and six months the bile duct ligation procedure was conducted by Dr Naiara Beraza, this procedure was conducted using a standard protocol described elsewhere under aseptic conditions on anaesthetised animals [146]. Following this procedure animal body weight and clinical signs were monitored for seven days following the induction of cholestasis. Furthermore, analgesia was administered prior to surgery and once a day for two days post-surgery.

Animals were sacrificed by exsanguination through cardiac puncture, under anaesthesia and seven days post-BDL. Blood was collected by this procedure, liver and intestinal samples were collected and sections were preserved by both formalin fixation in 10% formaldehyde solution for histochemical analysis and cryopreservation in liquid nitrogen. Intestinal contents were extracted and also cryopreserved. Samples were also collected of ileum and liver for FACS analysis in phosphate buffered saline (PBS) containing 2% foetal bovine serum (FBS). Serum was isolated from the collected blood by centrifugation and stored at -80°C for downstream analysis.

Faecal samples were collected throughout the experimental time course prior to antibiotic administration, following one week of antibiotic administration, three weeks post microbiota recovery from antibiotics, or pre-BDL and at time of sacrifice. These faecal samples were cryopreserved in either dry ice or liquid nitrogen.

The overall experimental schematic for each experiment is shown in figures 3.A, 4.A and 5.A.

2.2 Metagenomic analysis

2.2.1 Faecal DNA extraction

Bacterial DNA was extracted from approximately 15mg of cryopreserved mouse faecal sample using the MP Biomedicals® FastDNA spin kit for soil following manufacturer's instructions.

2.2.2 16S rDNA qPCR and Bacteroides thetaiotaomicron qPCR

DNA extracted from faeces was normalised to a concentration of 10ng/µl in nuclease free water and further diluted 10x in 5ng/µl salmon sperm DNA in sterile deionised

water. The 16S copy number of this DNA was then quantified by qPCR using SYBR select master mix and universal 16S primers the sequences of which are shown in table 1. A Viia 7 Real-Time PCR system was used and cycling conditions were as follows: 95°C 15 mins, 40 cycles of (94°C 45 secs, 52°C 45 secs, 72°C 2 mins) and 72°C 10 mins. The 16S copy number was then calculated from Ct using a standard curve from bacterial gDNA serially diluted to known concentrations in nuclease free water containing 5ng/µl salmon sperm DNA. To quantify the *Bacteroides thetaiotaomicron* (Bt) content of the gut bacteriome, a further qPCR was conducted, where required, on the extracted faeces, a standard curve of Bt genomic DNA was prepared from a stock of known concentration (10ng/µl sequentially diluted 2.5x to a final concentration of 0.00042 ng/µl). This standard curve along with the samples for Bt quantification were quantified by 16S quantification, as described above, to determine the 16S copy number. Samples were then quantified by Bt genomic DNA qPCR using primer sets and reaction parameters described elsewhere [147]. Bt genomic DNA copy number was then quantified in the samples by reference to the standard curve, and represented as a percentage of total 16S copy number in faeces.

2.2.3 Shotgun metagenomics

Bacterial DNA was extracted from faeces as described in section 2.2.1 and sample DNA normalised to 10ng/µl by dilution in nuclease free water. Samples were sent to Source bioscience for Illumina MiSeq 2x 150 base pair paired end sequencing. Raw data was processed by Dr Raymond Kiu. Raw reads (fastq) were quality-filtered and trimmed using fastp v0.20.0. After quality filtering steps as described above, Kraken v2.1.2 was utilised for taxonomic assignment for metagenome reads (Kraken2 standardDB), with confidence level set at 0.1 as recommended by the author of the software. Bracken v2.6.2 was then used to estimate the relative abundance of taxa at both genus and species level (-t set at 10 as recommended to reduce false positive) from Kraken2 reports. For taxonomic profiles, genus reads with <1000 across all samples were removed (with average 520,000 resultant genus reads across all samples). Data visualisation for NMDS and Shannon diversity index were conducted in R using the vegan and ggplot2 packages with the assistance of Vinicius Dias-Nirello. LDA analysis was conducted using the LEfSe galaxy module from the Huttenhower lab at a significance threshold of $P<0.01$ as described elsewhere [148].

2.3 LC-MS analyses

2.3.1 Bile acid content quantification

Bile acids were extracted from the liver and faecal samples of mice at time points shown in figure 2.1. 50mg of liver tissue or approximately 15mg of faeces was homogenised for 1 minute at 6000 RPM (Precellys® 24 Touch homogenizer– Bertin Technologies) in 500µl of ice cold 90% methanol using zirconium oxide beads (Fisher Scientific). Samples were centrifuged at 12000 x rpm for 10 minutes before pellets were discarded and 25µl of internal standard was added. Sample clean-up was conducted using an Oasis PRiME HLB µELution Plate (Waters). Mass spectrometry was performed by Mark Philo using the Agilent 1260 HPLC coupled to an AB Sciex 4000 QTrap triple quadruple mass spectrometer as described by our laboratory group previously [45]. The bile acids analysed are shown in Table 1 below.

Table 1. Bile acids quantified by LC-MS

Acronym	Full name
TCA	Taurocholic acid
TMCA	Tauromuricholic acid
THCA	Taurohyocholic acid
TDHCA	Taurodehydrocholic acid
TDCA	Taurodeoxycholic acid
TCDCA	Taurochendeoxycholic acid
TUDCA	Tauroursodeoxycholic acid
THDCA	Taurohyodeoxycholic acid
TLCA	Taurolithocholic acid
GCA	Glycocholic acid
GHCA	Glycohyocholic acid
GCDCA	Glycochendeoxycholic acid
GDCA	Glycodeoxycholic acid
GHDCA	Glycohyodeoxycholic acid
GUDCA	Glycoursodeoxycholic acid
GLCA	Glycolithocholic acid
α-MCA	Alpha-muricholic acid

β-MCA	Beta-muricholic acid
MCA	Muricholic acid
CA	Cholic acid
DCA	Deoxycholic acid
CDCA	Chendeoxycholic acid
HDCA	Hyodeoxycholic acid
UDCA	Ursodeoxycholic acid
LCA	Lithocholic acid

2.3.2 SCFA content quantification

SCFAs were extracted and analysed using a method described elsewhere in liver and colon model samples [149]. Briefly, SCFAs were extracted from approximately 15mg of faecal samples collected at the time points shown in figure 2.1. Samples were homogenised for 1 minute at 6000 RPM (Precellys® 24 Touch homogenizer– Bertin Technologies) in 300µl of 0.1% orthophosphoric acid (Lichropur) diluted in deionised water. Samples were centrifuged at 15000 RPM for at least 20 minutes or until the samples were clarified. Internal standards were added and the samples were transferred to HPLC vials (Chromex scientific). SCFA content was then quantified by LC-MS/MS by Dr Shikha Saha using an Agilent 6490 Triple Quad MS mass spectrometer (Agilent Technologies, Santa Clara, CA, USA) equipped with an Agilent 1290 HPLC system (Agilent Technologies, Santa Clara, CA, USA) using a method described by Dr Saha elsewhere [149].

2.4 Histochemical analyses

2.4.1 Tissue processing

Tissues were fixed in 10% formalin (Merck) immediately post-harvest and moved to 50% ethanol after one day fixation. Samples were then dehydrated using the Leica tissue processor following manufacturer's instructions and later embedded in paraffin wax. Embedded liver tissue sections were then cut at a thickness of 3µm using a Leica microtome.

2.4.2 Deparaffinisation of tissue sections

Tissues were deparaffinised in histoclear (Merck) for 10 minutes then rehydrated in a graduated ethanol series (100%, 80%, 70% absolute ethanol diluted in deionised water) for 2 minutes in each solution. Samples were then rehydrated in deionised water for at least 5 minutes.

2.4.3 Haematoxylin and eosin staining

Liver tissue sections were stained in with haematoxylin and eosin (H&E) to assess tissue pathophysiology. Following deparaffinisation, slides were immersed in haematoxylin for 5 minutes, followed by 1% HCL in 70% ethanol, washed in running water for 5 minutes, neutralised in 0.1% $NaCO_3$ solution in water for 15 seconds and again washed. Slides were then counterstained in eosin for 30 seconds and dehydrated as previous in graduated ethanol solutions (70%, 80%, 100% absolute ethanol diluted in water) for 2 minutes each and then immersed in histoclear for at least 10 minutes. Slides were then mounted using neomount.

2.4.4 Sirius red staining

Liver tissue sections were stained with sirius red to assess liver scarring resulting from cholestatic disease, Direct red 80 stains collagen red, whilst FCF fast green counterstains the parenchyma green. Following deparaffinisation, slides were immersed in sirius red solution one (0.01% FCF Fast green solution in saturated picric acid) for 15 minutes, after which they were immersed in solution 2 (0.04% FCF Fast green, 0.1% Direct red 80 solution in saturated picric acid) for 15 minutes. The sections were then dehydrated in a graduated ethanol series and immersed in histoclear as previous. Slides were then mounted as above.

2.4.5 CK19 immunohistochemistry

Liver tissue sections were stained with CK-19 to determine the extent of cholangiocyte and ductular proliferation in response to cholestasis, known as the ductular reaction, which is a common assessment of cholestasis progression [150]. Sections were deparaffinised as described above, endogenous peroxide activity was then blocked using 3% hydrogen peroxide in methanol (Fisher Scientific) for 10 minutes. Sections were rinsed in water, after which, antigen retrieval was conducted via microwave heating in

sodium citrate buffer (0.053% trisodium citrate dihydrate and 0.17% citric acid, pH 6.0 in water) for 20 minutes. Slides were cooled then washed for 3 x 5 minutes in PBS. Non-specific antibody binding was blocked by incubating for 1 hour in blocking buffer containing 10% goat serum, 0.1% Triton x100 and 1% BSA in PBS. Sections were incubated with CK-19 primary antibody (TROMA III, Developmental Studies Hybridoma Bank, University of Iowa) diluted 1:200 in antibody diluent (Dako) overnight at 4°C in a wet chamber. Sections were then washed for 3 x 5 minutes in PBS and incubated with anti-rat secondary HRP-conjugated antibody (#7077 - Cell Signalling) diluted 1:200 in antibody diluent (Dako) for 1 hour at room temperature. Finally, slides were washed for 3 x 5 minutes in PBS and developed with DAB+ chromogen system (Dako), then counterstained with haematoxylin, dehydrated and mounted as above.

2.4.6 F4/80 immunohistochemistry

Liver tissue sections were stained with F4/80 to determine the infiltration and localisation of macrophages within the liver. Sections were deparaffinised as described above, endogenous peroxide activity was then blocked using 3% hydrogen peroxide in methanol (Fisher Scientific) for 10 minutes. Sections were rinsed in water, after which, proteinase K was used for antigen retrieval. Slides were immersed in 20µg/ml proteinase K solution in TE buffer for 20 minutes at room temperature (Proteinase K PCR Grade, Roche Merck life sciences), TE Buffer: 50mM TRIS Base, 1mM EDTA, 0.5% Triton X-100, pH 8 diluted in water. Slides were cooled then washed for 3 x 5 minutes in PBS. Non-specific antibody binding was blocked by incubating for 1 hour in blocking buffer containing 10% goat serum and 0.1% Triton x100 in PBS. Sections were incubated with F4/80 primary antibody (abdSerotec MCA497BB Rat anti-mouse biotin) diluted 1:100 in antibody diluent (Dako) overnight at 4°C in a wet chamber. Sections were then washed for 3 x 5 minutes in PBS and incubated with anti-rat secondary HRP-conjugated antibody (#7077 - Cell Signalling) diluted 1:100 in antibody diluent (Dako) for 30 minutes at room temperature. Finally, slides were washed for 3 x 5 minutes in PBS and developed with DAB+ chromogen system (Dako), then counterstained with haematoxylin, dehydrated and mounted as above.

2.4.7 Slide imaging and image analysis

All of the above histochemical stains were brightfield imaged, H&E stains were imaged using a Leica M165C Stereomicroscope, multiple (3-5) representative images were taken

at 4x of each tissue section. The remaining histochemical stains were imaged using an Olympus BX60 microscope, multiple representative images were taken using the 10 (for Sirius red and CK19 stains) or 20x (for F4/80 stains) objective. Image analysis was conducted using Image J software, in the cases of H&E and CK19, stained areas were quantified manually and in the case of sirius red RGB stack images were quantified using the thresholding function.

2.5 Serum chemistry analyses

As a quantitative measure of liver damage and the extent of cholestasis, serum chemistry analysis was conducted to determine transaminase, alkaline phosphatase, and total bilirubin content. Serum was extracted from blood samples by centrifugation at 3000 RPM for at least 20 minutes at 4°C. This serum was then diluted 2x in sterile PBS if the mouse had not received BDL and 10x if BDL had been conducted and alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP) and total bilirubin (TBIL) content measured by RANDOX Daytona+ serum chemistry analyser following manufacturer's instructions.

2.6 Intestinal permeability assay

In order to assess intestinal permeability as a proximal measure of gut-liver antigen translocation, Fluorescein isothiocyanate (FITC)-Dextran was administered to mice which can be detected in serum if the gut epithelial layer is permeable. 2 hours prior to sacrifice, mice were administered with 200µl of 50mg/ml FITC-Dextran solution in PBS. As previously, at point of sacrifice blood was collected and serum isolated by centrifugation. Sera were diluted 8x from mice which had undergone BDL and 2x where BDL was not performed, these sera were then measured for fluorescence on a Clariostar plate reader at excitation 485nm, emission 528nm. The content of FITC-Dextran was calculated using a standard curve of increasing FITC-Dextran concentration.

2.7 qPCR- Liver and Bone marrow derived macrophages (BMDMs)

2.7.1 RNA extraction

In order to determine the response of the liver as a whole and macrophages to damage and inflammatory stimulus, quantitative polymerase chain reactions (qPCRs) were

conducted on cDNAs generated from RNA extracted from whole liver homogenate or BMDM cells to investigate the expression of various relevant genes.

In the case of liver, small sections of cryopreserved liver tissue were cut and homogenised using a Precellys® 24 Touch homogenizer (Bertin Technologies) for 1 minute at 6000 RPM in Qiazol lysis reagent (Qiagen), using zirconium oxide beads (Fisher scientific). In the case of BMDMs, Qiazol lysis reagent was added directly into the wells of the cell culture plates and the wells scraped using a sterile cell scraper to encourage cell lysis. Qiazol RNA samples were then phase separated by vortexing with chloroform after which, samples were centrifuged at 12000 x rpm for 10 minutes at 4°C. The aqueous phase containing RNA was collected and precipitated using isopropanol and samples were centrifuged at 12000 x rpm for 10 minutes at 4°C to obtain the RNA pellet. The pellet was washed twice in 70% ethanol by centrifugation at 12000 x rpm for 10 minutes at 4°C. RNA pellets were air dried and resuspended 1:20 in RNase free water (Merck).

2.7.2 cDNA generation by reverse transcription

RNA concentration was determined by nanodrop (Thermo scientific nanodrop 1000 spectrophotometer), 1 µg of RNA was taken and in the case of whole liver homogenate, was DNAse I (Roche) treated by manufacturer's instructions prior to Rt-PCR. The RNA aliquot was reverse transcribed using M-MLV reverse transcriptase (Invitrogen) to generate cDNA following manufacturer's instructions.

2.7.3 qPCRs

The generated cDNA was then used for qPCR to investigate the expression of various genes (Table 2). qPCRs were conducted using SYBR select mastermix (Thermo-Fisher scientific) following manufacturer's instructions, using the following cycling method: 95°C for 3 minutes, 40 cycles of (95°C 15 seconds, 60°C 1 minute) followed by a melt curve. Gene expression was normalised to TATA box binding protein (TBP) housekeeping gene expression and expressed as versus expression in control samples (ΔΔCt) unless otherwise stated.

2.8 Flow cytometry analysis

2.8.1 Liver immune cell flow cytometry analysis

In order to analyse the innate immune cell population of the liver, Flow cytometry analysis was conducted on liver samples. A small section of the liver right lateral lobe was isolated and placed in PBS 2% FBS on ice. These liver sections were then manually homogenised, and collagenase treated in 2.5% collagenase solution in PBS 2% FBS for 30 minutes at 37°C. The liver homogenate was then passed through a 70µm cell strainer, centrifuged at 1300 RPM for 10 minutes at 4°C and the supernatant discarded. The pellet is then resuspended in 35% Percoll (GE) diluted in PBS 2% FBS and centrifuged at 1700 RPM for 40 minutes at room temperature for immune cell isolation. The supernatant was again discarded, the pellet resuspended in 2mL red blood cell (RBC) lysis buffer 10x (Biolegend) diluted 10x in deionised water and incubated for 3 minutes at room temperature. 1mL of PBS 2% FBS was then added and the cells solutions centrifuged at 1300 RPM for 10 minutes at 4°C. Supernatant was discarded and the pellet was resuspended in 95µL of antibody solution (antibodies diluted 1:200 in antibody staining solution: 2% Goat, mouse, and human serum, 6.66% Bovine serum albumin (BSA) in PBS), the cells were incubated in this solution at 4°C in the dark for at least 30 minutes. The antibodies used are listed in table 3. 3mL of PBS 2% FBS was then added to the cell solutions and these were then centrifuged at 1300 RPM for 10 minutes at 4°C, the supernatant was then discarded, and the pellet resuspended in 220µL of PBS 2% FBS. Flow cytometry was conducted using a BD LSRFortessa and analysis using FlowJo software.

Table 2. Genes and associated primer sequences used for qPCRs.

Gene name	Forward primer sequence	Reverse Primer sequence
16S Uni	GTGSTGCAYGGYYGTCGTCA	ACGTCRTCCMCNCCTTCCTC
TBP	GAAGCTGCGGTACATTCCAG	CCCCTTGTACCCTTCACCAAAT
Tumour necrosis factor α (TNFα)	CCCTCACACTCAGATCATCTTCT	GCTACGACGTGGGCTACAG
Interleukin 1 β (Il1β)	GAAATGCCACCTTTGACAGTG	TGGATGCTCTCATCAGGACAG
Interleukin 6 (Il6)	TACCACTTCACAAGTCGGACCG	CTGCAAGTGCATCATCGTTGTTC

Chemokine (C-X-C motif) ligand 1 (CXCL1)	ACTCAAGAATGGTCGCGAGG	GTGCCATCAGAGCAGTCTGT
Chemokine (C-C motif) Ligand 2 (CCL2)	TTAAAAACCTGGATCGGAACCAA	GCATTAGCTTCAGATTTACGGGT
Chemokine (C-C motif) Ligand 3 (CCL3)	CTCCCAGCCAGGTGTCATTT	TGGACCCAGGTCTCTTTGGA
Chemokine (C-C motif) Ligand 3 (CCL4)	GCAACACCATGAAGCTCTGC	CCATTGGTGCTGAGAACCCT
Matrix metalloproteinase 9 (MMP9)	CAAAGGCCTCAAGTGGGACC	CATCGATCATGTCTCGCGGC
Matrix metalloproteinase 2 (MMP2)	ACAAGTGGTCCGCGTAAAGT	GTAAACAAGGCTTCATGGGGG

Table 3. Liver innate immune cell antibody panel for flow cytometry

Antibody	**Fluorophore**	**Supplier**
Cluster of differentiation (CD) 45	APC-Cy7	BD biosciences
CD11b	PE	BD Biosciences
F4/80	FITC	Miltenyi Biotec
Ly6G	PerCP	Miltenyi Biotec
Ly6C	VioBlue	Miltenyi Biotec
CD14	PE-Cy7	Biolegend
CX3CR1 (Or)	APC	Biolegend
CD68 (Or)	APC	Biolegend

2.8.2 Bone marrow isolated macrophage phagocytosis assays

To assess the clearance of cellular debris by macrophages, mature macrophages were isolated from bone marrow tissue and exposed to fluorescently labelled dead cell debris in vitro and fluorescence intensity analysed by flow cytometry. This analysis was conducted by Miss Katherine Hampton. Bone marrow cells were isolated as described in 2.9.1 and counted using a Cellometer T4 brightfield viability cell counter. 2 million cells were incubated with 1 million dead cells stained with pH rhodo (these cells were prepared as described in 2.9.2 and stained with pH rhodo by incubation with pH rhodo stain in the dark at 4°C for at least 30 minutes) for 2 hours in the dark at room temperature. These cells were then fixed with 4% paraformaldehyde (PFA) in PBS for 30 minutes, following which cells were washed in cell culture medium. The fixed cells were then incubated in cell staining solution containing F4/80-PE-cy7 antibody (Miltenyi Biotec) at a 1:100 concentration in cell culture medium for at least 15 minutes at 4°C. Cells were then washed and centrifuged, resuspended in 250µl of PBS and analysed by flow cytometry using the CyFlow Cube 6 (Sysmex, Milton Keynes, UK) .

2.9 *In Vitro* studies

2.9.1 Bone marrow cell isolation and macrophage differentiation

In order to assess the preconditioning of macrophages remote from the liver by antibiotic pretreatment, bone marrow cells were isolated, differentiated into macrophages and their response to stimulus assessed *in vitro.* Male WT mice were either untreated or treated with antibiotics and given a 3-week recovery period as described previously. These mice were then sacrificed, and leg bones collected for the isolation of bone marrow tissue. The bones were then flushed with Roswell Park memorial institute (RPMI) medium containing 10% FBS and 50 units/ml Penicillin-streptomycin (penstrep, Thermofisher scientific) through a 40µm cell strainer. Cells from mice of the same treatment group were then pooled and the suspensions centrifuged at 1000 RPM for 5 minutes. Cells were then resuspended in 10 ml RPMI 10% FBS, 50 units/ml penstrep containing 30% L929 conditioned media produced as described by our group elsewhere [151]. The cell density was then counted using a Countess II automated cell counter and the cell suspensions diluted to a density of 1 million cells per ml in RPMI, 10% FBS, 50 units/ml penstrep. These cells were then incubated at 37°C 5% CO_2

for four days at which point the cells were supplemented with RPMI 10% FBS, 50 units/ml penstrep and 30% L929 conditioned media. The cells were incubated for at least a further 3 days, exact time of incubation depending on differentiation and attachment. Macrophages were then detached using ice cold PBS and seeded into uncoated multi-well culture plates for future experiments.

2.9.2 BMDM experiments

BMDMs were seeded at a concentration of 0.5 million cells/ml into 12 or 6 well uncoated culture plates and incubated overnight at 37°C, 5% CO_2 in RPMI without L929 conditioned media. Cells were serum starved for at least four hours prior to incubation with 100ng/ml LPS in PBS or 1.5 million dead cells/ml PBS for up to 24 hours.

Dead cells were prepared by Miss Katherine Hampton by the following method. Bone marrow tissue was isolated as described in 2.9.1 and incubated at a super-confluent density to induce cellular stress in RPMI 50 units/ml penstrep for 1-2 days at 37°C, 5% CO_2 . Cytosine arabinoside (Ara-c) was added to the cells to a concentration of 411µM and incubated for 24 hours at 37°C, 5% CO_2. The cells were then collected, centrifuged at 16,000RPM for 10 minutes and supernatant discarded, the pellet was then resuspended in PBS and centrifuged at 150RPM for 5 minutes and supernatant discarded to remove whole cells. The pellet was then resuspended in at 16,000RPM for 10 minutes, the supernatant discarded, and the pellet resuspended in PBS and apoptotic bodies counted manually using a haemocytometer. The cells were then centrifuged and resuspended to the desired concentration.

2.10 *Bacteroides thetaiotaomicron* (Bt) culturing and administration

Bt culturing was conducted by Dr Regis Stentz, *Bacteroides thetaiotaomicron* VPI-5482 (a strain widely used as a model commensal bacterium for investigating host-microbe interactions [152]), using a method described elsewhere [153]. Briefly, Bt was cultured in anaerobic conditions, at 37°C in BHI medium (Oxoid) supplemented with 15µM hemin. The Bacterial culture was centrifuged and resuspended in PBS to a concentration of $0.5x10^9$ colony forming units (CFU)/ml. Mice were administered with 200µl of this suspension, for a final dose of $1x10^8$ CFU, 1 day prior to BDL. In these experiments, faeces samples were collected prior to Bt administration pre-BDL and 1 day post Bt administration also pre-BDL as described previously.

2.11 Graphical figure production

Graphical figures were created with BioRender.com and where figures are adapted from existing figures in the literature this is acknowledged in the figure legend.

2.12 Data analysis and statistics

Data is expressed as mean ± standard error of mean (SEM) statistical significance was calculated using Welch T-Test or Brown-Forsyth and Welch ANOVA as appropriate and, unless otherwise stated, with an appropriate post-test applied. All analysis was conducted using GraphPad Prism software unless otherwise specified. Where required, advice on statistical analysis was provided by George Savva (QIB).

2.13 Experiment repeats and data shown

The data shown in each chapter is from one representative experiment unless otherwise stated, each analysis / assay was conducted on each experiment unless otherwise stated and the representative data is from the same representative experiment unless otherwise stated.

Chapter Three.
Analysis of gut microbial recovery post-antibiotics and the downstream effects upon metabolite production and cholestatic liver disease progression in youth.

3.1 Introduction

Throughout one's life antibiotics of many types may be administered for a variety of reasons at a variety of stages and in a variety of combinations, which will inevitably lead to a degree of dysbiosis in the gut microbiome. This dysbiosis will have immediate impacts upon many pathways including, among others, energy metabolism, immunity and neurological function, impacts which have been associated with many subclinical dysfunctions [13,26,29,31,54,94,154,155]. Recent studies have investigated the impact of antibiotic treatment, both at the time of administration and over time following withdrawal. What these studies have made evident is that whilst microbial biomass seemingly recovers to pre-administration levels, there are varied effects upon population composition, a general reduction in microbial diversity, and increased lateral gene transfer (of which AMR genes are the most significant). In some cases these changes are irreversible into the longer term [20,22,26,35,37,144]. The disruption to gut microbial populations arising from antibiotic use often constitutes a significant perturbing factor to the complex network of interdependencies between taxa in the gut microbiome. This network functions as a component in various host metabolic and signalling processes, hence, any long-term alteration to the gut microbiome caused by antibiotic treatment will likely have a detrimental impact upon host processes, an impact determined by treatment conditions. The gut-liver axis is comprised of many such host process and is therefore highly likely to be affected by antibiotic generated dysbiosis. To briefly summarise what has been mentioned previously, the gut-liver axis is the network of signalling and metabolic pathways between the gut and its microbial inhabitants- the gut microbiota- and the liver [17,44,46].

A primary component of the gut-liver axis is the enterohepatic circulation of bile acids. Bile acids are released from the liver post-prandially and transported via the bile ducts to the gut, where their main function is the emulsification of lipids from diet to facilitate lipid reabsorption [49]. However, bile acids have many secondary functions which centre upon their antimicrobial and eubiotic effects in the gut microbiome, functions which include microbial population regulation, use as metabolites and conversion into secondary bile acids for the purposes of inter-species competition [17]. The metabolism of bile acids by the microbiota also helps to facilitate their enterohepatic circulation, the process by which 95% of bile acid content is recycled by the liver following reuptake from the gut [49].

Upon reaching the gut, conjugated bile acids are first deconjugated, primarily by bacterial bile salt hydrolases (BSHs) and the taurine or glycine conjugate is metabolised by the bacteria [17]. The deconjugated bile acid may then be recirculated. However, some of these bile acids are then metabolised further into secondary bile acids whereon the majority are excreted in the faeces (approximately 5% of the total bile acid pool) [17].

In addition to the functions mentioned above, bile acids are also important signalling molecules, who's primary receptor, FXR, is responsible for the regulation of bile acid synthesis. This occurs by intracellular signalling in hepatocytes following ligation by agonistic bile acids or by promoting FGF15/19 production in the gut, which then act as the regulatory signal after arriving at the liver. Additionally, both FXR and TGR5 (another bile acid receptor) are expressed on several immune cells, including dendritic cells and macrophages, where ligation can modulate inflammation [56]. Bile acids can have both FXR antagonistic activity (such as in the case of tauromuricholic acids) or agonistic activity (such as in the cases of deoxycholic acid and lithocholic acid). FXR antagonism generally increases bile acid synthesis and has pro-inflammatory effects, whereas agonism reduces bile acid synthesis and has anti-inflammatory effects [56].

In addition to bile acids, short chain fatty acids (SCFAs) have several roles within the gut-liver axis. SCFAs in the gut are microbial metabolites generated from indigestible dietary fibre which can constitute a secondary energy source for intestinal epithelial cells and other cells distant from the gut once they have reached the circulation [63,156]. This makes SCFAs central to metabolic homeostasis and hence disruption to their content is implicated in many metabolic dysfunctions, obesity, diabetes mellitus and non-alcoholic fatty liver disease in particular [156,157]. In addition to their role in energy metabolism, SCFAs play a significant role in immunity. The three most abundantly produced SCFAs- acetate, propionate and butyrate- play roles in innate immune function and are generally considered to have anti-inflammatory properties, effects which can be tissue specific [16,62,64,155,158,159].

Cholestatic disease is the accumulation of bile acids to cytotoxic concentrations, generated by biliary obstruction and has been considered a disease arising from dysfunction within the gut-liver axis [160]. The accumulation of cytotoxic concentrations of bile acids causes cell death, which stimulates an immune response resulting in inflammation. As cholestatic disease presently has no restorative treatment, the disease

then progresses to fibrosis and eventual cirrhosis, this leads to organ failure requiring transplantation [109,112].

There are many experimental models of cholestasis in mice, including transgenic (MDR 2 -/-), chemical (DDC- 3,5-Diethoxycarbonyl-1,4-Dihydrocollidine- diet) and surgical (Bile duct ligation) whereby the common bile duct is ligated to prevent bile outflow. Human cholestatic disease presents as either PSC (Primary sclerosing cholangitis) or PBC (Primary biliary cholangitis). Although presentation of the disease can vary, the common coincidence of IBD (inflammatory bowel disease) in PSC-IBD, which has a distinct hepatic aetiology [112], is evidence that there is 1. A bidirectional transmission of dysfunction and 2. intestinal factors are determinants of disease presentation. In addition to PSC-IBD, cholestatic disease is also known to reduce the integrity of the intestinal epithelium without IBD presentation, this increases intestinal permeability and promotes antigenic translocation to the liver from the gut [45,113,161]. The causative mechanisms of both PSC and PBC are under scrutiny and are various, although autoantigens raised against components of the biliary tree appear prevalent, regardless of aetiology [109,112]. Recent studies suggest that these autoantigens arise owing to changes in gut and bile resident bacterial populations driving inflammation towards the biliary tree [109,112]. Antibiotics, as a significant disruptor to the gut microbiota must therefore be considered as a potential influencing factor of cholestatic progression.

The most common clinical course of action upon hospitalisation owing to cholestatic disease, is the immediate administration of broad-spectrum antibiotics such as vancomycin. This ameliorates the risk of bacterial sepsis from reduced intestinal barrier function and hepatic gatekeeping. Human studies have investigated the immediate effect of antibiotic treatment upon both the intestinal microbiota and in cholestasis. These studies indicate an improved clinical outcome, driven by improvements in liver pathophysiology, in patients with PSC when vancomycin or metronidazole are administered [162,163]. However, whilst this clear requirement for antibiotics to ameliorate infection risk and apparent improvement in patient outcome has been documented, the effect of antibiotic administration prior to disease onset remains unaddressed.

Further investigations into the impact of antibiotics upon cholestasis, have demonstrated that antibiotic administration early in cholestasis progression or immediately prior to induction increases the severity of cholestatic injury. One of these studies investigated the immediate effect of antibiotic induced microbial depletion in

the MDR 2 -/- transgenic mouse model of cholestasis. Findings showed that antibiotics perturbed bile acid recirculation and that this effect arose from microbial depletion. This, in turn, promoted increased bile acid accumulation, cytotoxicity and periductular leakage of bile into the parenchyma, the cumulative effect of which was to increase damage, fibrosis and inflammation [103]. A second study using a similar model of antibiotic treatment in the MDR2 -/- model demonstrated similar pathophysiological results such as increased liver damage and fibrosis in antibiotic treated cholestatic mice, with the most impactful results elicited when vancomycin was administered. They further identified that the prevalence of *E.faecalis* and *E.coli* marked accelerated hepatic damage and fibrosis, while *Lachnospiraceae spp.* had a eubiotic effect centring on microbial population recovery and SCFA production [164]. Finally, another study recapitulated these previous results, demonstrating increased hepatic damage and inflammation in BDL mice immediately post-antibiotics. They also highlighted a differential expression of genes key to fatty acid processing in the liver, between BDL and antibiotic treated BDL mice by RNA seq [165].

It is evident from the studies mentioned above that the en masse depletion of close to the entire microbial community severely disrupts the gut-liver axis and lead to complications in biliary metabolism. While this provides important context for my investigation, the object of this study is not the *immediate* impact of antibiotics upon cholestatic disease, but instead upon the effects of long-term alteration to the gut microbiota caused by antibiotic treatment *prior to* cholestatic disease onset or induction. Hence, this thesis seeks to determine the extent to which antibiotic induced alterations to the microbiome are retained once bacterial biomass has recovered and the effect they have upon cholestatic disease.

3.2 Hypothesis and aims

I hypothesise that the retention of antibiotic induced changes to gut bacterial communities and the associated change to microbial metabolites results in worsened cholestatic liver disease pathophysiology in young mice. Furthermore, I hypothesise, that antibiotics induce changes to macrophage inflammatory and fibrotic responses which may contribute to the worsened disease pathophysiology.

This chapter aims to:

1. Characterise the bacterial community present in the gut throughout the recovery of bacterial biomass following antibiotic depletion.
2. Characterise the faecal and hepatic bile acid pool and the faecal SCFA profile throughout the administration of antibiotics and the progression of cholestatic disease in antibiotic treated mice in comparison to untreated mice.
3. Illustrate the effect of sustained antibiotic induced bacterial population change upon the progression of cholestatic disease and injury.
4. Demonstrate the antibiotic induced alteration of the inflammatory response to cholestasis, post bacterial biomass recovery, in vivo and of macrophages to pro-inflammatory stimulus in vitro.

The structure of the mouse treatment and sampling is shown in the figure below to aid in data interpretation and general understanding (Fig 3.A).

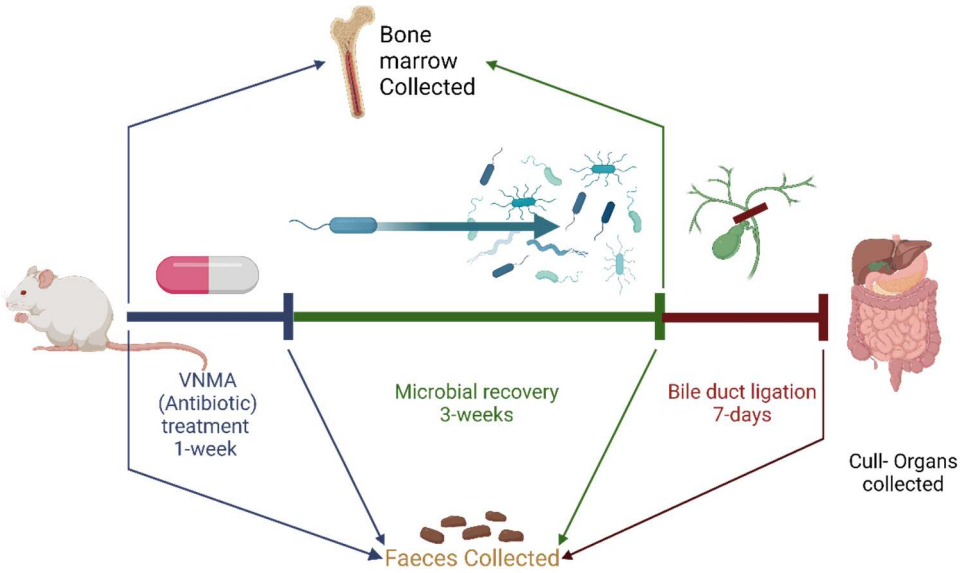


Figure 3.A. Experimental and sampling schematic; chapter three. *Antibiotics (VNMA) were administered for one week, following which the microbiome was allowed to recover for three weeks. After which, BDL was conducted, and cholestatic disease progressed for a further week. Animals were then culled, and samples collected terminally. Faeces was collected throughout this time course at the timepoints indicated. Furthermore, bone marrow was collected terminally at the indicated timepoints in separate animals for macrophage derivation. This experiment was conducted 3 times (excluding bone marrow collection, performed once). Data shown is from one representative experiment unless otherwise stated.*

Created with BioRender.com

3.3 Results

3.3.1 VNMA depletes the faecal microbiota and whilst biomass recovers within three weeks post-administration, composition is significantly altered.

To determine the impact of antibiotic-induced population change upon cholestatic disease I first sought to elucidate the disruption of microbial populations. Faecal samples were collected at points throughout the period of antibiotic treatment and recovery (pre-treatment, immediately following antibiotic administration for one week and 3 weeks following final administration) (Fig 3.A), DNA was then extracted from approximately 15mg of faecal sample and 16S copy number normalised to sample mass.

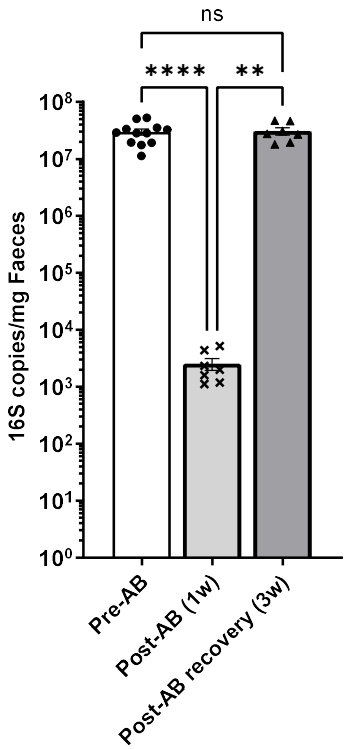


Figure 3.1. Faecal universal 16S qPCR showing depletion of bacterial biomass following antibiotic treatment and recovery after 3 weeks. *Antibiotics significantly deplete bacterial biomass over 1 week and biomass recovers to approximately pre-treatment levels following 3 weeks recovery. Ct was converted to copy number by reference to a standard curve. Data is represented on a log scale for ease of viewing. ** = P<0.01, **** = P<0.0001 Brown-Forsyth and Welch ANOVA. Data shown is from a representative experiment of 3 repeats.*

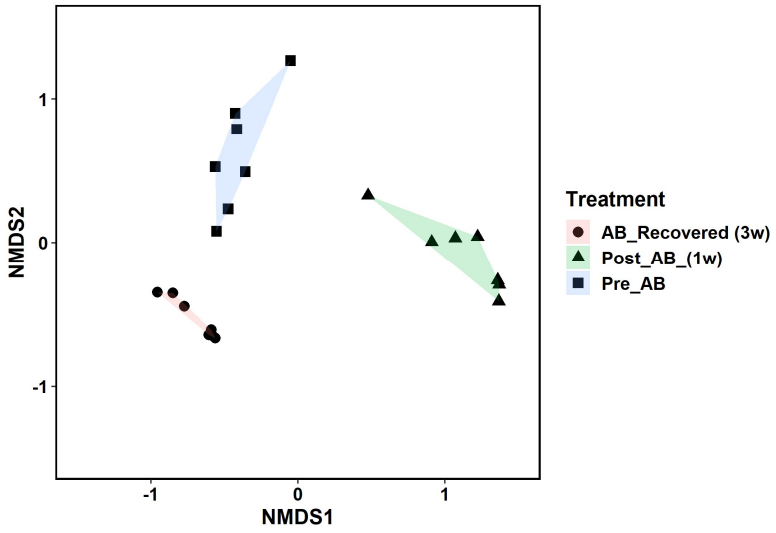
I first performed a qPCR to determine the quantity of 16S gene copies present in the faeces, normalising copy number to sample mass. It is evident that through 1 week of VNMA treatment (Post-AB (1w)) 16S copy number/mg faeces is depleted from a pre-treatment (Pre-AB) level of approximately 300,000,000 to approximately 20,000, which recovers to approximately pre-treatment levels following 3 weeks of recovery (Post-AB recovery (3w)) (Fig 3.1). This data demonstrates that while bacterial biomass is severely depleted by one week of VNMA treatment, there is recovery to pretreatment levels within 3 weeks, indicating no long-term effect to gut bacterial population size.

Having determined that bacterial population recovers over the defined recovery period, I then sought to investigate the impact upon population composition. The DNA from the faecal samples was sequenced by shotgun metagenomics, raw reads were quality filtered and trimmed, taxonomic assignment and metagenome reads conducted using Kraken 2 and Bracken then used to estimate relative taxa abundances. These species and genus abundance matrices were then analysed by alpha (Shannon) diversity (Fig 3.2a-b), beta (Bray-Curtiss dissimilarity) diversity between treatment groups (Fig 3.2 c) and LEfSe (Linear discriminant analysis of effect size) to show which specific genera (Fig 3.2 d) and species (Fig S1) are significantly increased or decreased in population size between treatment groups.

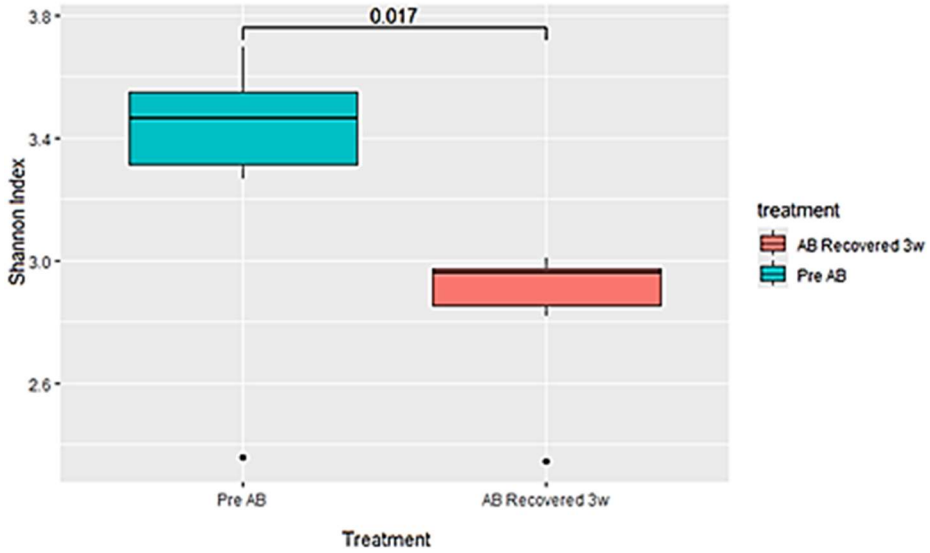
Firstly, it is evident there is a reduction in faecal bacterial diversity between pre-treatment and antibiotic recovered mice (Fig 3.2a), with a significant reduction of approximately 0.5 in Shannon index. Excluding one pre-treatment outlier, there is no overlap in Shannon index between these groups, with antibiotic recovered faecal samples displaying a reduction in both species richness and evenness (Fig 3.2 b). Analysis of Bray-Curtiss dissimilarity demonstrates that the composition of bacterial populations prior to antibiotic treatment are relatively diverse, having a loose though distinct clustering, with antibiotic treatment immediately driving a large change in composition as expected. However, following recovery, the composition fails to recover to its initial composition, clustering distinctly from the pre-treated samples, though relatively internally similar, clustering tightly (Fig 3.2c). Finally, the LEfSe analysis shows several key genera depleted following antibiotic treatment. Particularly noteworthy genera being: *Bacteroides, Parabacteroides, Alistipes* and *Phocaecicola* as they are associated with beneficial outcomes in liver disease and are correlated with reduced inflammation both in the gut and more generally [166–175]. Others show a population increase, notably *Staphylococcus, Campylobacter* and *Streptococcus* have a large increase in population

size. These genera encompass a number of species which are potential opportunistic pathogens or associated with worsened outcomes in liver disease [176–180] (Fig 3.2d).

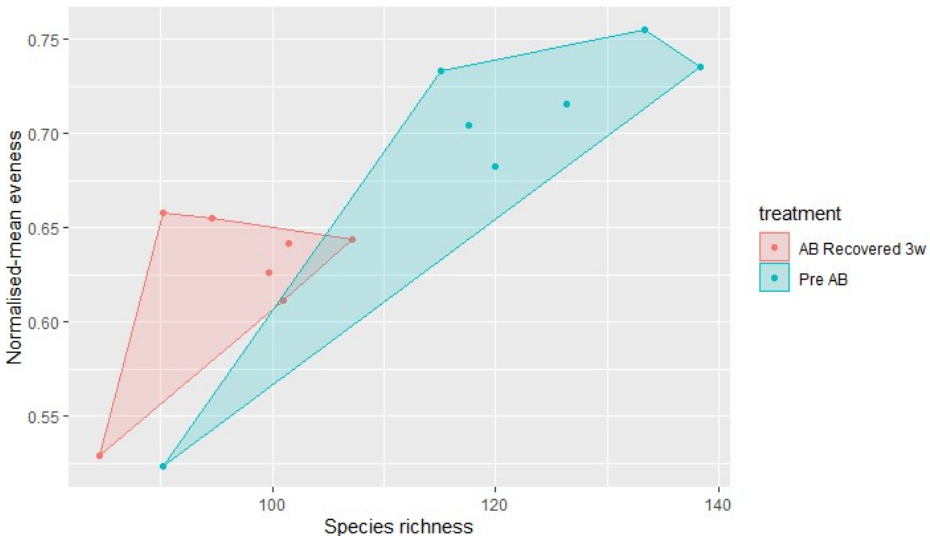
A.



B.



C.



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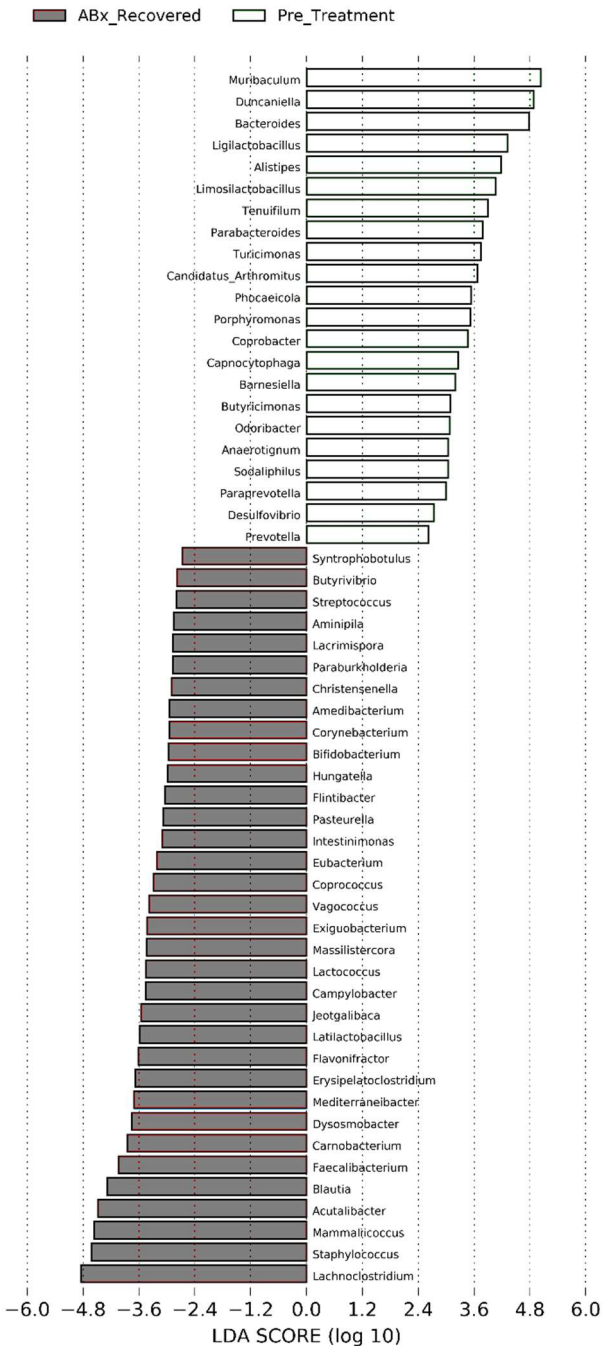

Figure 3.2 Comparative analysis of faecal bacterial population compositions in young mice throughout antibiotic treatment and recovery analysed by shotgun metagenomics. *NMDS plot of β diversity (Bray-curtiss) demonstrates distinct clustering of pre-treatment, post-antibiotic and antibiotic recovered bacterial populations (A). Shannon index measurement showing significant reduction in population diversity following antibiotic recovery (P measured by Wilcoxson Rank-sum test) (B), with a reduction in both population evenness and richness (excluding one pre-treatment sample) (C). Linear discriminant analysis plot demonstrating differential enrichment of several key genera between pre-treatment and antibiotic recovered mice, significance value threshold P<0.01 (D). This analysis was conducted once.*

Overall, these data indicate that while bacterial population size may recover following

antibiotic treatment, population composition is negatively affected. Diversity is significantly reduced, and population composition changed, with a higher proportion of genera associated with worse outcomes in liver diseases and a decreased population of those associated with improved disease.

3.3.2 Antibiotic recovered mice produce an altered pool of faecal bile acids, with an increase in FXR antagonists with a coincident decrease in agonists.

The primary function of the gut microbiota in the gut-liver axis is the metabolism and recirculation of bioactive compounds such as bile acids. Hence, having determined that the microbiota is significantly altered by antibiotic treatment, I then sought to determine the impact this had upon biliary processing. As previously, faecal samples were collected throughout the antibiotic treatment course and post-recovery (Fig 3.A), and bile acids were extracted and quantified by LC-MS.

Analysis showed that, post antibiotic recovery, the faecal biliary pool was altered with an increased prevalence of TMCA, a primary bile acid and FXR antagonist [79], and a decreased prevalence of DCA and LCA, secondary bile acids and potent FXR agonists [79].

Overall, this represents a decrease in secondary bile acids produced by microbial metabolism and a concomitant increase in primary, unprocessed bile acids (Fig 3.3a). Despite this apparent alteration in FXR agonist vs antagonist ratio, there is no change in total bile acid content, suggesting a lack of alteration in biliary outflow from the liver (Fig 3.3b).

3.3.3 Antibiotic recovered mice produce a somewhat altered hepatic bile acid pool, dominated by Tauromuricholic acid.

To further investigate this alteration to bile metabolism, the hepatic pool of bile acids was then quantified by LC-MS. Cryopreserved liver tissue samples were obtained, bile extracted, and bile acid content measured by LC-MS with content normalised to sample mass.

Analysis showed a predominance of TMCA in both pre-treatment and post-recovery, with an increase post-recovery to approximately 90% of total content. There is also a

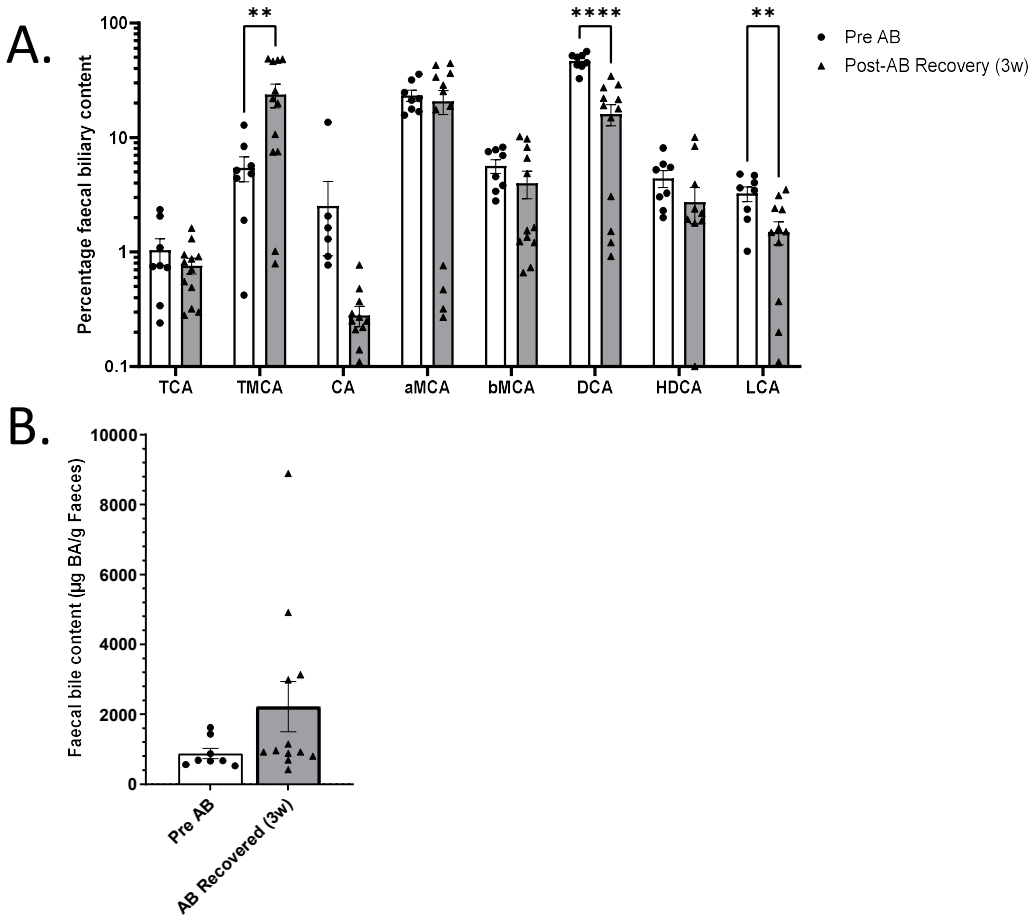

Figure 3.3 LC-MS quantification of faecal biliary content in pre-treatment and antibiotic recovered faeces. *Graph showing bile acid content as a percentage of total faecal bile acid content (A) TMCA content is significantly increased following antibiotic recovery, while DCA and LCA are significantly reduced, data shown on a logarithmic scale for ease of viewing. Graph showing total bile acid content which remains unaffected following antibiotic recovery excluding select samples (B) ** = P<0.01, **** = P<0.0001. Welch T-test. Data shown is from a representative experiment of 3 repeats.*

reduction in the content of TCA and TDCA, though the latter is only present in relatively trace amounts (Fig 3.4a). Total hepatic bile content remains unaffected by antibiotic treatment and recovery (Fig 3.4b).

These data indicate that while faecal biliary content is altered in antibiotic recovered mice, comprising of a greater content of FXR antagonists and a lesser of agonists, there is no alteration in total faecal bile acid content. Additionally, there is a significant increase in TMCA, with an associated reduction in other primary bile acids but with no change in total hepatic pool. This suggests that microbial metabolism of bile acids in the gut is altered, generating a reduced quantity of secondary bile acids, this then may have

an impact upon synthesis of bile acids to produce a greater proportion of TMCA vs TCA and TDCA in the liver, while there is no impact upon quantity of bile acid generated or outflow to the gut.

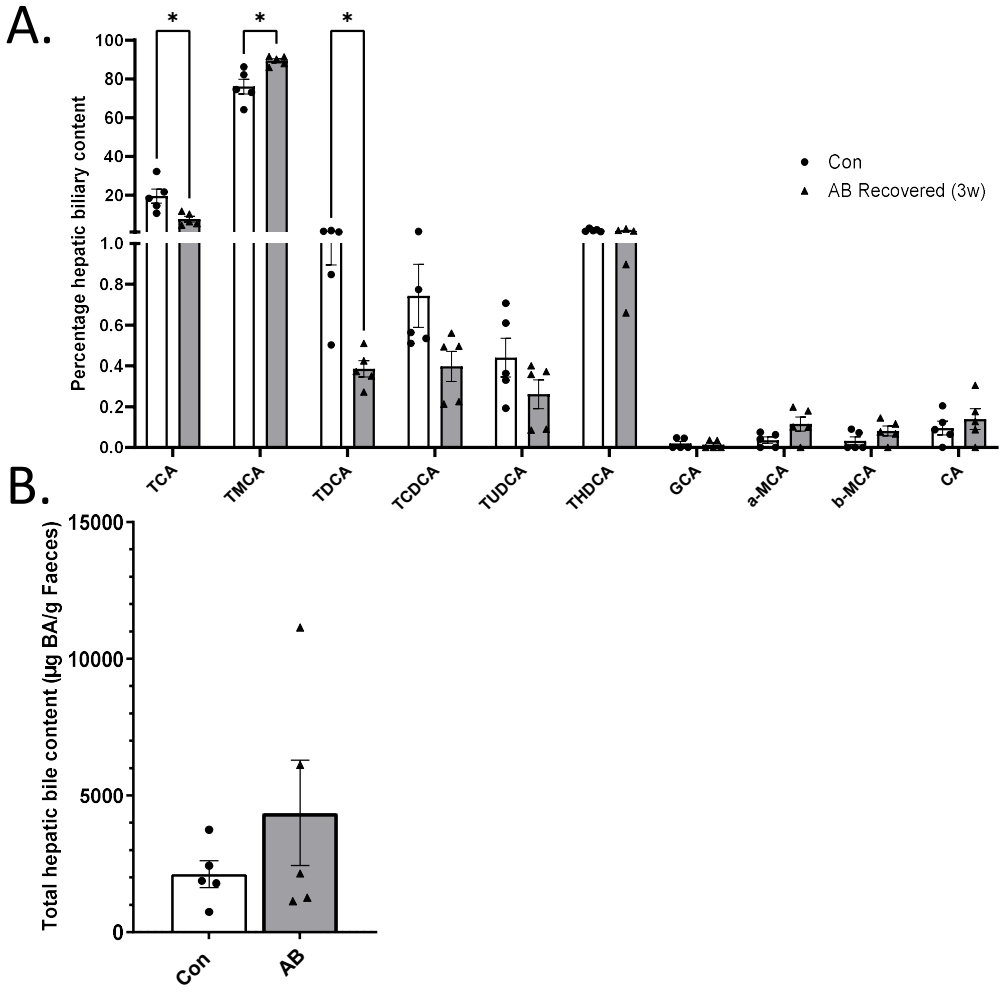

Figure 3.4 LC-MS quantification of hepatic biliary content in pre-treatment and antibiotic recovered liver samples. *Graph showing bile acid content as a percentage of total hepatic biliary content (A). TMCA content is significantly increased following antibiotic recovery, while TCA and TDCA are significantly reduced, data shown on a split scale (0-1%, 1-100%) for ease of viewing. Graph showing total bile acid content which remains unaffected following antibiotic recovery excluding select samples (B) * = P<0.05. Welch T-test. Data shown is from a representative experiment of 3 repeats.*

3.3.4 Antibiotic treatment led to reduced faecal SCFA content, with acetate and propionate being depleted to the greatest extent.

Having identified alterations in faecal biliary content, I then sought to investigate potential deviations in the content of SCFAs in the faeces. As previously, SCFAs were extracted from faecal samples and quantified by LC-MS.

SCFA content was reduced consistently following antibiotic recovery, with acetate and propionate displaying the greatest reduction from approximately 2 mg/g to 0.6 mg/g respectively (Fig 3.5). This data suggests that the capacity of the recovered microbiota to generate SCFAs is severely impaired.

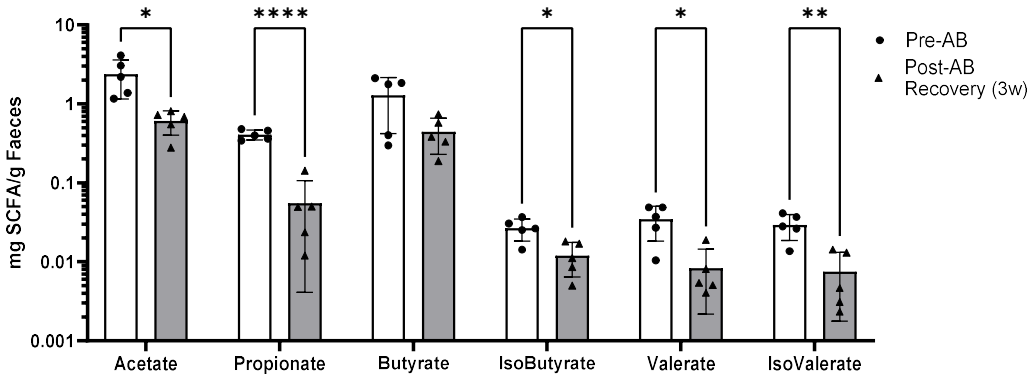

Figure 3.5 LC-MS quantification of the faecal SCFA content in pre-treatment and antibiotic recovered mice. *All SCFAs are reduced in antibiotic recovered mice while in butyrate this is not statistically significant. * = P<0.05, ** = P<0.01, **** = P<0.0001. Welch T-test. Data shown is from a representative experiment of 3 repeats.*

3.3.5 Following bile duct ligation there is an increase in bacterial population with antibiotic recovered mice displaying an altered population composition.

In order to determine the effect of microbial community change upon cholestasis severity it was first necessary to determine how the microbiome continues to change following BDL and particularly the differential effects upon the antibiotic recovered bacterial community. BDL is a common, acute, model of cholestatic liver disease in which the common bile duct is ligated to prevent biliary outflow, which leads to bile accumulation progressing to cholestatic disease [181]. 16S qPCR upon DNA extracted from faecal samples collected following 7 days BDL showed that in comparison to healthy mice, BDL mice displayed an approximately 3x increase in faecal 16S copy number, though this was not statistically significant when comparing pre-treatment to BDL only (Fig 3.6). This is to be expected given the common cooccurrence of small intestinal bacterial overgrowth (SIBO) with cholestasis [182]. In addition to an alteration to population size, there is a change to composition dissimilarity (Fig 3.7). Between control and BDL, this difference is slight, with a large overlap in clustering between groups, whereas between antibiotic recovered and antibiotic recovered BDL (ABBDL) groups, in

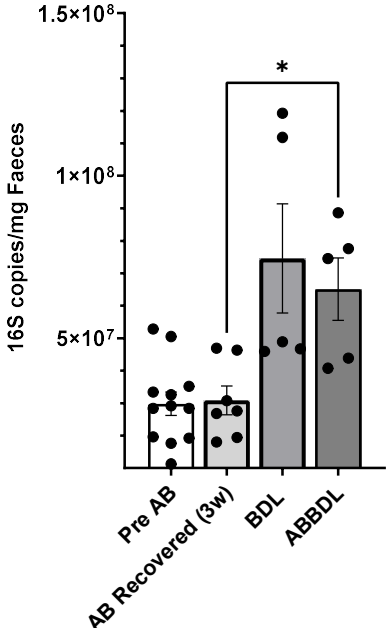

Figure 3.6. Faecal universal 16S qPCR showing expansion of bacterial population following bile duct ligation. *Cholestasis induced by BDL greatly increases faecal bacterial population size. 16S copy number was calculated with reference to a 16S standard curve. * = P<0.05, Brown-Forsyth and Welch ANOVA. Controls shown are the same as Fig 3.1. Data shown is from a representative experiment of 3 repeats.*

the majority of samples there is a shift in composition shown by NMDS plot of Bray-Curtiss dissimilarity (Fig 3.7a). This composition is distinct from that of antibiotic recovered, pre-treatment and BDL samples. Whilst it is evident that there is a reduction in diversity measured by Shannon index, from pre-treatment (Pre-AB) to recovered (AB recovered (3w)) samples, there is no reduced diversity observed between untreated BDL samples and antibiotic pretreated BDL (ABBDL) samples (Fig 3.7b), though it appears that there is a large reduction in species richness and evenness in a subset of antibiotic recovered BDL samples in comparison to the majority of untreated BDL samples (Fig 3.7c). When investigating specific alterations to genera measured by LDA, the most notable change is the reduced proportion of *Bacteroides* and *Alistipes* in antibiotic recovered BDL samples (Fig 3.7d).

Overall, these data recapitulate that cholestasis has a significant impact upon bacterial biomass, causing a degree of bacterial overgrowth. Furthermore, at a genus level, this data affirms the previous results showing the reduction in *Bacteroides*, *Alistipes* and *Phocaecicola* in antibiotic recovered mice when compared to untreated, a change seemingly unaffected by the induction of cholestasis, though differences in Parabacteroides are not seen following BDL. The lack of difference in diversity must also

be noted, although this appears to be owing to a small number of outliers in the BDL (BDL only) and ABBDL (3-week antibiotic recovered BDL) groups seen by the clustering of most BDL and ABBDL samples when viewed by richness and evenness.

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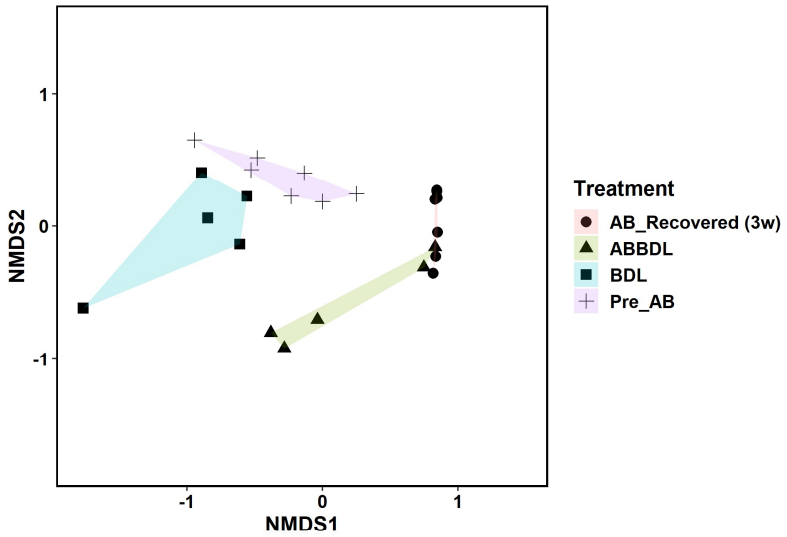

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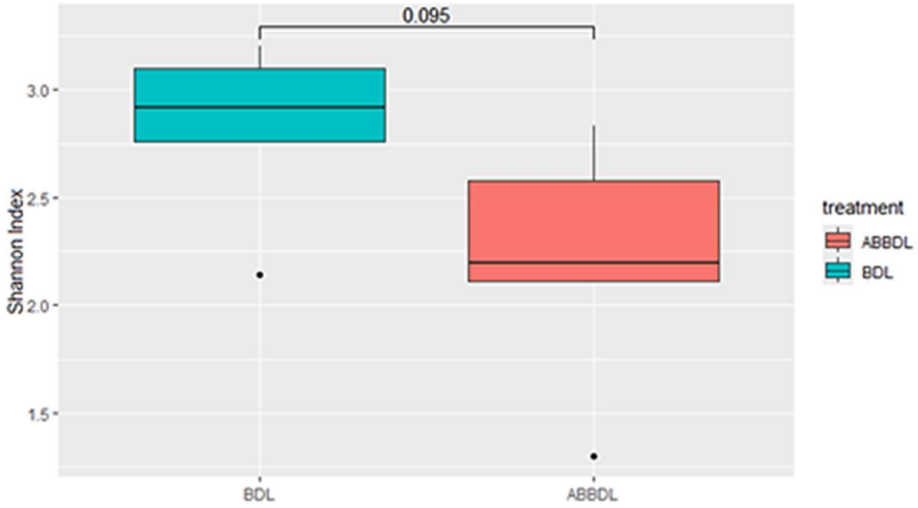

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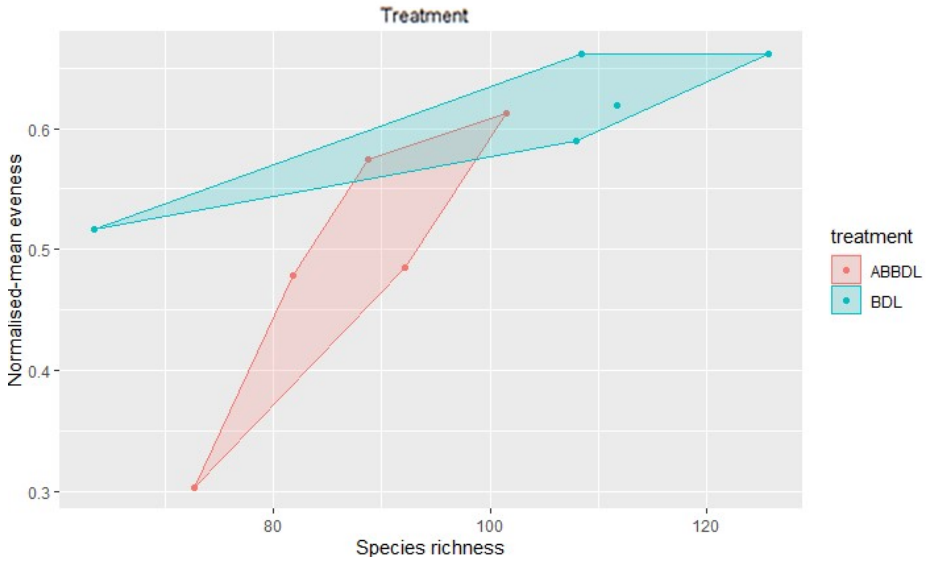


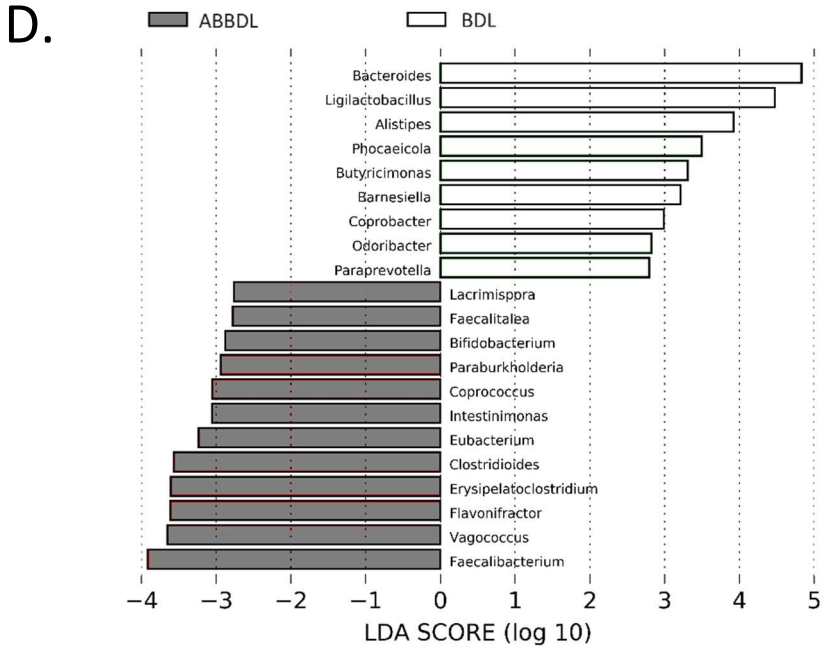


Figure 3.7 Comparative analysis of faecal bacterial population compositions between BDL and ABBDL mice analysed by shotgun metagenomics. *NMDS plot of β diversity (Bray-curtiss) demonstrates distinct clustering of Control, antibiotic recovered, BDL and antibiotic recovered BDL gut bacterial populations (A). Shannon index measurement showing reduction in population diversity between BDL and antibiotic pre-treated BDL gut bacterial populations, though not significant (P measured by Wilcoxson Rank-sum test) (B), with a general reduction in both population evenness and richness (C). Linear discriminant analysis plot demonstrating differential enrichment of several key genera between BDL and antibiotic recovered BDL mice with a P value threshold of P>0.01 (D). This analysis was conducted once.*

3.3.6 Antibiotic pretreatment has no effect upon hepatic biliary content following BDL.

While it is evident that cholestatic disease will have an impact upon hepatic biliary content, the impact of antibiotic pretreatment has not been studied. In order to investigate the impact of antibiotic pretreatment upon post-BDL hepatic biliary content, a comparative analysis between pre-treatment, BDL and antibiotic pre-treated BDL hepatic bile acids was performed.

Results show that BDL produces a significantly altered bile acid pool, with TMCA increasing from 65% to near 100% of content. This reduces the relative content of other primary bile acids such as TCA and CA. Furthermore, there is a reduction in the trace quantities of secondary bile acids, such as TDCA, TUDCA and THDCA. However, there are

no statistically significant differences between BDL and antibiotic pre-treated BDL samples (Fig 3.8).

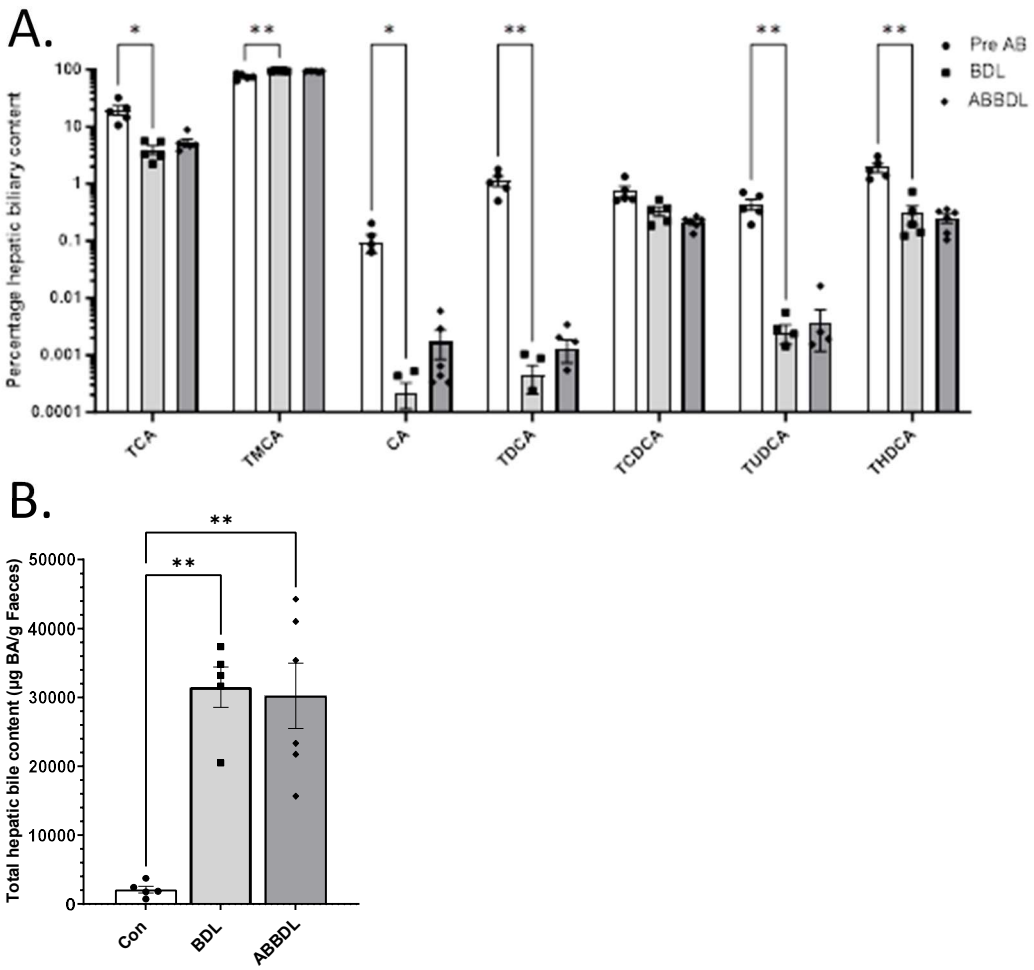


Figure 3.8 LC-MS quantification of hepatic biliary content in pre-treatment, BDL and antibiotic recovered BDL liver samples. *Graph showing bile acid content as a percentage of total hepatic biliary content (A). TMCA content is significantly increased following BDL, while TCA, CA, TDCA, TUDCA and THDCA are significantly reduced (though they are only present in trace amounts), data shown on a logarithmic scale for ease of viewing. Graph showing total hepatic bile acid content which is increased by BDL but unaffected by antibiotic pre-treatment. (B) * = P<0.05, ** = P<0.01. Welch T-test. Data shown is from a representative experiment of 3 repeats.*

3.3.7 BDL reduces the SCFA output of the microbiota, with a greater reduction in the faecal SCFA content of antibiotic pretreated BDL mice.

As has been shown in this thesis and in the literature [116,183], BDL somewhat alters the composition of the gut microbiota, which could be expected to alter the production of SCFAs in the gut. Given this potential disruption, the cumulative effect of antibiotic

treatment and BDL could further reduce or disrupt faecal SCFA content which I then sought to investigate. SCFA content was quantified by LC-MS in faecal samples collected from mice pre-treatment (Pre-AB) and following BDL in untreated (BDL) and antibiotic pre-treated (ABBDL) mice. Analysis showed that BDL has a negative impact upon faecal SCFA content, significantly reducing the quantities of propionate, butyrate and valerate. Antibiotic pretreatment led to a greater reduction in propionate and butyrate from pretreatment, though this was not statistically significant between ABBDL and BDL only, however valerate is significantly reduced though only present in relatively trace amounts (Fig 3.9). This demonstrates that the relatively minor change in bacterial population composition produced by BDL generates a reduction in SCFA output and that this effect is intensified by the additional changes from antibiotic pretreatment shown previously.

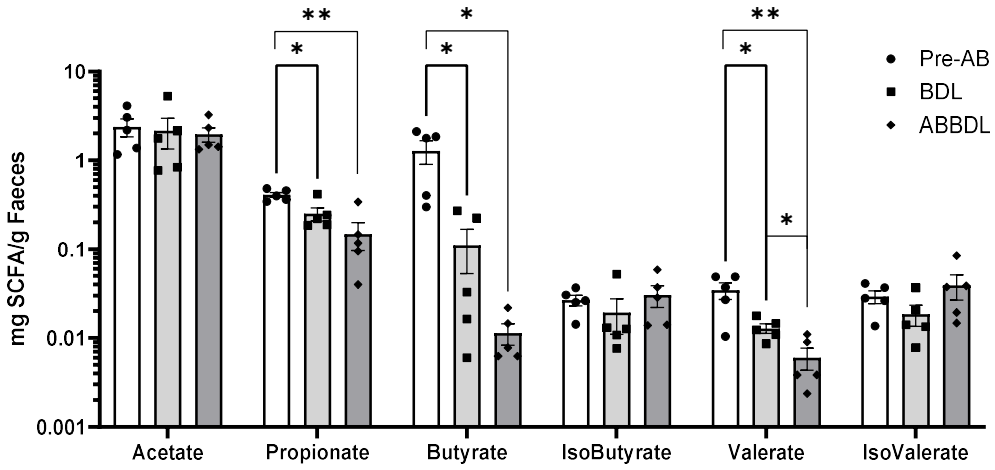


Figure 3.9 LC-MS quantification of the faecal SCFA content of pre-treatment, BDL and antibiotic recovered BDL mice. *Faecal propionate, butyrate and valerate are reduced in BDL mice in comparison to pre-treatment mice, with a greater reduction in antibiotic pre-treated BDL mice. * = P<0.05, ** = P<0.01. Welch T-test. Data shown is from a representative experiment of 3 repeats.*

3.3.8 Antibiotic pretreatment increases fibrosis resulting from cholestatic disease, while hepatic damage and the ductular reaction remain unaffected.

Having identified several alterations in both key bacterial populations and their metabolites involved in the gut-liver axis, noted for their roles in liver diseases, I then sought to investigate how these changes may impact upon cholestatic disease progression. The accumulation of bile acids in the liver results in cytotoxicity and direct hepatic damage. Therefore, in order to investigate the impact of antibiotics on the

progression of cholestatic disease I first sought to analyse the extent of liver damage. Briefly, liver samples were fixed in formalin, embedded in paraffin and sections cut using a microtome. These sections were then rehydrated and haematoxylin and eosin stained. The sections were imaged using brightfield microscopy and necrotic tissue area was then quantified manually using image j software. This analysis showed a slight though not statistically significant increase in necrosis resulting from antibiotic treatment prior to BDL (Fig 3.10).

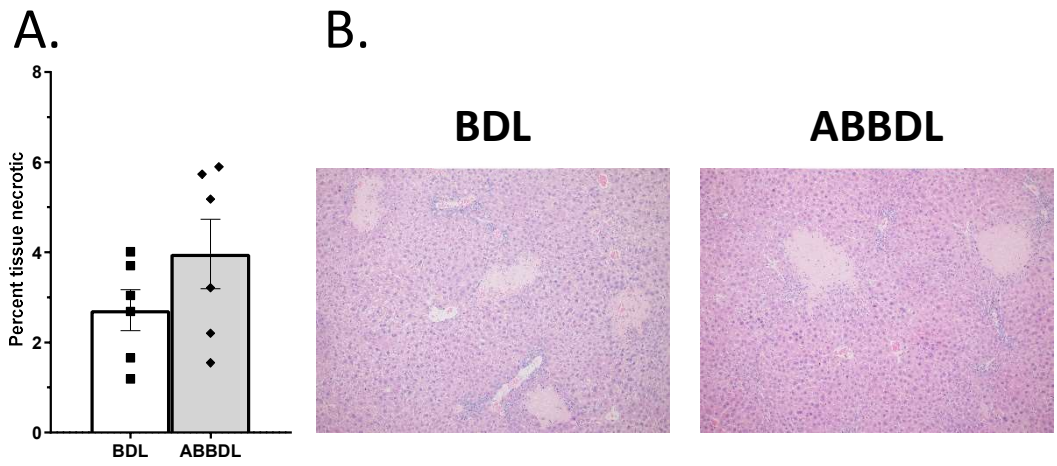


Figure 3.10 Haematoxylin and eosin visualisation of tissue necrosis in BDL and antibiotic pretreated BDL livers. *Manual quantification of necrotic areas shows a slight but not statistically significant increase in necrosis (A), Representative images are shown right (B). Significance measured by Welch T-test. Data shown is from a representative experiment of 3 repeats.*

In addition to histological analysis, transaminase content in serum was measured as a quantitative measure of damage. Serum was extracted from blood samples by centrifugation and diluted 10-fold in the case of BDL and ABBDL mice and 2-fold in control or antibiotic recovered mice. These diluted sera were then analysed using a RANDOX Daytona+ clinical chemistry analyser. Owing to a shortage of serum from several samples in our original experiment, the serum data is somewhat unreliable. These data display an increase in ALT to a greater extent than AST, though the statistical significance must be treated with a degree of scepticism (Fig 3.11).

Following cellular death arising from bile toxicity, the liver responds through cholangiocyte activation and ductular proliferation, known as the ductular reaction [150,181]. The extent of the ductular reaction in cholestasis is a measure of the severity and progression of cholestatic disease. In order to assess the extent of the ductular reaction in our samples, I performed an immunohistochemical stain on formalin fixed, paraffin embedded liver samples, staining for CK19 - a marker present on proliferating

cholangiocytes. These stains were then quantified manually using image j (Fig 3.12). The analysis showed a slight but statistically insignificant increase in CK19 staining between BDL and antibiotic pre-treated BDL mice.

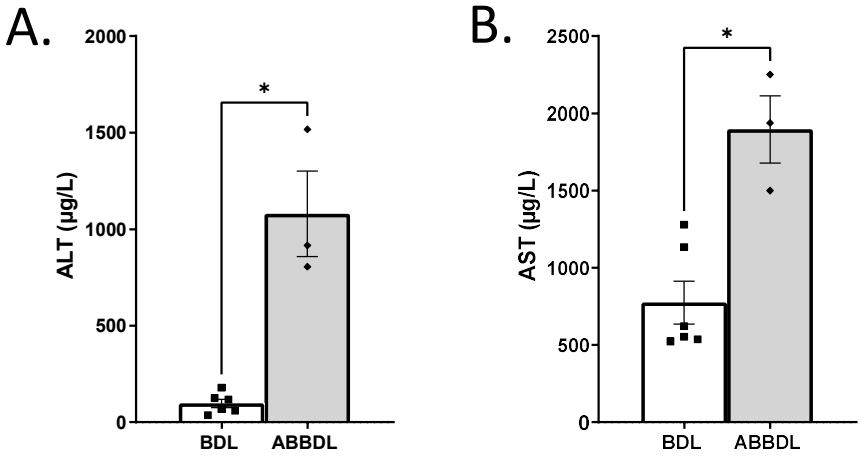


Figure 3.11 Serum transaminase quantification (Alanine transaminase and Aspartate transaminase) by RANDOX Daytona+. *ALT levels (A) and AST levels (B) are elevated in ABBDL samples compared to BDL only. * = P<0.05 Welch T-test. Data shown is from a representative experiment of 3 repeats.*

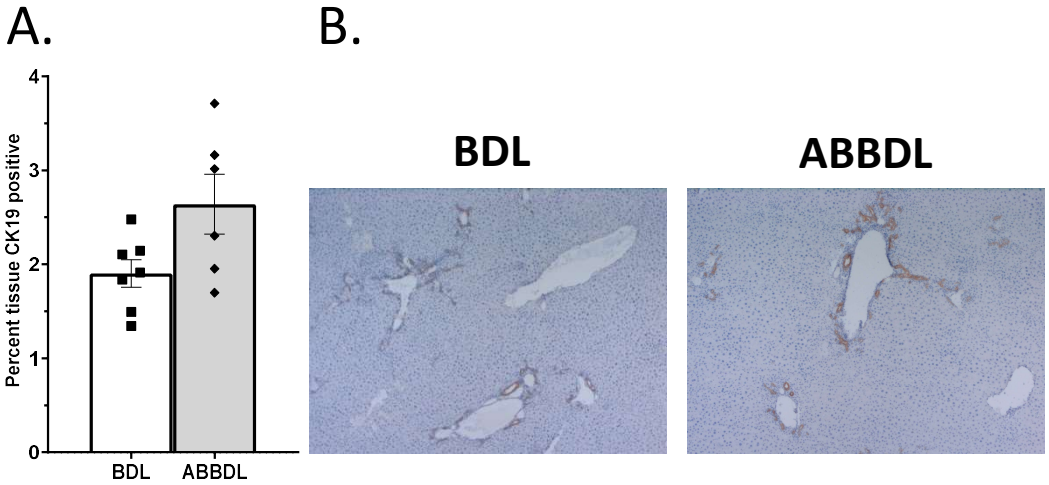


Figure 3.12 CK19 immunohistochemical staining showing cholangiocyte proliferation in BDL and antibiotic pre-treated BDL livers. *Manual quantification of stained area shows a slight but not statistically significant increase in ductular proliferation (A), Representative images are shown right (B). Significance measured by Welch T-test. Data shown is from a representative experiment of 3 repeats.*

As a further and quantitative analysis of the extent and severity of cholestasis I then analysed serum alkaline phosphatase and total bilirubin content. Serum alkaline phosphatase quantification being a routine measurement method of the extent and severity of cholestatic disease [150]. Furthermore, total serum bilirubin indicates the extent of cholestasis by the leakage of bile into the bloodstream following buildup in the bile duct obstructed liver. Data showed no significant differences between BDL and ABBDL in

either ALP or total bilirubin, reaffirming that the extent of cholestasis was unaffected by antibiotic pre-treatment (Fig 3.13).

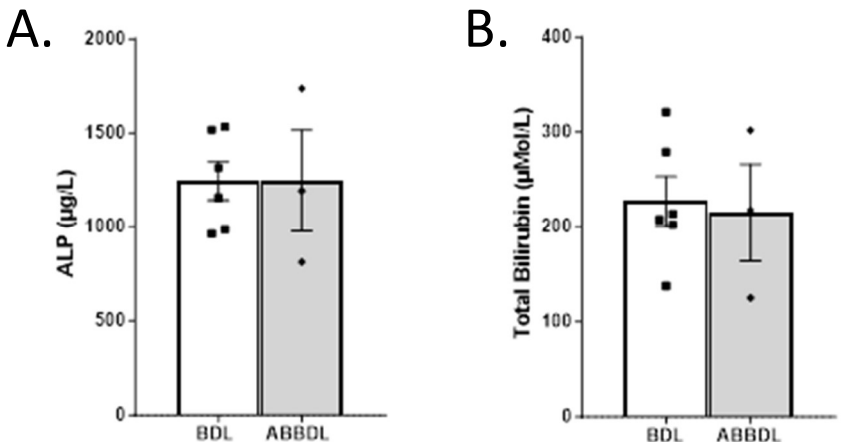


Figure 3.13 Serum Alkaline phosphatase and total bilirubin quantification. *ALP levels (A) and total bilirubin levels (B) are unaffected by antibiotic pre-treatment in BDL mice. Significance measured by Welch T-test. Data shown is from a representative experiment of 3 repeats.*

Having identified the extent of both necrosis and the ductular reaction I then investigated the effect of antibiotic pre-treatment upon the extent of fibrotic scarring during cholestatic disease. Fixed and paraffin embedded liver samples were Sirius red stained, and images were quantified for percent red stained area using an automated image j macro. Data demonstrated that antibiotic pre-treated mice have significantly increased fibrosis arising from cholestasis, displaying an approximate 50% increase in scarring in comparison to untreated BDL mice (Fig 3.14).

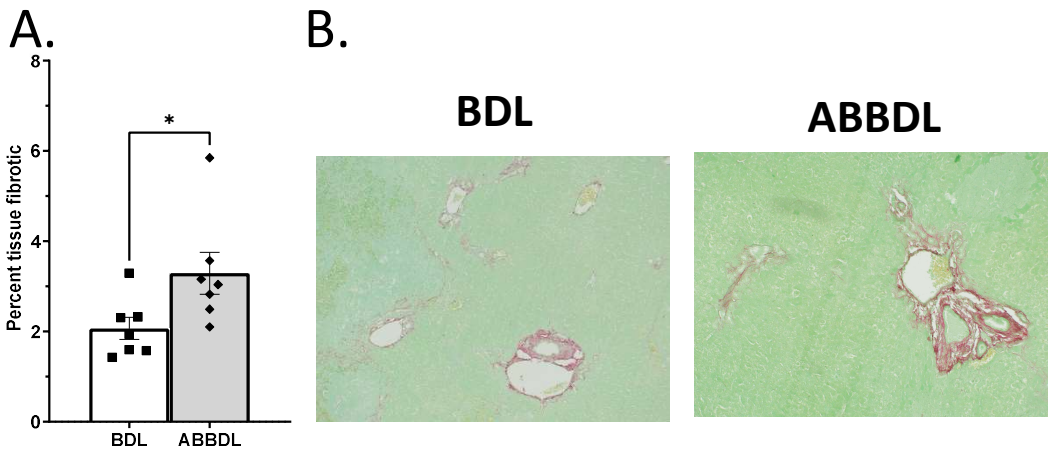


Figure 3.14 Sirius red staining showing fibrotic deposition (red staining) in BDL and antibiotic pre-treated BDL mice. *Macro quantification of stained area shows a statistically significant increase in fibrosis given antibiotic pretreatment (A). Representative images are shown right (B). *=P<0.05 Welch T-test. Data shown is from a representative experiment of 3 repeats.*

Overall, these data demonstrate that while antibiotic treatment has limited to no effect upon hepatic damage, the ductular reaction and the severity of cholestasis, there is a significant increase in fibrosis resulting from said damage and cholestatic injury following BDL. This suggests that antibiotic treatment and recovery leaves the resilience of liver tissues to biliary cytotoxicity unaffected, though the response to cellular damage results in increased fibrotic deposition. The aim of further investigation was therefore, to elucidate the mechanism by which fibrosis was promoted following antibiotic pre-treatment.

3.3.9 Antibiotic pretreatment does not affect the level of intestinal permeability following BDL.

My next objective was to investigate the alterations to liver immunity which may produce the observed increase in fibrosis and potential increase in damage. Firstly, I sought to determine the inflow of immune stimuli from the gut, this initial investigation will inform further analyses of the stimulus to which immune cells are responding. In order to demonstrate the degree of intestinal permeability, mice were administered with FITC-dextran by oral gavage two hours prior to sacrifice, whole blood was then extracted, serum isolated by centrifugation and FITC content measured by relative fluorescence on a plate reader. Results demonstrate that BDL significantly increases

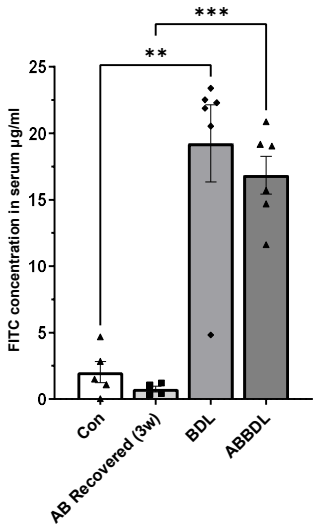


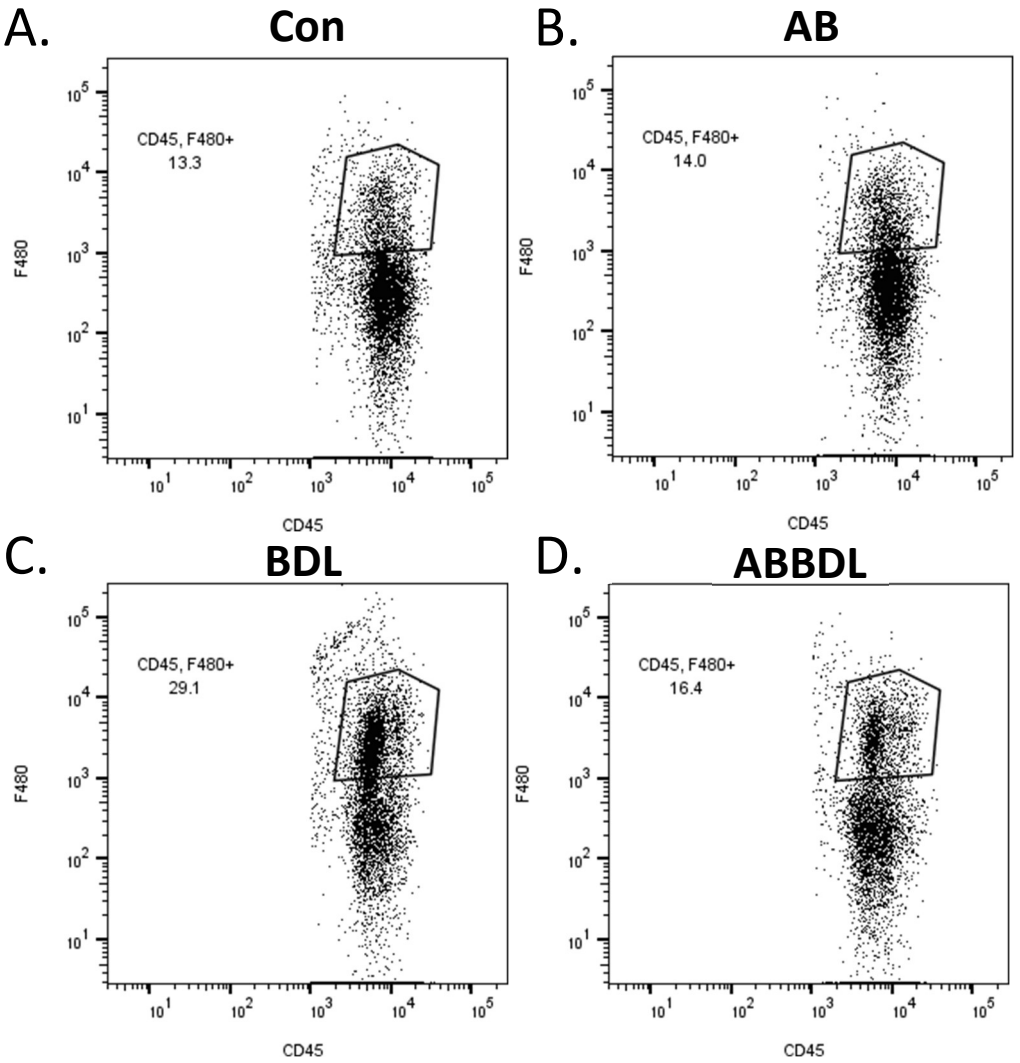
Figure 3.15 FITC-Dextran content in serum following BDL untreated and antibiotic pretreated mice. *BDL significantly increases FITC content in serum, suggesting increased intestinal permeability which is well documented. However antibiotic recovered mice display no significant alteration to permeability. Statistical testing conducted by Brown-forsyth and Welch ANOVA. ** = P<0.01, *** = P<0.001. Data shown is from a representative experiment of 3 repeats.*

intestinal permeability (measured by FITC-Dextran content in serum), though there is no further significant effect given antibiotic pre-treatment (Fig 3.15).

3.3.10 Antibiotic pretreatment reduces monocyte/macrophage infiltration into the cholestatic liver, while cytokine expression remains mostly unaffected in vivo.

Once it had been determined that gut permeability to antigenic stimulus which may arrive at the liver is consistent between antibiotic pretreated BDL and untreated BDL mice, I then investigated the innate immune cell population of the liver. Focus was placed in particular upon the innate immune population, particularly macrophages given their role in wound and damage resolution and the initiation of fibrosis both in cholestatic liver disease and more generally [45,66,121,127,133,184–186]. In order to investigate this, immune cells were isolated from whole liver tissue samples, stained using fluorescently labelled antibody markers and analysed by flow cytometry. Data indicates that the quantity of F4/80^{+} macrophages present in the antibiotic pre-treated cholestatic (ABBDL) liver is significantly reduced to near control levels (Fig 3.16). Furthermore, the quantity of Ly6C Hi cells is also significantly reduced to in antibiotic pre-treated cholestatic livers suggesting that the reduction in macrophage population is driven by a reduction in infiltrating monocytes (Fig 3.17).

In order to confirm the reduced infiltration of macrophages in ABBDL mice compared to BDL only, I then performed an immunohistochemical analysis of liver sections, staining for F480 to further investigate the presence and localisation of macrophages in the cholestatic liver. Analysis showed a lower amount of F4/80 stained tissue in antibiotic pre-treated BDL livers when compared to BDL alone (Fig 3.18). Furthermore, there appears to be a change in macrophage clustering given antibiotic pre-treatment, whereby macrophages are more dispersed in the parenchyma and show reduced grouping when compared to BDL only. These observations and data suggest that antibiotic pre-treatment reduces macrophage infiltration into the cholestatic liver indicated by reduced Ly6C^{Hi} cells within the F480+ population, which drives an overall reduction in macrophage population shown by reduced F480+ populations in addition to reduced F480 staining. Additionally, it appears that the macrophages present in the liver are impaired in their chemotactic behaviour suggested by the reduction in clustering of F480+ cells in immunostained liver tissue sections (Fig 3.18).



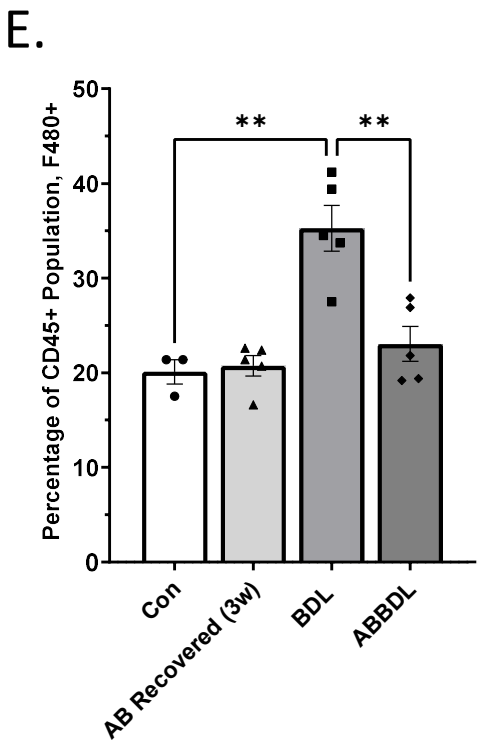
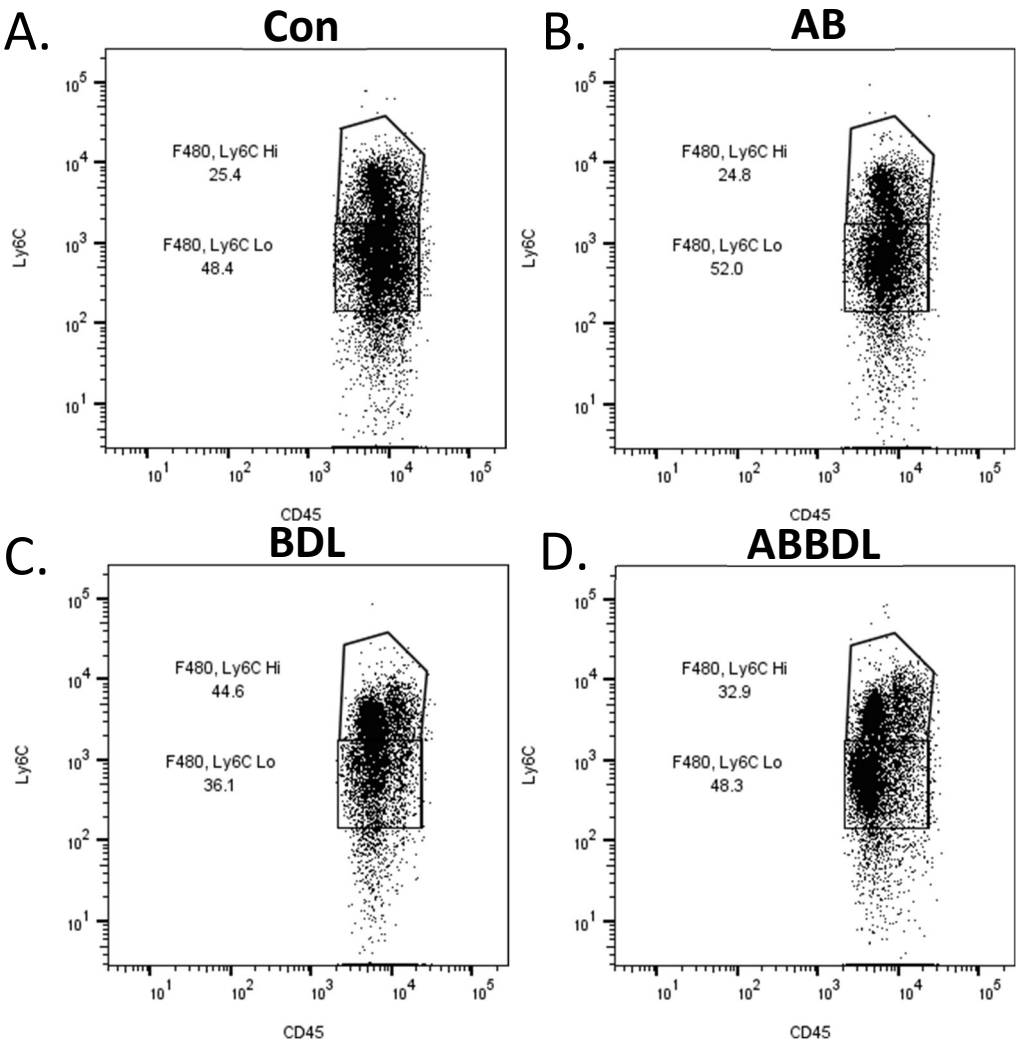


Figure 3.16 Flow cytometry analysis displaying F480+ populations in the liver.

*Flow cytometry plots showing F480+ cell populations in Control (A), Antibiotic recovered (B), BDL (C) and Antibiotic pre-treated BDL (D) Livers. Graph showing F480+ cell population as a percentage of CD45+ cell population in the Liver (E) ** = P<0.01 Brown-Forsyth Welch ANOVA. Antibiotic pre-treatment has no effect upon basal levels of F480+ populations (A,B,E), BDL significantly increases the F480+ population from control levels (A,B,C,E), while antibiotic pre-treatment significantly reduces the F480+ population following BDL to near control levels. Data shown is from a representative experiment of 3 repeats.*



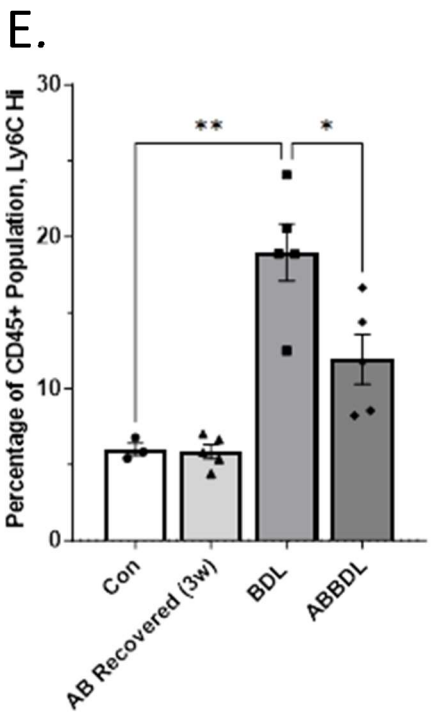


Figure 3.17 Flow cytometry analysis displaying F480+, Ly6C populations in the liver. *Flow cytometry plots showing Ly6C Hi and Lo cell populations previously screened for F480 staining in Control (A), Antibiotic recovered (B), BDL (C) and Antibiotic pre-treated BDL (D) Livers. Graph showing F480+, Ly6C Hi cell population as a percentage of CD45+ cell population in the Liver (E) * = P<0.05, ** = P<0.001 Brown-Forsyth Welch ANOVA. Antibiotic pre-treatment has no effect upon basal levels of Ly6C Hi populations (A,B,E), BDL significantly increases the Ly6C Hi population from control levels (A,B,C,E), while antibiotic pre-treatment significantly reduces the Ly6C Hi population following BDL to near control levels. Data shown is from a representative experiment of 3 repeats.*

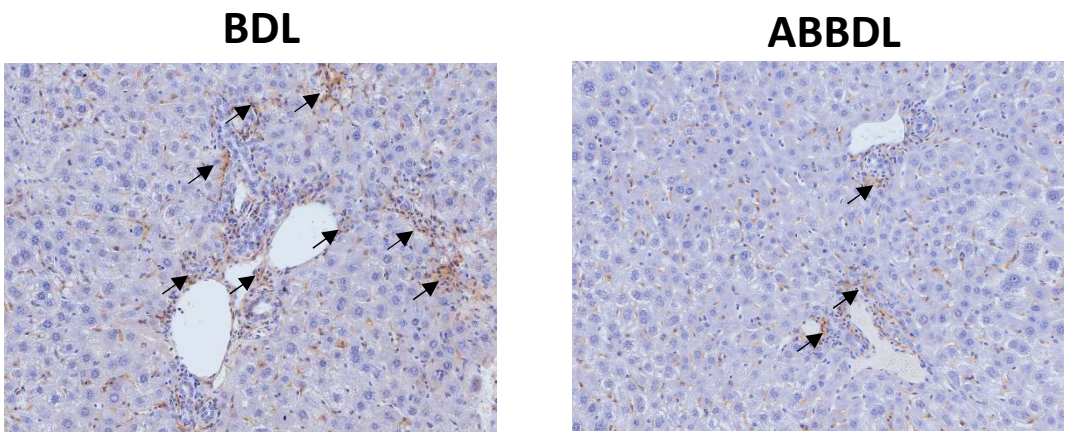


Figure 3.18 F4/80 immunohistochemical staining of cholestatic liver tissue sections.
Representative images of F4/80 stained liver tissue sections showing that antibiotic pre-treatment significantly reduces F4/80 staining, F4/80 stained cells also appear to be more dispersed, with fewer and smaller discernible clusters of staining (indicated by arrows). This analysis was conducted twice, images shown are from one representative experiment.

Given the results of the flow cytometry analysis, I then sought to analyse how this altered macrophage infiltration in ABBDL mice might impact upon cytokine expression and by extension disease pathophysiology during cholestasis. RNA was extracted from whole liver tissue, quantified and cDNA generated. This cDNA was then analysed for the level of various cytokines by real-time qPCR. Results demonstrated no significant differences in cytokine expression between ABBDL and BDL only mice, however there was a noticeable increase in Il1β and Il6 in various antibiotic pre-treated BDL samples though not statistically significant (Fig 3.19).

This data demonstrates that despite the significant decrease in macrophage infiltration, there appears to be no associated reduction in pro-inflammatory cytokine expression in antibiotic pretreated mice. Furthermore, the lack of decisive change in chemokine expression would suggest a decrease in sensitivity to chemoattractants. Furthermore, the lack of change in pro-inflammatory cytokine expression, taken together with the reduction in total macrophage population would imply a more pro-inflammatory polarisation conferred upon the macrophages present to produce an equivalent level of total cytokine expression. Alternatively, other cell types may be affected and are responsible for the inflammation not being produced by the macrophages absent in ABBDL mice in comparison to BDL-only mice.

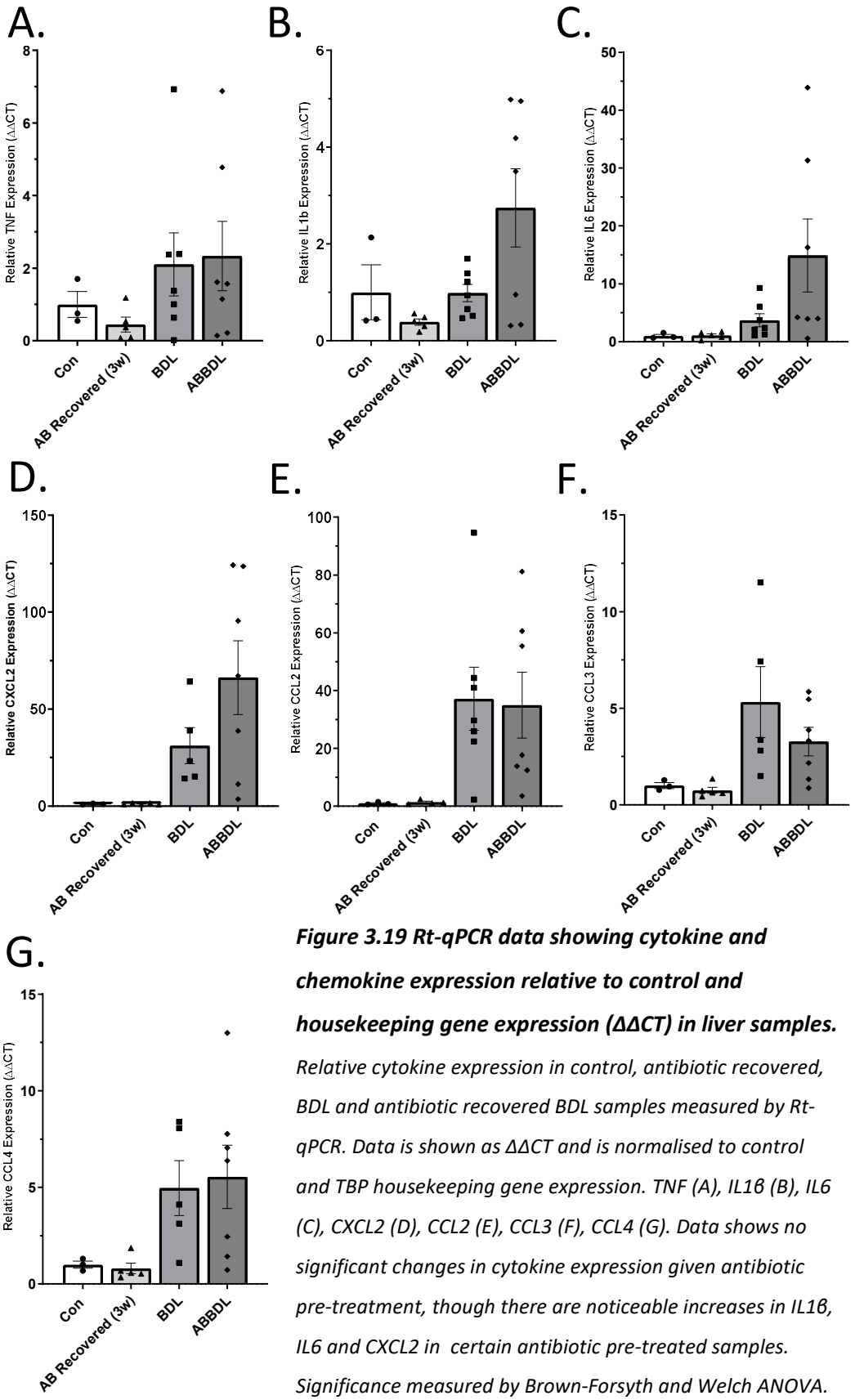


Figure 3.19 Rt-qPCR data showing cytokine and chemokine expression relative to control and housekeeping gene expression (ΔΔCT) in liver samples.

Relative cytokine expression in control, antibiotic recovered, BDL and antibiotic recovered BDL samples measured by Rt-qPCR. Data is shown as ΔΔCT and is normalised to control and TBP housekeeping gene expression. TNF (A), IL1β (B), IL6 (C), CXCL2 (D), CCL2 (E), CCL3 (F), CCL4 (G). Data shows no significant changes in cytokine expression given antibiotic pre-treatment, though there are noticeable increases in IL1β, IL6 and CXCL2 in certain antibiotic pre-treated samples. Significance measured by Brown-Forsyth and Welch ANOVA. Data shown is from a representative experiment of 3 repeats.

3.3.11 In comparison to macrophages from untreated mice, those from antibiotic pretreated mice display a more pro-inflammatory, less phagocytic response to LPS stimulus and an altered response to dead cells in vitro.

In order to investigate these seemingly contradictory results whereby cytokine expression remains consistent, though macrophage infiltration is reduced, and to confirm this nascent hypothesis; that macrophages must therefore have a more pro-inflammatory phenotype, I investigated cytokine expression in isolated macrophages in response to LPS and dead cells; both relevant to the progression of cholestatic liver disease in vivo. Briefly, bone marrow HSCs were extracted from control and 3-week antibiotic recovered mice and those from each treatment group were pooled to achieve the required cell count for each well. These HSCs were then differentiated into BMDMs, stimulated with pro-inflammatory stimulus (LPS or dead cells from untreated bone marrow tissue) and cytokine expression analysed by Rt-qPCR. The results of this analysis showed significant increases in the expression of TNF, IL1β, IL6 and CXCL2 in response to LPS stimulus throughout the 6-hour experiment time course in BMDMs from antibiotic recovered mice in comparison to those from untreated mice (Fig 3.20 A-D).

As a further and separate analysis, mature macrophages were extracted from bone marrow and their phagocytic potential assessed by engulfment of *E.coli* pH Rhodo bioparticles. This model was used to investigate the alteration to the behaviour of liver infiltrating macrophages in response to translocated bacterial stimulus. This analysis showed a significant reduction in phagocytic potential in macrophages extracted from antibiotic recovered mice (Fig 3.20 E).

In response to dead cell stimulus there was no significant change in IL1β and IL6. CXCL2 on the other hand, displayed significantly increased expression throughout the experiment time course and TNF at 1 hour, with noticeable though statistically insignificant increases at 3 and 6 hours post dead cell addition (Fig 3.21 A-D). Furthermore, CCL2 expression was significantly reduced between BMDMs from control and antibiotic recovered mice at both three and six hours and noticeably reduced (though not significantly) both basally and at one hour. There was no change in CCL2 expression following dead cell addition in antibiotic recovered macrophages (Fig 3.21 E). Perhaps most pertinent to the increase in fibrotic deposition following antibiotic

recovery, MMP9 expression is heavily reduced by antibiotic pre-treatment both basally and in response to dead cell stimulus (Fig 3.21 F).

Taken together, these results appear to confirm the hypothesis that macrophages in antibiotic pretreated mice have a greater pro-inflammatory response to stimulus. Furthermore, the result perhaps most pertinent to the increased hepatic fibrosis seen in antibiotic pretreated cholestatic mice, is the reduction in MMP9 expression both basally and following dead cell exposure, which may be a partial explanation for this apparent increase in fibrotic deposition [121,127,131,132,184,187]. Additionally, the reduction in CCL2 expression may be a partial explanation for the reduction in macrophage recruitment to the cholestatic liver. Finally, the increased expression of CXCL2 in both LPS and DC stimulated BMDMs is interesting though seemingly contradictory. CXCL2 expression in macrophages is usually associated with M2 polarisation [188] and is involved in the recruitment of neutrophils. This may be a by-product of the reduced phagocytic capacity of the macrophages which may compensate by recruiting other phagocytes or could represent a more complex polarisation which has increased CXCL2 expression and low phagocytic capacity but high pro-inflammatory cytokine expression.

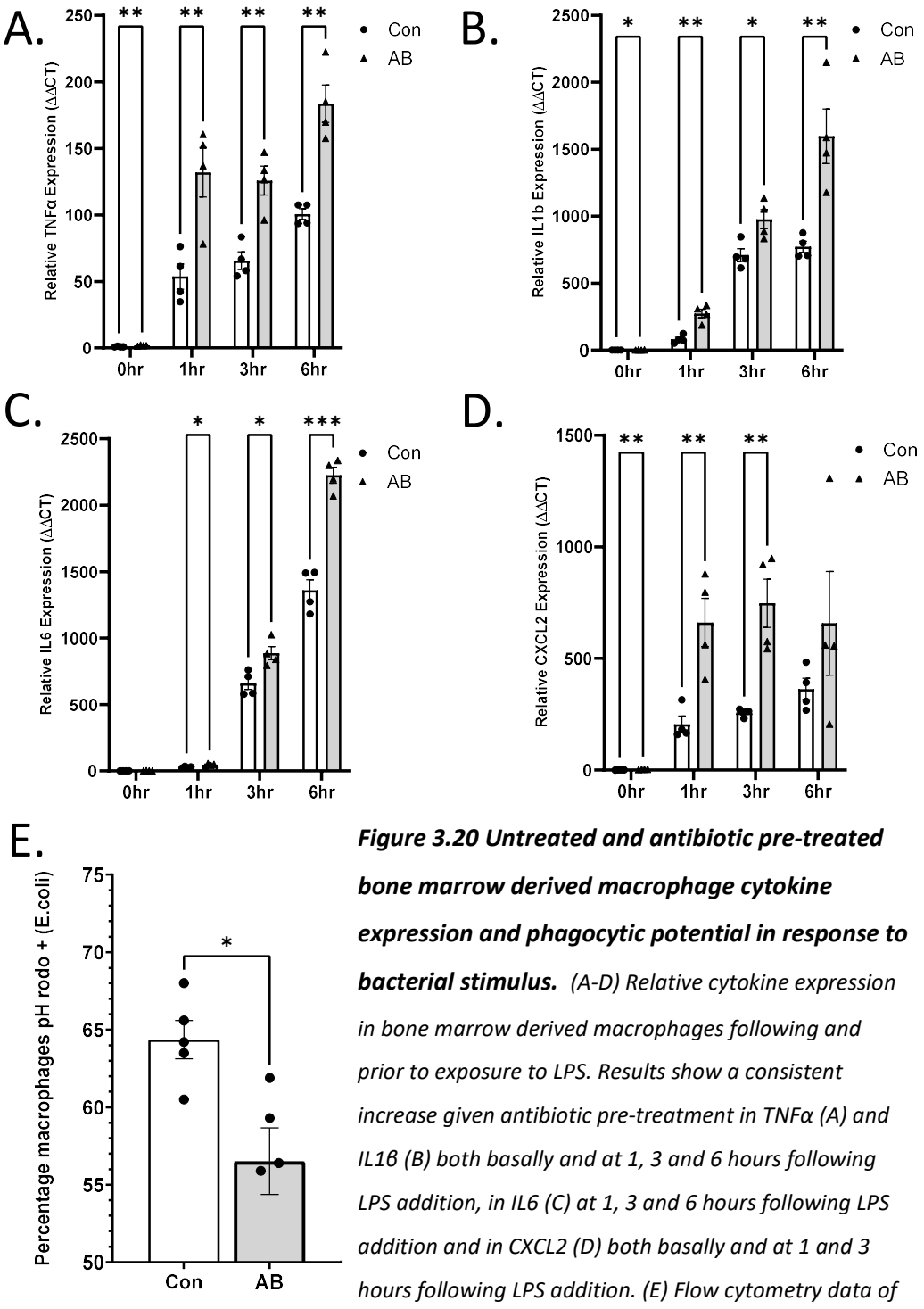


Figure 3.20 Untreated and antibiotic pre-treated bone marrow derived macrophage cytokine expression and phagocytic potential in response to bacterial stimulus. *(A-D) Relative cytokine expression in bone marrow derived macrophages following and prior to exposure to LPS. Results show a consistent increase given antibiotic pre-treatment in TNFα (A) and IL1β (B) both basally and at 1, 3 and 6 hours following LPS addition, in IL6 (C) at 1, 3 and 6 hours following LPS addition and in CXCL2 (D) both basally and at 1 and 3 hours following LPS addition. (E) Flow cytometry data of pH Rodo stained E.coli bead presence in F480+ cells. Data shows reduced phagocytosis of these beads in macrophages from antibiotic recovered mice, compared to control. * = P<0.05, ** = P<0.01, *** = P<0.001. Welch T-test. A-D data points represent a single well, E, data points represent macrophages from a single mouse. This experiment was conducted once.*

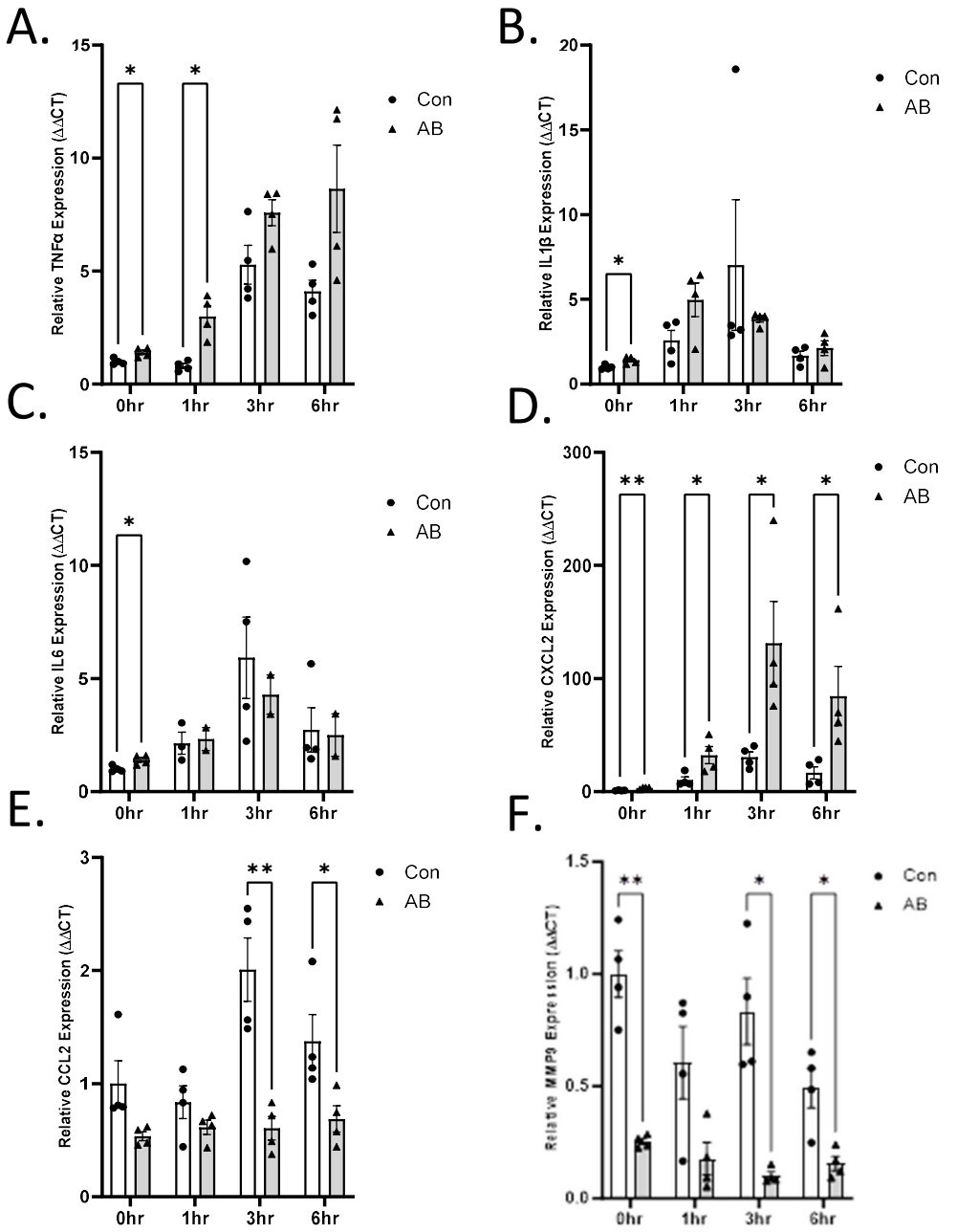


Figure 3.21 Untreated and antibiotic pre-treated bone marrow derived macrophage cytokine expression and phagocytic potential in response to dead cell stimulus.

*Relative cytokine expression in bone marrow derived macrophages following and prior to exposure to dead cells. Results show an increase, given antibiotic pre-treatment, in TNFα (A) both basally and at 1 hour which are statistically significant and at 3 and 6 hours though not significant. IL1β (B) and IL6 (C) show no significant changes following dead cell exposure. CXCL2 (D) expression show consistent and significant increases throughout the experiment time course both basally and following dead cell addition. Both CCL2 (E) and MMP9 (F) show significantly reduced expression at both 3 and 6 hours in antibiotic recovered macrophages, with MMP9 expression also being reduced basally. * = P<0.05, ** = P<0.01. Welch T-test. Each data point represents a single well. This experiment was conducted once.*

3.4 Discussion

Until this point, the effect of antibiotics upon the severity of various liver diseases, and cholestasis in particular, has focused upon these effects immediately following or during antibiotic administration. This investigation is highly pertinent, given the frequent cooccurrence of liver disease and systemic infection owing to the dual effect of increased intestinal permeability and decreased liver immune sentinel activity [150]. However, there is a pressing question which this study seeks to address, that being, as mentioned previously, the effect of antibiotic treatment post microbial recovery. Antibiotic treatment has been shown to have a persistent effect upon the composition of the intestinal microbiota[20,22,157,189–191,23,26,29,37,140,142–144], though the majority of these studies focus upon the impact of single antibiotics [140], which necessarily have specific impacts upon particular communities within the microbiota. Our aim was to investigate the generalised impact of microbial depletion and recovery. Further investigations could draw from this the specific microbial alterations which impact cholestatic disease.

3.4.1 The implications of notable alterations in the intestinal microbiome generated by antibiotic pretreatment

To investigate the impact of VNMA upon gut intestinal bacterial communities we first defined the resultant changes in both total bacterial biomass and community composition. Initially, results demonstrated a decrease in total bacterial biomass and community diversity following antibiotic treatment, with subsequent recovery to pre-treatment levels after three weeks. However, after bile duct ligation, there was a notable increase in bacterial biomass, indicative of small intestinal bacterial overgrowth (SIBO) often associated with cholestasis[182]. SIBO has been linked through meta-analysis to several comorbidities in cirrhotic liver diseases like ascites and hepatic encephalopathy. However, no clear association has been found between SIBO and disease severity despite increased GI symptoms during illness [182]. Notably, the lack of significant increase or decrease in bacterial overgrowth between untreated and antibiotic pre-treated BDL samples suggests that SIBO may not be a determining factor for increased disease severity in antibiotic-pretreated mice.

Whilst we observed a recovery in gut bacterial biomass, shotgun metagenomic analysis displayed substantial changes in population composition between antibiotic recovered and untreated mice shown by Bray-Curtis dissimilarity. The surface level manifestation

of this change is the reduction in alpha diversity following antibiotic recovery prior to BDL comprised of a reduction in both population richness and evenness.

A study in neonatal mice also showed a similar reduction in diversity one week post antibiotic withdrawal [26], the study identified two distinct and stable populations which arise from treatment and recovery. There was a noticeable, though not statistically significant, decrease in diversity in both compositions after 72 weeks. A meta-analysis on the impact of antibiotic treatments up to fourteen days post-administration in children has corroborated these mouse model findings [144]. Their analysis showed a distinct split in findings, with many studies displaying a reduction in alpha diversity following antibiotics, but with a substantial minority showing no significant differences. Most correlational analyses of microbial diversity in liver disease have investigated the effects of liver dysfunction upon microbial diversity, rather than the reverse. The impact of diversity upon cholestasis has yet to be investigated in depth, although reductions in bacterial diversity have been noted in human patients with PSC [41]. This chapter may indicate a link between reduced pre-BDL microbial diversity and increased cholestatic severity.

On the other hand, the prevalence of specific microbes in the microbiota during cholestasis has received a greater degree of investigation, with various functional genera and species having been associated with increased liver disease severity [41,51,193–198,114,116,117,164,170,172,180,192]. Our investigation of the composition of the gut microbiome showed the populations of many genera and species being depleted and expanding post antibiotic recovery. The key genera highlighted in our analysis, depleted following antibiotic treatment and recovery are *Bacteroides*, *Parabacteroides*, *Alistipes* and *Phocaeicola*.

The genus *Bacteroides* has frequently been identified as a key genus in the microbiota, consisting of several highly functional and generally beneficial species [199]. Indeed, the *Bacteroides* : *Firmicutes* ratio is considered a somewhat reliable proximal measurement of the health and dysbiosis of the microbiota generally, with a low *Bacteroides : Firmicutes* ratio having been associated with various diseases most significantly obesity. There is however, a more complex relationship in liver disease. Fibrosis in NASH, for example, has been correlated with both low and high levels of both *Bacteroides* and *Firmicutes* [195]. Our analysis demonstrated a large scale reduction in *Bacteroides* and expansion of *Firmicutes* following antibiotic recovery and retained post-BDL (Fig S3).

Bacteroides spp. have been shown to be protective in various liver diseases. Generally speaking this effect has been shown to be reliant upon regulation of energy metabolism and maintenance of intestinal barrier function, mediated by the generation of SCFAs and signalling via released cellular components [85,169,170,172,195]. Additionally, Bacteroides species are key to the gut-liver axis, functioning as central producers of deconjugated bile acids, reducing FXR antagonism from conjugated bile salts and producing deconjugated bile acids for recirculation [138,175]. While bile salt hydrolase enzymes (BSH) are widely distributed within gut microbial bacteria, many of the productive genera are depleted following antibiotic recovery. *Bacteroides spp.* are key BSH producers and are heavily depleted by our antibiotic regimen and fail to recover post-recovery. Hence, their reduction would be expected to greatly disrupt bile cycling and FXR activity. This is directly relevant to the reduction of deconjugated bile acids in the intestine and reduction of SCFA production I observed in young antibiotic recovered mice.

One species in particular, *Bacteroides thetaiotaomicron* (Bt), within the genus *Bacteroides* is depleted following antibiotic recovery (Fig S1). Bt demonstrates remarkable anti-inflammatory properties. Reconstitution of Bt produces restorative effects in IBD, by reducing inflammation and improving gut barrier function [171]. The anti-inflammatory effects of this species appear to be mediated, at least in part, by outer-membrane vesicles, generated both in homeostatic conditions and more so in times of stress- such as antibiotic administration [200,201]. These vesicles are trafficked widely throughout the host organism, with high concentrations detectable in the liver [202].

The related genus *Parabacteroides* was also heavily depleted following antibiotic recovery and shares many key properties with Bacteroides, relevantly BSH activity [173]. A 2023 study has demonstrated that *Parabacteroides distansonis* has a significant impact upon hepatic fibrosis in mice with chemically induced hepatic fibrosis. Additionally and especially pertinent to this study, they also saw reduced *P.distansonis* in human cholestasis patients with increased fibrosis [173]. They linked this effect to BSH activity which reduced FXR activation, in turn reducing toxic primary bile acid accumulation in the liver, reducing damage and attenuating fibrosis. It must be noted that as: A. we see no difference in *Parabacteroides* levels between BDL and antibiotic pre-treated BDL mice and B. we see no alteration in TCDCA levels in the liver, we likely have a distinct mechanism at work in our model. However, there may be an effect of preconditioning prior to BDL as we see *P.distansonis* is depleted following antibiotic recovery prior to BDL (Fig S1).

Similarly, *Phocaeicola dorei* has received some attention in its effect upon liver disease outcomes particularly NASH and metabolic dysfunction. I observed a depletion of *P.dorei* following antibiotic recovery both prior to and following BDL (Fig S1,2). A 2022 human FMT study highlighted *P.Dorei* as a key microbe for the deconjugation of bile acids whose population size correlated strongly with deconjugated CDCA and LCA concentration in the gut [175]. *P.dorei* is an important producer of bile salt hydrolases and produces various secondary bile acids as metabolic byproducts [175]. Furthermore, a 2024 study highlighted *Phocaecicola vulgatus* as a protective species in a murine model of metabolic-associated steatotic liver disease (MASLD), as with *P.dorei* this species is also depleted following antibiotic recovery both prior to and post-BDL (Fig S1,2). Reconstitution of *P.vulgatus* in antibiotic depleted ApoE -/- (NAFLD model) mice reduced lipid deposition, serum transaminase levels and most relevantly the level of fibrosis in the liver. They identified reduction in histone acetylation, mediated by the metabolite 3-hydroxyphenylaceticacid, as the alleviating mechanism. The importance of this cannot be understated, as it suggests a potential pre-conditioning mechanism in hepatocytes for increased hepatic damage and fibrosis when *Phocaeicola. spp* is lost following antibiotic recovery [203].

The loss of the beneficial gut bacterial species observed in antibiotic recovered mice, prior to and or following BDL (Fig S1,2) could constitute a viable explanation for the increase in hepatic fibrosis seen in antibiotic pretreated BDL mice. What is certain is that there is a substantial loss of several bacterial species which slow the progression of various liver diseases, particularly reducing or slowing the progression of fibrosis resulting from cytotoxic damage. The results from a previous study by Awoniyi *et al* resemble our observations. The study links microbial population change (though different microbes are impacted) to the severity of cholestatic fibrosis and increased inflammation, highlighting SCFA production as being key to these increases in severity markers. Furthermore, the increases in hepatic damage, inflammation and fibrosis produced by vancomycin treatment were attenuated by the supplementation of an acetate, propionate and butyrate cocktail [164], all SCFAs significantly reduced following antibiotic recovery in our analyses. However, these effects are observed when antibiotics are administered immediately prior to the induction of cholestasis in MDR -/- mice.

3.4.2 The association of microbial population change, metabolite content change and cholestatic disease pathophysiology.

Highlighted in the previously mentioned studies was the role of key bacterial species in the generation of beneficial metabolites and bile acid recirculation. The loss of *Bacteroides*, *Parabacteroides*, *Phocaecicola* and various other metabolically active genera would be expected to reduce the content of both SCFAs and secondary and deconjugated bile acids. Our results demonstrated a significant reduction in all SCFAs following antibiotic recovery, BDL generated a reduction in propionate and butyrate from pretreatment levels with ABBDL displaying a greater reduction. A seminal previous study in this area by Zarrinpar *et al* showed the immediate impact of AIMD (Antibiotic induced microbial depletion)- defined as repeated treatment with vancomycin, neomycin, metronidazole, ampicillin (VNMA) and amphotericin B over a period of 13-30 days [63]. Whilst this treatment is of a greater timespan than ours and lacks a recovery period it displays striking similarities, especially in the reduction of SCFAs and secondary bile acids, with a cooccurrence of increases in primary, conjugated bile acids.

Our results, in the main, resemble the results of Zarrinpar *et al*. after one week of VNMA treatment prior to recovery and display that they are somewhat retained following three weeks of microbial recovery. The significance of these results lies in the role of these metabolites as determinants of liver disease severity. Whilst we see limited differences in hepatic bile acid content, there is a distinct shift in intestinal bile acids from antibiotic recovered mice. This reduction was constituted of a reduction in secondary and deconjugated bile acids and an increase in primary, conjugated bile acids. This alteration is not reflected in the liver bile acid pool, ruling out a switch in primary bile acids towards TMCA in lieu of TCA and TDCA. However, even a gut localised alteration in FXR stimulation may impact hepatic metabolism and will have impacts upon the physiology of the gut.

Our data displayed a clear increase in the proportion of FXR antagonists in the faeces of antibiotic pretreated mice (TMCA) and a reduction in FXR agonists (DCA and LCA). FXR activity in the gut is central to the gut-liver axis, directing the regulation of bile acid synthesis and cycling. FXR agonism in the gut stimulates FGF15 production in mice, which is trafficked to the liver where it has an inhibitory effect upon bile acid synthesis and general liver metabolism [204]. This would suggest that the significant increase in FXR antagonists in the gut would promote bile acid synthesis following antibiotic recovery.

However, we do not see alterations in either gut, liver or serum total biliary pool suggesting that a further modulatory pathway is involved, preventing this.

The local effects of bile acids in the gut and upon innate immune cells may also impact the condition of the liver during cholestasis. FXR agonists have previously been shown to improve gut barrier function, with a high prevalence of FXR antagonists promoting bacterial translocation to the liver [44,58,64,84]. Generally, this has a negative impact upon liver disease progression, with translocated bacteria such as Enterococcus and Escherichia or endotoxin having severely detrimental effects upon the progression of various liver diseases including cholestasis [58,64,100,103,187]. However, our results show no change in intestinal permeability, modelled by serum FITC content, following antibiotic recovery either in health or during cholestasis. Though, it must be noted that the bacteria which are enriched following antibiotic recovery may have a differential impact upon the liver during cholestasis. Furthermore, the modulatory effects of FXR stimulation arising from bile acid signalling in the serum may affect the activity of liver infiltrating immune cells deriving from the circulation.

Results from Schneider *et al* 2021 show a central role for disruption of FXR signalling immediately following antibiotic administration in driving worsened cholestasis severity [103]. Similarly to the data in this chapter, their data show, that antibiotic depletion produced a domination of the faecal biliary pool with TMCA and a total loss of all FXR agonistic bile acids, which drives FGF15 repression. Conversely, they show increased hepatic bile accumulation. Altered bile accumulation promoted increased periductular infarcts, associated damage and disease progression, produced by breakdown in bile duct barrier function. This phenotype was rescued by exogenous FXR agonist treatment. However, without a hepatic bile independent, FXR dependent, mechanism for cholangiocyte barrier dysfunction and fibrosis the mechanism described by Schneider et al. is unlikely to be a driving mechanism in our model. This is owing to our observed lack of increased biliary accumulation and increased hepatic necrosis in antibiotic pretreated BDL mice.

FXR and TGR5 are the classical bile acid receptors and are known to be expressed by many cell types within the immune system, most prominently macrophages and Kupffer cells. Generally speaking, FXR agonist activity in macrophages promotes M2 polarisation and anti-inflammatory effects [205], reducing leukocyte infiltration in both liver disease and IBD [79]. FXR activation leads to SHP expression, which then acts as a repressor for

various pro-inflammatory cytokines and chemokines such as IL1β, TNFα and CCL2 [79]. TNFα and IL1β have both been shown in this chapter to have significantly increased expression in BMDMs derived from antibiotic recovered mice versus untreated mice. Furthermore, TGR5 agonism promotes anti-inflammatory effects in macrophages, TGR5 is most highly expressed in gut-localised monocytes and macrophages, with secondary bile acids being the primary ligands [79]. Hence, with a reduction in secondary bile acids and an increase in FXR antagonists in the faeces, likely reflected in the wider circulation, this may promote a pro-inflammatory phenotype post-antibiotic recovery in tissues remote from the gut. It may also explain some of the increases in pro-inflammatory cytokine expression observed in antibiotic recovered BMDMs. However, it is somewhat puzzling that when macrophages are isolated from conditions of potentially high FXR antagonism, the pro-inflammatory phenotype is maintained, which is demonstrated by the in vitro BMDM data. As neither FXR nor TGR5 activation in macrophages have been shown to produce any epigenetic change, one must conclude that either a separate mechanism is at work in driving this pro-inflammatory polarisation/differentiation of macrophages or that a novel mechanism involving bile acid induced epigenetic change is at play.

Disruption to bacterial SCFA production may provide an alternative or coincident mechanism by which a pro-inflammatory phenotype is promoted in antibiotic pretreated macrophages. The SCFAs acetate, propionate and butyrate have all been shown to be key bacterial metabolites both in energy homeostasis and in organism wide immune modulation [16,62,63,158,159]. Until recently SCFAs were generally acknowledged to impart an anti-inflammatory effect upon innate immune cells, mediated by GPCR detection and signalling [159]. Our data shows clearly that following antibiotic treatment and recovery, both acetate and propionate are significantly reduced prior to BDL. Following BDL, both propionate and butyrate are significantly reduced, to a greater degree given antibiotic pretreatment.

Microbiota derived acetate functions primarily as a secondary energy source in homeostatic conditions, being used to supplement glucose in energy metabolism. It is imported into host cells, converted into acetyl-CoA and used for ATP production by classical respiration, it can also be used as a metabolite in protein acetylation and for fatty acid biosynthesis [206].

The role of microbially derived acetate in cholestasis has not been studied in depth, though given its role in the progression of other liver diseases, it is difficult to identify why this may be, especially given its centrality to hepatocyte metabolism. However, it has been identified as a potent modulator of immune cell activity, including in macrophages [158,196,206]. The mechanism by which acetate modulates immune cell activity has been hotly debated. Many studies suggest that acetate has no impact upon histone acetylation and instead plays a signalling and metabolic role in regulating the production of cytokines [158], while others demonstrate a clear increase in hyper acetylation of histones, particularly H3 and H4 via inhibition of histone deacetylases, demonstrated in many innate immune cells, including macrophages [206].

In any case, acetate appears to have a tissue and environment dependent impact upon macrophage behaviour [206]. In the GI tract, acetate promotes an anti-inflammatory effect, down regulating the Nf-κB pathway and preventing pro-inflammatory cytokine production generally, while promoting NLRP3 dependent inflammasome formation and the associated increase in IL-1β [206]. While in the respiratory tract it has been demonstrated to prevent M2 polarisation of alveolar macrophages and generate a pro-inflammatory phenotype with a greater phagocytic capacity [158]. However, it must be noted that M2 polarisation in the respiratory tract is associated with worsened allergic asthma [207] so it may be said that preventing M2 polarisation drives an anti-inflammatory outcome in this context. Furthermore, acetate plays an important role in the microbiota as a precursor for various microbes in the synthesis of more complex SCFAs, particularly propionate and butyrate [206]. Hence, the reduction in gut acetate content produced by our antibiotic treatment likely plays a role in the reduction in both propionate and butyrate antibiotic recovered mice display. Both SCFAs also play a role in macrophage inflammatory profile discussed below.

In the liver disease context, microbially derived acetate has been shown to protect against NAFLD-HCC (NAFLD associated hepatocellular carcinoma) formation by inhibiting hepatocyte production of IL6 via GPCR (G-protein coupled receptor) binding [196]. Given the complex role of acetate in inflammation and the lack of its study in immune cells in the liver inflammatory or cholestatic context, it is difficult to determine what impact it may have upon disease progression. However, given the import of microbially derived acetate by hepatocytes in the liver and its metabolic importance in NAFLD [196], one may hypothesise that hepatic acetate content may impact histone acetylation in liver resident and infiltrating macrophages.

Propionate was shown to be significantly reduced following antibiotic recovery, both prior to and following BDL induced cholestasis. Similarly to acetate, exogenous propionate can be taken up by host tissues and used in various metabolic processes. In the liver its role is most prominent in gluconeogenesis for which propionate is a metabolite and like acetate prevents steatosis and insulin resistance in NAFLD by suppressing de novo lipid synthesis and fatty acid uptake [156,208].

In macrophages propionate, like acetate, has a complex effect. It has been shown to suppress M2 polarisation in alveolar macrophages [158] and has also been shown to imprint an antimicrobial program when bone marrow cells are exposed during differentiation in vitro [18]. This programming improves the protection of macrophages against various pathogens by various mechanisms, generally improving intracellular capacity for the destruction of microbes. Whilst there is evidence that propionate may have an antimicrobial, M2 inhibitory effect, it has also been shown to be anti-inflammatory in various contexts [18,62,158].

There has been very little study of propionate's role in liver disease outside of NAFLD, however it has come under recent scrutiny in the context of atherosclerosis, where supplementation with propionate is protective in the development of atherosclerotic lesions. It has been shown to both reduce the uptake of free cholesterol from the gut, promote reduction in generalised inflammation by the modulation of regulatory and helper-T cells and to attenuate fibrotic deposition in the nascent atherosclerotic lesion [209–211]. Whilst the role of T-cell modulation cannot be ignored in these studies, it was also shown that F480+ cell (macrophage) infiltration into the lesions was reduced by propionate treatment, which had a knock-on effect upon the level of fibrosis and whilst it was shown that Treg depletion abrogated the beneficial effects of propionate supplementation, it did not abolish them [209]. The link between these studies and cholestatic liver disease lies in two studies, the first of which highlights free cholesterol and ox-LDL in inflammation and fibrosis progression in fatty liver disease [212]. Accumulation of oxLDL in the parenchyma and sinusoidal compartments generated increased levels of fibrosis in human liver tissue sections. Given that hypercholesterolemia is a common co-morbidity of cholestasis and propionate inhibits cholesterol uptake in the gut, reduced propionate generated by antibiotic treatment combined with BDL preventing biliary outflow, may lead to excess cholesterol accumulation in the cholestatic liver, which has been associated with worse disease prognosis [213]. The second of these studies demonstrates that cholesterol metabolism is

significantly perturbed by AIMD in the context of BDL, during which fibrosis is significantly increased [165].The perturbation drives a reduction in genes associated with HDL production and an increase in those associated with vLDL (very Low Density Lipoprotein) production[165]. Furthermore, and highly relevantly, hepatic stellate cells are highly sensitive to cholesterol accumulation and, in response to localised free cholesterol accumulation, increase fibrotic deposition [184,212]. Hence the role of propionate in this mechanism may be pertinent to cholestasis and fibrosis and begs further study.

Butyrate, unlike both acetate and propionate, has a more well-defined role in both inflammation generally and in the context of liver disease. Our results show a more modest and statistically insignificant decrease in butyrate following antibiotic recovery, which may be linked to the expansion of various butyrate producing genera following recovery such as *Intestinimonas* and *Faecalibacterium*. However, following BDL, there is a greater degree of butyrate reduction in antibiotic pre-treated BDL mice than BDL only when compared to pre-treatment levels. Though, here again there is no significant difference between the levels of butyrate between ABBDL and BDL. Hence, we cannot say conclusively that butyrate is reduced by antibiotic treatment in the long term.

3.4.3 The implications of changes to immune function generated by antibiotic pretreatment during cholestatic liver disease progression

The gut epithelium is well recognised as an immune educational interface, with microbiota composition playing a central role in organism-wide immune function and environmental conditions [70]. Beneficial compositions, high in genera such as *Bacteroides*, *Bifidobacterium*, *Akkermansia* etc.- commensals in short - have a tolerogenic effect, whereas dysbiotic compositions may have a pro-inflammatory effect [27]. Whilst, as cells of the innate immune system, the capacity of adaptive behaviour in macrophages is limited, there is capacity for altered behaviour in response to consistent stimulus, not only in terms of polarisation [158,188,205,214], but also arising from alternative differentiation in the bone marrow [215]. In addition to the direct communication and 'education' at the gut epithelium, the effects of altered SCFA and bile acid content enumerated above will have a wide range of impacts upon host immunity and macrophage behaviour specifically. This impact during cholestasis is observed in our data.

In this study the most notable impact of antibiotic pretreatment upon the immune response to cholestasis is the reduction in macrophage infiltration to the liver following BDL. This is demonstrated by the reduction in F480+ cells detected by flow cytometry and reduction in F480 staining. Reduction in macrophage recruitment to the cholestatic liver has previously been shown to reduce the severity of cholestatic injury in the murine MDR2 -/- model of PSC [186]. Pertinently to our in vitro results, this reduction in macrophage recruitment was achieved by inhibition of the CCL2-CCR2 pathway of recruitment and chemotaxis. We observe a similar reduction in CCL2 expression in response to dead cell stimulus in vitro in macrophages from antibiotic recovered mice. This directly contradicts our data showing reduced macrophage infiltration, yet with increased fibrosis. In this study it was shown that both pro and anti-inflammatory macrophages accumulate in the cholestatic liver and localise to the peribiliary space, with a greater predominance of pro-inflammatory macrophages in the latter stages of disease. A potential explanation for the observation of reduced infiltration and increased fibrosis is the preconditioning of infiltrating macrophages, by antibiotic treatment generated changes in bile acid and SCFA content, which then arrive (though in reduced numbers) at the cholestatic liver.

What is immediately evident in our data is that BMDMs from antibiotic pretreated mice in comparison to untreated mice are primed for a pro-inflammatory response, especially in reaction to LPS stimulus. Whilst we see no increase in intestinal permeability following antibiotic recovery, the increase in permeability following BDL will result in increased bacterial translocation, to which antibiotic pretreated mice will be more likely to raise a more inflammatory response. This then begs the question of why we do not see this reflected in the whole liver homogenate qPCR data. The answer may lie in the reduced infiltration of macrophages following antibiotic recovery, this likely reduces the total inflammatory response within the liver. The question then becomes: given that there is no overall change in inflammation, what relevance does this bear upon worsened disease severity? The answer I believe is two-fold, firstly, it has been shown that macrophages play a dual-role in the progression of liver fibrosis- with pro-inflammatory macrophages exacerbating the progression of fibrosis [127]. Depletion of macrophages during fibrosis progression alleviates the liver fibrotic burden in CCL4 treated mice. However, depletion during resolution abrogates ECM degradation and scarring persists [133]. Hence, reduction in macrophage number is not an unalloyed good. The in vitro qPCR data presented here demonstrates a reduction in MMP-9 expression in BMDMs in

response to dead cell stimulus. MMP9 is an essential enzyme involved in tissue remodelling, wound repair and matrix degradation, the reduction in which will drive worsened fibrotic deposition [132]. Additionally, the increased expression of IL1β, TNFα and IL6 in these cells, each of which have profibrotic effects upon HSCs, would be expected to have a profibrotic effect in this context [127]. Furthermore, investigating the F480 staining closer it is evident that despite the reduction in macrophage infiltration, there are still noticeable clusters of macrophages, localised in the periductular space. Localisation of macrophages in this compartment during cholestasis has been shown to be a marker of worsened cholestatic disease progression [186] and given their more inflammatory behaviour, with reduced MMP expression, this may accelerate disease to a greater degree than in untreated BDL mice. The accumulation of pro-inflammatory macrophages to a greater degree than anti-inflammatory is associated with the later stages of cholestasis progression [186].

It appears that the main differentiator of the behaviour of antibiotic pre-treated macrophages is in their chemotactic and phagocytic potential. They are less able to phagocytose bacterial stimulus and to attract further macrophages to the site of insult by CCL2 induced macrophage chemotaxis. The increase in CXCL2 is likely to increase the attraction of phagocytes, with an unimpaired capacity for phagocytosis, to compensate for the reduction in macrophage phagocytosis. An alternative explanation may be that the macrophages have an alternative polarisation, pro-inflammatory but with low phagocytic potential and macrophage recruitment.

3.4.4 Summary

The results shown in this chapter demonstrate that antibiotic pretreatment accelerates the progression of cholestatic liver disease. Furthermore, they suggest that this effect is driven by changes to gut bacterial population composition and worsened pathophysiology is possibly mediated by alterations to the inflammatory response to injury or insult. As mentioned previously, investigations into the immediate impact of antibiotics have shown a similar physiological impact on the damage arising from antibiotic treatment during, or prior to, the induction of cholestasis [103,164,165]. Our results contribute to this field by demonstrating that these impacts can be retained following recovery of the microbiota. Furthermore, we highlight several key mechanisms which may drive this alteration, such as altered bile acid or SCFA signalling and reduced bacterial tolerogenesis.

3.5 Further work

This chapter presents the narrative that antibiotic driven, long-term microbial population change produces alterations in metabolite production. These changes have well documented mechanisms by which they can produce the changes that we observe in macrophage behaviour. These changes are retained despite a removal from the biological conditions which differentiate antibiotic pretreated and untreated mice. The BMDMs from each group are differentiated in an identical in vitro environment which suggests that they are permanently, or persistently, affected by a change arising from antibiotic pretreatment. Given the known epigenetic changes produced by SCFA content change and the persistence of the effects observed, it is likely that an epigenetic mechanism is at play. In order to confirm and identify these particular mechanisms, one could investigate the acetylation, or otherwise, of macrophage histone proteins isolated from the liver by FACS or in BMDMs. To further investigate the affected pathways western blots could be used to identify the quantities of various proteins involved in the Nf-κB and inflammasome pathways which have been linked to SCFA mediated epigenetic changes [158,164,206]. Additionally, to confirm that these effects are driven by changes in microbial metabolite content exogenous SCFAs or FXR agonists could be supplemented during antibiotic recovery to investigate whether they have a restorative effect upon worsened cholestatic fibrosis given antibiotic pretreatment. The associated assays could then be performed to investigate macrophage behaviour.

The effect of propionate supplementation upon cholesterol metabolism and the role cholesterol may play in worsened cholestasis following antibiotic recovery could then be investigated. We have performed a preliminary study using propionate supplementation where we observed improved survival but failed to rescue the antibiotic recovered profibrotic phenotype. It must be noted however, that this propionate was only provided from one day prior to BDL and for the 7 days post BDL, while intestinal propionate content is shown to be reduced from the point immediately following antibiotic withdrawal. Therefore, if the impact of propionate results from conditioning by epigenetic changes [158] then treatment immediately prior to BDL may not reverse these changes immediately resulting in changes to cholestatic disease pathophysiology. Cholesterol burden and metabolism in the liver could be investigated by immunohistochemistry and qPCR or RNA seq. Macrophages could be isolated from the cholestatic liver by FACS and their cholesterol content and lipid burden investigated by

Oil Red O or lipidtox staining, their lipid handling pathways by qPCR or RNA seq and the differential behaviour based upon cholesterol and lipid burden by various means such as qPCR, RNA seq and phagocytosis and caspase assays in vitro.

Finally, the effect of various genera and species of bacteria has been noted in various liver diseases, including cholestasis. Whilst the effect of one species in particular is investigated in the following chapter (*Bacteroides thetaiotaomicron*), there are various other functional species depleted following antibiotic recovery. One in particular *Parabacteroides distansonis* stands out as demanding further investigation. As mentioned previously, a 2023 study found *P.distansonis* abundance to be directly associated with hepatic fibrosis, both in a mouse model of chemically induced fibrosis, but also and most relevantly in juvenile human PSC [173]. A future study would reconstitute *P.distansonis* either immediately following antibiotic treatment, or immediately prior to BDL, and the effects of reconstitution analysed using the methods described in this chapter.

3.6 Conclusion

Taken together, the results of this chapter demonstrate that though bacterial biomass recovers following antibiotic treatment and recovery, there is a long-term impact upon the composition of the gut bacteriome. The changes to population composition have a knock-on effect upon the generation of microbial metabolites which are central to the gut-liver axis, bile acids and short chain fatty acids. These changes then have a modulatory effect upon the inflammatory response of macrophages to stimulus, an effect which is independent of the immediate environmental preponderance of metabolites, demonstrated by the behaviour of ex vivo macrophages. The alteration to immune activity then appears to alter the recovery of the cholestatic liver to injury, by exacerbating fibrotic deposition.

The focus of future work would be to identify the mechanism by which altered microbial composition and metabolite production produces alterations in macrophage behaviour. It is well known that SCFAs alter histone acetylation which drives reduced inflammation, future experiments would likely focus upon this and investigate acetylation levels in macrophages. Furthermore, in vivo tissue staining could identify the polarisation or inflammatory state of macrophages localised to the periductular space in macrophages. Additionally, the role of cholesterol and lipid metabolism in this disease state could be a

novel and impactful area of research. Finally, reconstitution of specific genera and species of bacteria to alleviate disease state is investigated in the following chapter. However, the reconstitution of other species and bacterial metabolites might highlight the particular bacterial and metabolite depletions driving antibiotic worsened disease or potential therapeutic, eubiotic, strategies.

Chapter Four.
Bacteroides thetaiotaomicron reconstitution and its restorative effects following antibiotic recovery in cholestatic liver disease.

4.1 Introduction

In the previous chapter we investigated the impact of antibiotic treatment and recovery upon the microbiota and its output of metabolites, how this then impacts upon the pathophysiology of cholestatic disease and how worsened disease pathophysiology may be mediated by disruption to the inflammatory response. We saw reductions in the populations of many beneficial genera, most pertinently *Bacteroides.* This change to the intestinal microbiota produced knock-on reductions in SCFA (particularly propionate) production and an increase in conjugated bile acids in the intestine, with a concomitant decrease in deconjugated and secondary bile acids. Combined, these effects constitute a possible trigger for the increased fibrotic deposition and inflammatory disturbance or 'priming' for increased pro-inflammatory responses and decreased macrophage infiltration and bacterial phagocytosis. One of the most significantly depleted genera was Bacteroides, with *Bacteroides thetaiotaomicron* (Bt) being one of the species which comprised this genus-wide depletion.

Bacteroides thetaiotaomicron is a highly functional species within the microbiome. It is a non-motile, gram-negative obligate anaerobe which produces acetate, lactate, succinate and propionate by digesting various host derived glycans and diet derived indigestible metabolites, such as complex starches and pectins [152,216]. It has a highly dynamic cell surface, utilising compounds derived from its environment and detecting host and microbiome signals to alter its interactions within the microbiome. It does this by changing its cell surface and by metabolite, small molecule and outer membrane vesicle release [152]. Furthermore, Bt has been shown to initiate immunological and epithelial development when introduced into a germ-free mouse model [152,216].

As was mentioned in the discussion of the previous chapter, Bt possesses the capacity to produce outer membrane vesicles (OMVs) [202]. OMVs are spheroidal proteoliposomes produced by various species of bacteria. These proteoliposomes can contain many cytosolic components including DNA, RNA, proteins and metabolites and are decorated with various components of the bacterial outer membrane [200]. OMVs possess various properties, components and cargo which modulate host activity and the microbiome and are trafficked widely throughout the host organism once transported across the gut epithelium [200,202,217].

Bt OMVs are internalised by intestinal epithelial cells by endocytosis, trafficked throughout these cells and across the basolateral membrane transmigrating across the gut epithelium by modulating the tight junction barrier [202]. Following this transmigration, the OMVs are widely distributed. The greatest concentration of OMVs following oral administration (approximately 90% of the total detected OMVs) are detected in the GI tract, specifically in the small intestine, stomach, colon and caecum. Though the vast majority of OMVs escaping the gut arrive at the liver, approximately 10% of the total detected OMVs [202].

The effects of OMVs on the tissues to which they are transported are multifarious. In the context of IBD the effect is restorative, promoting reduced inflammation and improved gut barrier integrity [171]. Bt has been shown to reduce inflammation during IBD by antagonising Nf-κB via intracellular signalling in gut epithelial cells [167], which drives reduced TNFα and interferon gamma expression [168]. In BMDMs, preconditioning with Bt OMVs promotes an M2 phenotype, driving an increase in IL10 and a reduction in TNFα expression, produced by epigenetic changes through histone methylation [218]. Whilst this may be the case, Bt also possesses the capacity to increase production of bactericidal peptides and ensure efficient destruction and clearance of enteropathogens, mediated by effects on Paneth cells [216].

Given the anti-inflammatory phenotype produced by Bt OMVs in macrophages driven by epigenetic change [218] and the accumulation of OMVs in the liver, the effect of OMVs on macrophages appears antagonistic towards the mechanism proposed in the previous chapter for worsened cholestasis pathology. The proposed mechanism being macrophage driven increase in fibrosis, produced by epigenetic alteration through the effects of altered propionate, acetate and bile acid content. Furthermore, Bt's restorative role in steatotic liver diseases relates to the potential role of free cholesterol in increased cholestatic fibrosis following antibiotic recovery. Hence, loss of this bacterium may drive a more pro-inflammatory response whilst its reconstitution may reduce inflammation and ameliorate the profibrotic phenotype resulting from cholestasis post-antibiotic recovery.

In the liver disease context Bt has been shown to drive reduced steatosis and improved outcomes in both non-alcoholic fatty liver disease (NAFLD) and alcoholic steatohepatitis [169,170,172], this effect is driven by metabolic regulation in NAFLD, reducing lipid metabolism and deposition, reducing lipaemia and steatosis [170,172]. This reduction in

steatosis then alleviates the inflammatory burden upon the liver, preventing macrophage and monocyte infiltration, which then alleviates pathophysiology and fibrosis [170,172].

It is the capacity of Bt for immunological modulation and propionate and acetate production, in addition to its depletion following antibiotic recovery, that led me to suspect that its reconstitution may alleviate the effects of antibiotic pretreatment on cholestatic liver disease.

4.2 Hypothesis and aims

I hypothesize that Bt reconstitution in young, antibiotic pretreated mice results in restoration of the production of certain SCFAs. Furthermore, I hypothesise that Bt reconstitution results in alleviation of antibiotics' profibrotic effect during cholestatic liver disease pathophysiology and that this may be mediated, at least in part, by changes to macrophage behaviour.

This chapter aims to:

1. Illustrate the depletion of Bt and its restoration, or lack thereof, following recovery from antibiotic treatment and the expansion of the Bt population following reconstitution.
2. Demonstrate the impact of Bt reconstitution after antibiotic recovery upon the faecal SCFA profile.
3. Show the effect of Bt reconstitution following antibiotic recovery upon the progression of cholestatic disease.
4. Highlight the impact of Bt upon the antibiotic altered immune response during cholestatic disease.

The structure of the mouse treatment and sampling is shown in the figure below to aid in data interpretation and general understanding (Fig 4.A).

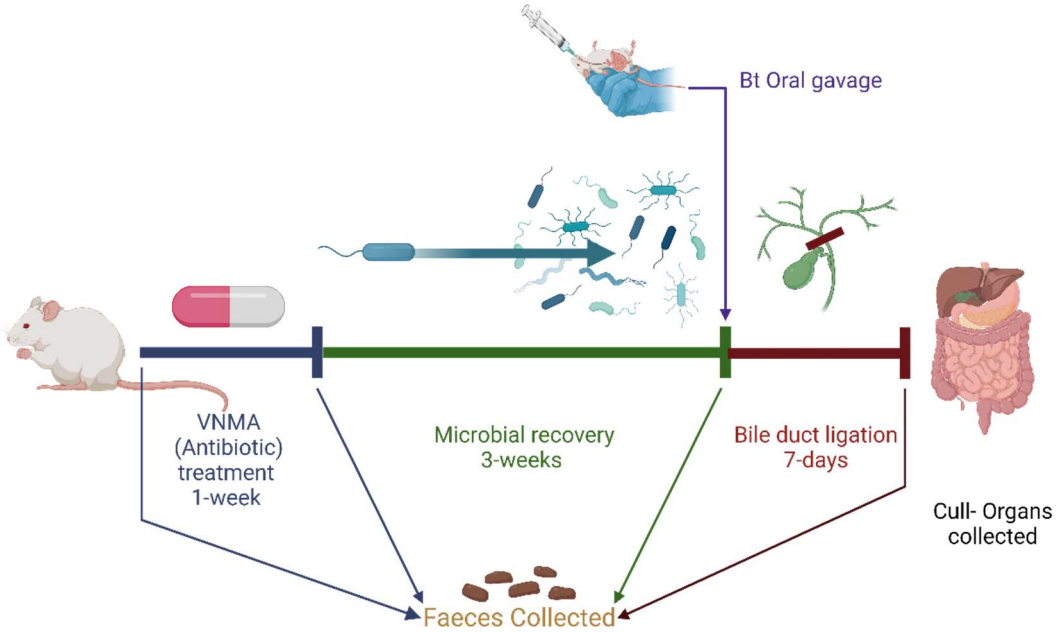


Figure 4.A. Experimental and sampling schematic; chapter four. *Antibiotics (VNMA) were administered for one week, following which the microbiome was allowed to recover for three weeks. Bacteroides thetaiotaomicron (Bt) was then administered by oral gavage one day prior to, BDL. Cholestatic disease then progressed for a further week. Animals were culled at 1-week post-BDL, and samples collected terminally. Faeces was collected throughout this time course at the timepoints indicated. This experiment was conducted twice, data shown is from one representative experiment unless otherwise stated.*

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4.3 Results

4.3.1 Bacteroides thetaiotaomicron reconstitution post antibiotic recovery results in a restoration of the Bt population to pretreatment levels.

I firstly sought to confirm that oral gavage of Bt reconstituted the bacterium into the microbial community of the gut. Bt was cultured overnight by Dr Regis Stentz as described elsewhere [153] and 200 µL of a suspension of $0.5x10^9$ CFU/ml in PBS was administered to antibiotic pretreated mice by oral gavage. Faecal pellets were collected throughout the experiment time course (Fig 4.A) (as described in the previous chapter) and DNA extracted from a defined quantity of faeces. 16S copy number was then

quantified by qPCR using universal 16S rRNA primers and Bt content then determined by qPCR using Bt genomic DNA primers as described elsewhere [147]. Ct was then converted to copy number by comparison to standard curves and the percentage of total bacterial population consisting of Bt calculated.

Results of the Bt genomic DNA qPCR demonstrated that Bt is lost following antibiotic treatment and recovery despite general recovery of total bacterial biomass. However, following gavage, Bt population is reconstituted (Fig 4.1). These results demonstrate that the oral gavage of $1x10^8$ CFU of Bt results in recolonisation of the intestinal microbiome with Bt to equal or greater levels than pretreatment.

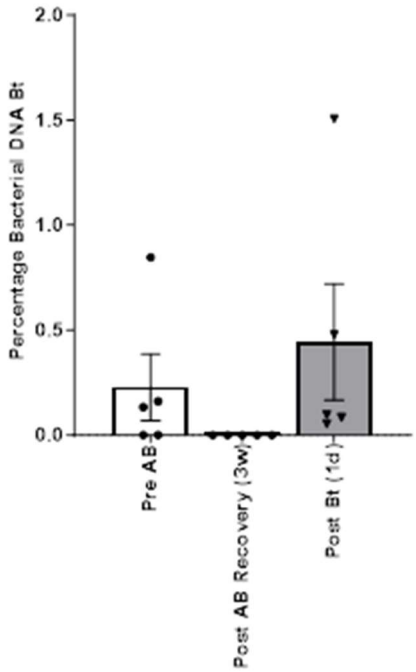


Figure 4.1. Faecal Bt population size represented as percentage of total bacterial population. *Antibiotics significantly deplete Bt population which fails to recover following the three-week recovery period. 1 day post gavage (Post-Bt 1d) Bt colonises the gut to a population approximately equal to or greater than the pretreatment level, which then can expand or deplete following one week of cholestasis induced by BDL (Post Bt BDL 1w). Data points represent paired values throughout the experiment time course. Data shown is from a representative experiment of 2 repeats.*

4.3.2 Bt reconstitution following antibiotic pretreatment increases propionate content following BDL, other SCFAs remain unaffected.

Given Bt's capacity for the generation of SCFAs, I sought to investigate the alteration to faecal SCFA content following Bt reconstitution. As previously, SCFAs were extracted from a defined quantity of faecal samples collected throughout the experiment time course and SCFAs extracted and quantified by LC-MS. Analysis of samples collected longitudinally throughout the experiment showed that one day post gavage (AB recovered (3w) Bt(1d)) the content of most SCFAs is reduced in comparison to Bt naive antibiotic recovered samples, excluding propionate which remains constant. However, propionate content is increased significantly after one week of Bt colonisation post-BDL (ABBtBDL) (Fig 4.2).

These results demonstrate that Bt reconstitution does not restore most intestinal SCFA levels following antibiotic recovery after one day, though propionate content is significantly increased given one week of Bt colonisation post-BDL.

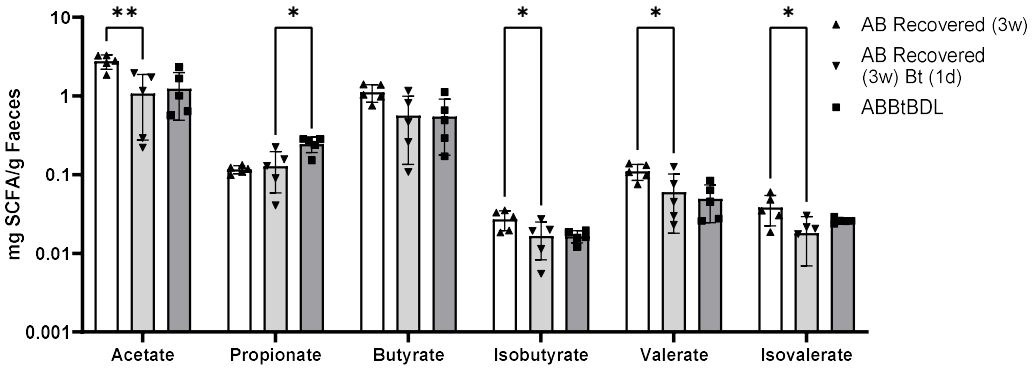


Figure 4.2 LC-MS quantification of the faecal SCFA content in antibiotic recovered mice post Bt reconstitution and BDL. *Most SCFAs are reduced in antibiotic recovered mice one day post Bt gavage (AB recovered (3w) Bt (1d)) which remains constant following one week of cholestasis (ABBtBDL). Propionate on the other hand is significantly increased following Bt gavage over one week following BDL. Data points represent paired samples. * = P<0.05, ** = P<0.01. Paired T-test. Data shown is from a representative experiment of 2 repeats.*

4.3.3 Bt reconstitution alleviates the increase in fibrosis generated by antibiotic pre-treatment, with limited effect upon both necrosis and the ductular reaction.

Having confirmed that Bt gavage leads to microbiome colonisation with Bt at levels equal to or greater than the pretreatment population and significantly increases propionate within one week, I investigated its impact on cholestatic disease pathophysiology.

Firstly, I sought to characterise the effect of Bt upon liver damage following BDL. As described in the previous chapter, formalin fixed liver tissues were embedded in paraffin, sections cut and haematoxylin and eosin stained. Necrotic areas were quantified manually in Image J in a semi-blinded manner and average necrosis per liver calculated from multiple representative images. Furthermore, blood was collected, serum extracted by centrifugation and transaminase content determined using a serum chemistry analyser.

Both histology and serum analysis data shows that antibiotic pretreatment drives somewhat, though not statistically significant, increased damage or necrosis following BDL, this is recapitulated here. However, Bt reconstitution has no effect on this, showing no change in necrosis (Fig 4.3), or in ALT (Fig 4.4 a), though there is a slight increase in

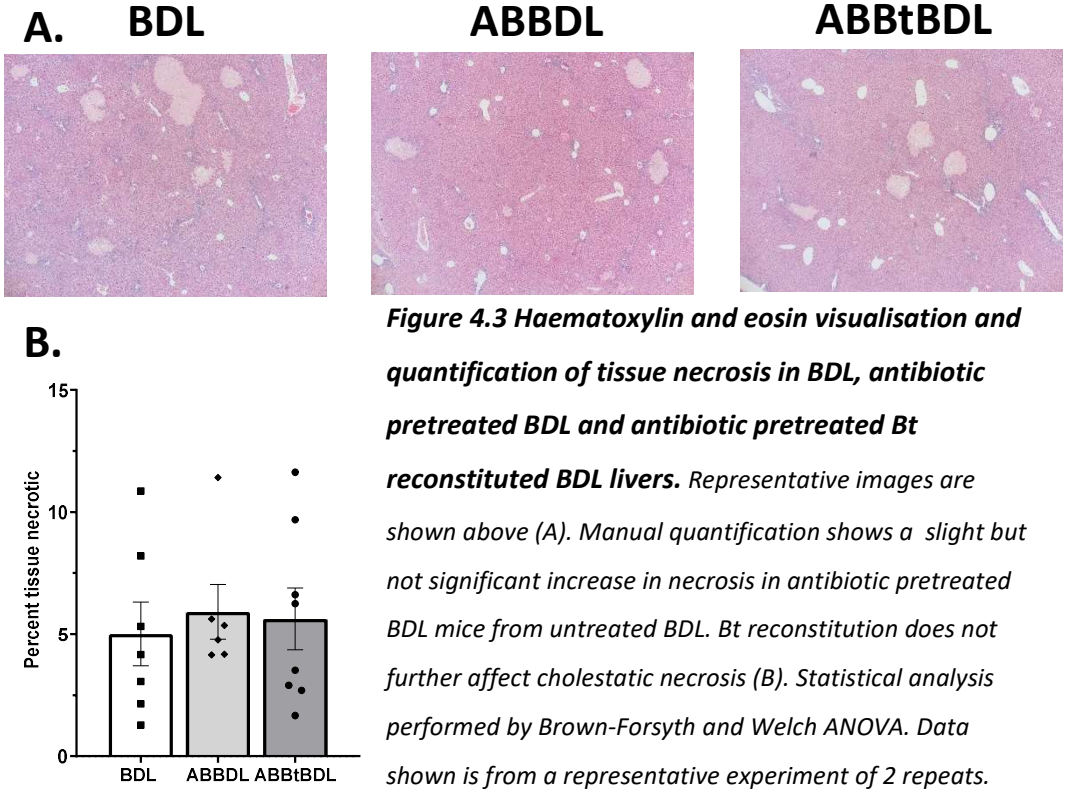


Figure 4.3 Haematoxylin and eosin visualisation and quantification of tissue necrosis in BDL, antibiotic pretreated BDL and antibiotic pretreated Bt reconstituted BDL livers. *Representative images are shown above (A). Manual quantification shows a slight but not significant increase in necrosis in antibiotic pretreated BDL mice from untreated BDL. Bt reconstitution does not further affect cholestatic necrosis (B). Statistical analysis performed by Brown-Forsyth and Welch ANOVA. Data shown is from a representative experiment of 2 repeats.*

AST (Fig 4.4 b), but not statistically significant between either untreated BDL or antibiotic recovered BDL.

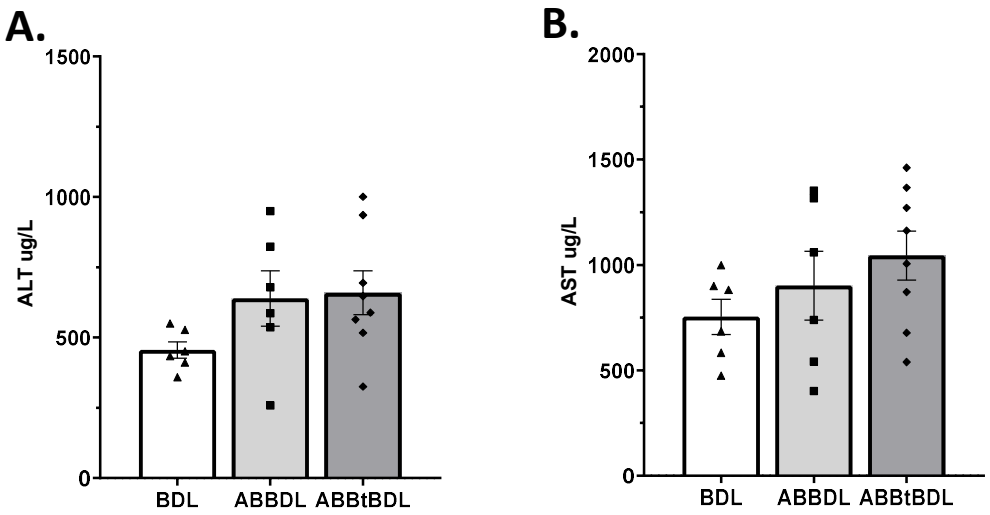


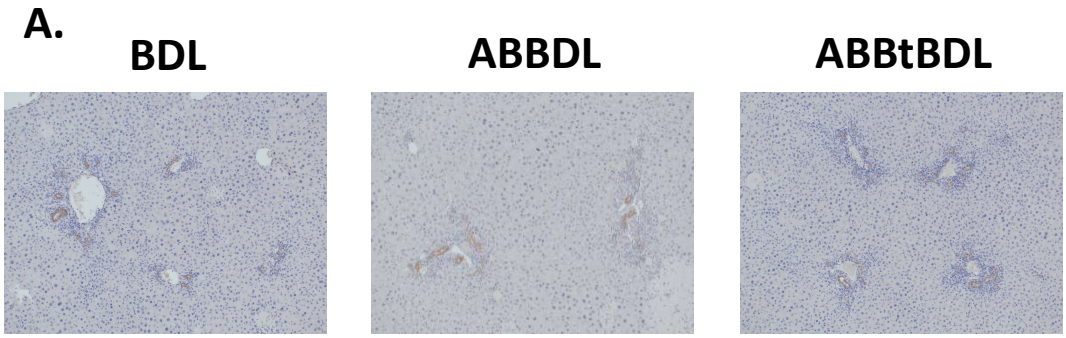
Figure 4.4 Serum transaminase quantification (Alanine transmainase and Aspartate transaminase) by Randox Daytona+. *ALT levels (A) and AST levels (B) are elevated in ABBDL samples compared to BDL only though this is not statistically significant. In ALT this is unaffected by Bt reconstitution, however in AST there may be a slight further increase. Statistical testing conducted by Brown-Fortsyth and Welch ANOVA. Data shown is from a representative experiment of 2 repeats.*

After having determined the effect of Bt reconstitution upon necrosis I then sought to characterise the effect upon the ductular reaction in response to BDL in antibiotic pretreated mice. Formalin fixed liver tissue samples, embedded in paraffin, were sectioned and immunostained for CK19, a marker for cholangiocyte proliferation [150]. Results demonstrated that, as previously, CK19 staining increased somewhat given antibiotic pretreatment, though not to a statistically significant degree, Bt reconstitution had no further effect upon CK19 expression (Fig 4.5).

As a further quantitative measure of the extent of cholestasis I then investigated alkaline phosphatase and total bilirubin content in serum. Results showed that serum alkaline phosphatase is somewhat reduced by antibiotic pretreatment prior to BDL, which is abrogated by Bt reconstitution, though no difference is statistically significant (Fig 4.6 a). Total bilirubin also follows this trend, whereby antibiotic pretreatment decreases total serum bilirubin which is abrogated by Bt reconstitution (Fig 4.6b).

Once I had confirmed that the extent of cholestatic damage, the ductular reaction and serum ALP and total bilirubin are mostly unaffected by Bt reconstitution, I then investigated the extent of fibrosis resulting from cholestasis. Tissue sections were sirius red stained and fibrotic staining quantified by automated image J macro. The data

revealed that, as in the previous chapter, antibiotic pretreatment significantly increases cholestatic fibrosis, it further demonstrated that this is attenuated to near untreated levels by Bt reconstitution (Fig 4.7).



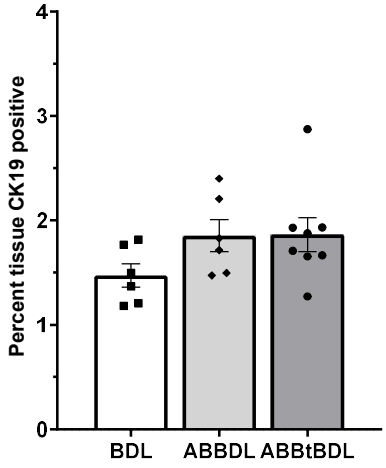


Figure 4.5 CK19 immunohistochemical staining showing cholangiocyte proliferation in BDL, antibiotic pretreated BDL and antibiotic pretreated Bt reconstituted livers. *Manual quantification of stained area shows a slight but not statistically significant increase in ductular proliferation given antibiotic pretreatment, which is unaffected by Bt (B), Representative images are shown above (A). Statistical significance measured by Brown-Forsyth and Welch ANOVA. Data shown is from a representative experiment of 2 repeats.*

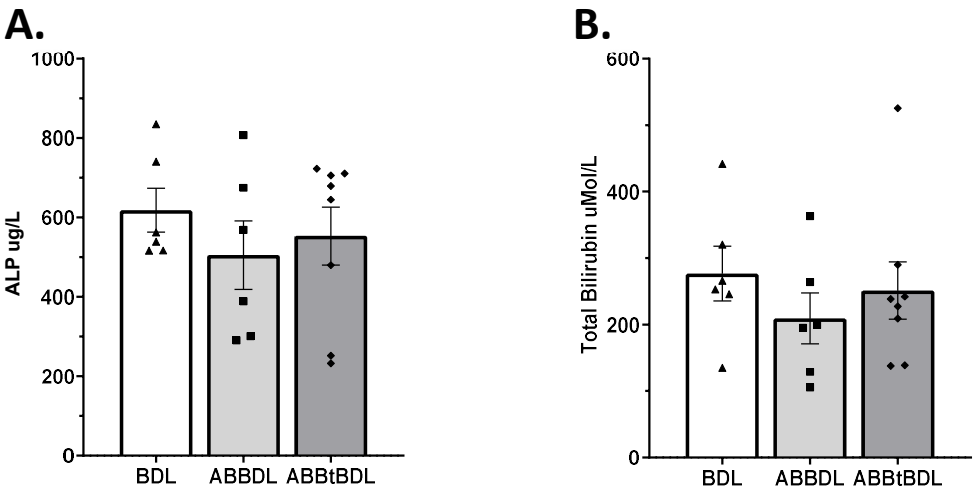


Figure 4.6 Serum Alkaline phosphatase and total bilirubin quantification by Randox Daytona+. *ALP levels (A) and total bilirubin levels (B) are somewhat decreased by antibiotic pretreatment prior to BDL which is attenuated by Bt reconstitution, though no difference is statistically significant. Statistical significance measured by Brown-Forsyth and Welch ANOVA. Data shown is from a representative experiment of 2 repeats.*

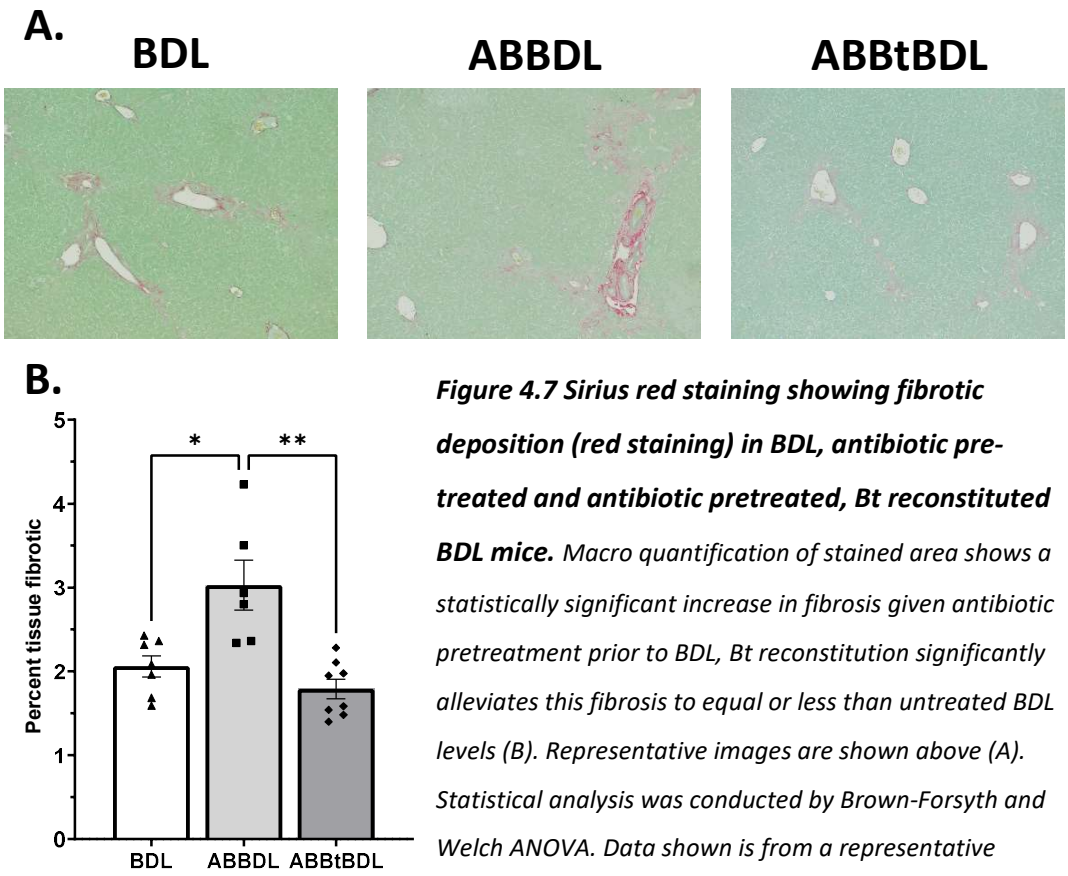


Figure 4.7 Sirius red staining showing fibrotic deposition (red staining) in BDL, antibiotic pre-treated and antibiotic pretreated, Bt reconstituted BDL mice. *Macro quantification of stained area shows a statistically significant increase in fibrosis given antibiotic pretreatment prior to BDL, Bt reconstitution significantly alleviates this fibrosis to equal or less than untreated BDL levels (B). Representative images are shown above (A). Statistical analysis was conducted by Brown-Forsyth and Welch ANOVA. Data shown is from a representative experiment of 2 repeats.*

Collectively these results demonstrate that Bt reconstitution has limited impact upon the extent of biliary cytotoxicity, characterised variously by the extent of hepatic necrosis, levels of serum transaminases, alkaline phosphatase, and bilirubin and by the ductular reaction. However, in terms of the significant increase in cholestatic fibrosis driven by antibiotic pretreatment, Bt reconstitution prevents this increase in fibrosis and may even very slightly decrease the extent of fibrosis from the untreated BDL baseline.

4.3.4 Bt reconstitution does not affect intestinal permeability following antibiotic pretreatment.

In order to investigate the driver behind the Bt colonisation dependent alleviation of cholestatic fibrosis I first investigated the ability of gut luminal contents to translocate to the liver by means of intestinal permeability. This constitutes a potential driver of increased fibrosis as translocated gut luminal content constitutes a pro-inflammatory stimulus, detrimental to the progression of cholestatic liver disease. To assess intestinal permeability FITC-dextran was administered to mice two hours prior to sacrifice, blood extracted post-sacrifice, serum isolated by centrifugation and FITC content assessed by fluorescence. Results of this analysis showed that Bt has no effect upon intestinal

permeability following BDL in antibiotic pretreated mice, though a slight statistically insignificant, decrease from untreated cholestatic mice remains (Fig 4.8).

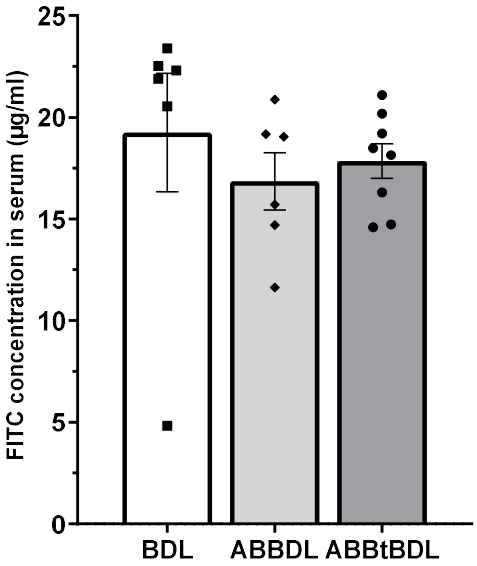


Figure 4.8 FITC-Dextran content in serum following BDL in untreated, antibiotic pretreated and antibiotic pretreated Bt reconstituted mice. *BDL significantly increases FITC content in serum, suggesting increased intestinal permeability which is well documented. However antibiotic recovered mice display no significant alteration to permeability. Significance calculations calculated by Brown-Forsyth and Welch ANOVA. Data shown is from a representative experiment of 2 repeats.*

4.3.5 Bt reconstitution restores macrophage infiltration and perivenular and periportal clustering in the cholestatic liver.

In the previous chapter (Fig 3.16-18) we saw a clear reduction in macrophage infiltration into the cholestatic liver of antibiotic recovered mice, I also observed by immunohistochemistry that the macrophages of these mice present displayed less perivenular and periductular clustering. Given this observation, I then sought to investigate the effect of Bt reconstitution upon macrophage infiltration and chemotactic activity. In order to investigate this, I conducted an F4/80 immunohistochemical stain upon liver sections from BDL, ABBDL and ABBtBDL treated mice. These stains confirmed the previous data showing a reduction in macrophage infiltration to antibiotic pretreated BDL mice when compared to BDL only. Furthermore, Bt reconstitution appears to restore the BDL only phenotype, whereby F4/80 staining appears to show equal periductular and perivenular clustering and total F4/80 staining in BDL and ABBtBDL livers, and the staining appears to be clustered around the periductular compartment or areas of necrotic damage (Fig 4.9).

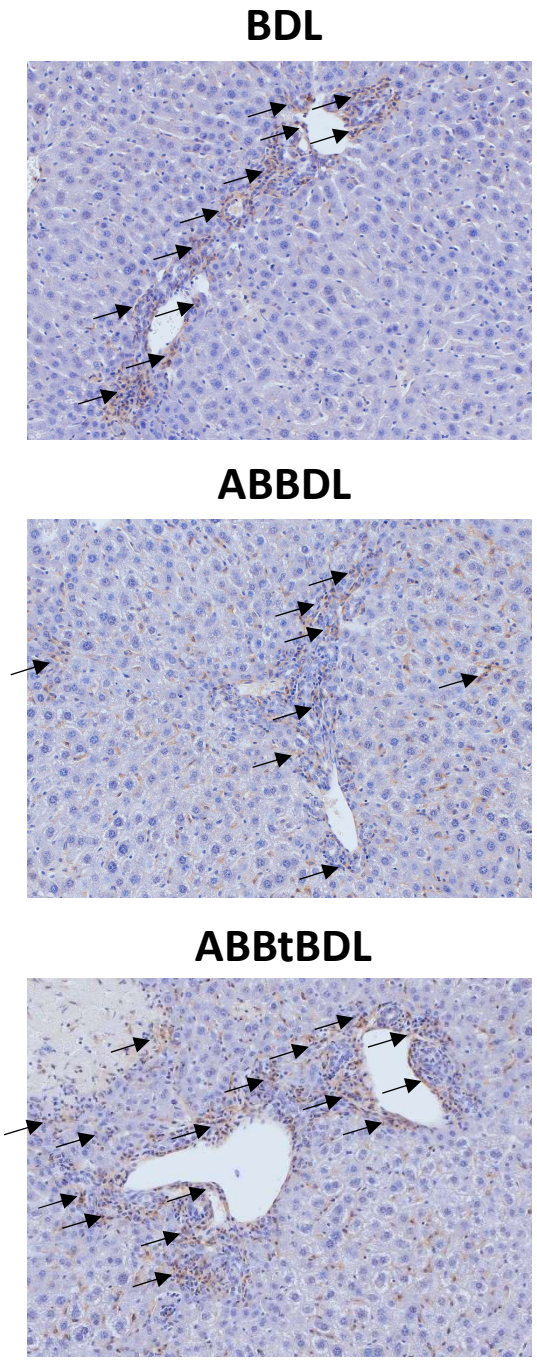


Figure 4.9 F4/80 immunohistochemical staining of cholestatic liver tissue sections.

Representative images of F4/80 stained liver tissue sections showing that antibiotic pre-treatment significantly reduces F4/80 staining, F4/80 stained cells also appear to be more dispersed, with fewer and smaller discernible clusters of staining (indicated by arrows). Bt reconstitution appears to restore untreated BDL levels of macrophage infiltration and clustering. This analysis was conducted once.

4.3.6 Bt reconstitution does not affect liver inflammatory cytokine expression following BDL.

After investigating effect of Bt treatment upon the innate immune cell infiltration to the liver following Bt reconstitution prior to BDL, I then sought to analyse the expression of cytokines in response to BDL. Similarly to the previous chapter there is no alteration to the expression of TNFα, Il1β or Il6 between antibiotic pretreated or antibiotic pretreated, Bt reconstituted BDL mice when measured by qPCR in liver homogenate RNA (Fig 4.10 A-C). Furthermore, there is no alteration in either CCL2 or CXCL2, expression between BDL, ABBDL or ABBtBDL (Fig 4.10 D,E). This data is largely in

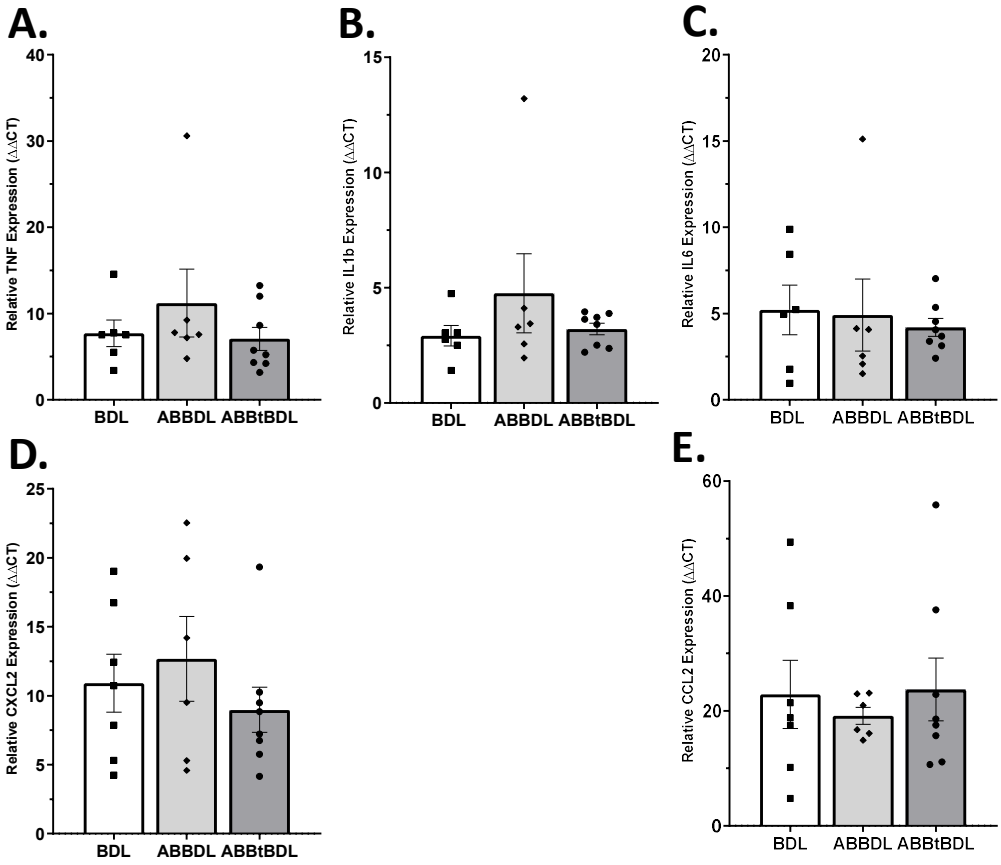


Figure 4.10 Rt-qPCR data showing cytokine and chemokine expression relative to control and housekeeping gene expression (ΔΔCT) in liver samples. *Relative cytokine expression in BDL, antibiotic recovered BDL (ABBDL) and antibiotic recovered Bt reconstituted BDL (ABBtBDL) samples measured by Rt-qPCR. Data is shown as ΔΔCT and is normalised to control and TBP housekeeping gene expression. TNF (A), IL1β (B), IL6 (C), CXCL2 (D), CCL2 (E). Data shows no significant changes in cytokine expression given antibiotic pre-treatment. Significance measured by Brown-Forsyth and Wech ANOVA. Data shown is from a representative experiment of 2 repeats.*

alignment with the data shown in the previous chapter whereby differences in cytokine expression are not seen at the level of the whole liver, which is unaffected by Bt reconstitution. This appears to correlate with the previous chapter, whereby, despite decreased macrophage infiltration in antibiotic pretreated mice there is equal pro-inflammatory cytokine expression, suggesting that the macrophages present are more pro-inflammatory. The fact that ABBtBDL animals display equal levels of macrophage infiltration to BDL only animals without increased pro-inflammatory cytokine expression suggests an anti-inflammatory effect upon infiltrating macrophages.

4.3.7 Bt reconstitution restores MMP2 expression following antibiotic reconstitution, though fibrolysis related genes remain unaffected.

Having determined that overall cytokine expression is unaffected by Bt reconstitution, I then sought to investigate the expression of fibrolysis related genes by qPCR as previous. The data showed that both TIMP3 and MMP9 (Fig 4.12 A,C) expression are unaffected by both antibiotic pretreatment and Bt reconstitution, although MMP2 expression is significantly reduced in antibiotic recovered mice and restored by Bt

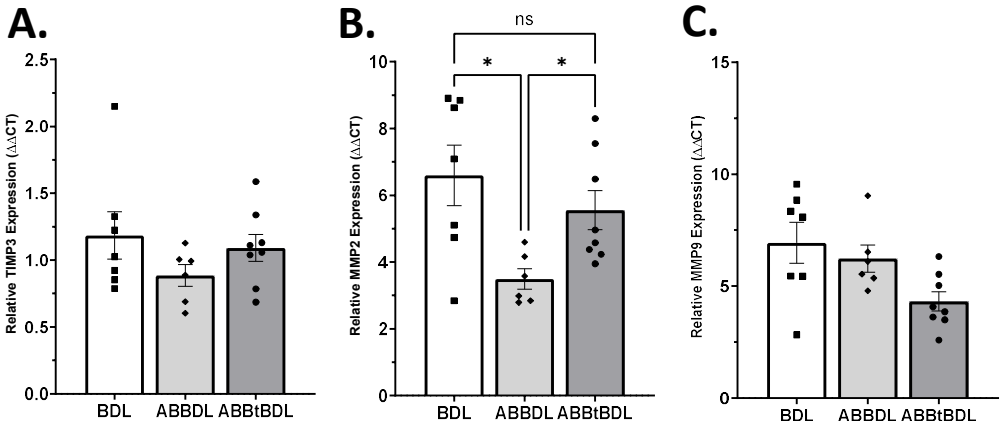


Figure 4.11 Rt-qPCR data showing TIMP3, MMP2 and 9 expression relative to control and housekeeping gene expression (ΔΔCT) in liver samples. *Relative TIMP3 (A), MMP2 (B) and MMP9 (C) expression in BDL, antibiotic recovered BDL (ABBDL) and antibiotic recovered Bt reconstituted BDL (ABBtBDL) samples measured by Rt-qPCR. Data is shown as ΔΔCT and is normalised to control and TBP housekeeping gene expression. Data shows a significant reduction in MMP2 expression given antibiotic pre-treatment and a restoration in expression in Bt reconstituted mice, similar changes in expression are shown in TIMP3 while not statistically significant. MMP9 appears reduced given antibiotic pretreatment and further reduction given Bt reconstitution, though no differences are statistically significant. Significance measured by Brown-Forsyth and Wech ANOVA. This analysis was conducted once.*

reconstitution to untreated BDL levels (Fig 4.12 B). This data suggests that Bt restores macrophage MMP2 fibrolytic activity in response to cholestatic disease following antibiotic pretreatment which may partially explain the amelioration of worsened fibrosis resulting from antibiotic induced microbial population change.

4.3.8 Antibiotic pretreatment prior to BDL reduces macrophage phagocytic capacity towards dead cell stimulus, restored by Bt reconstitution.

In addition to the analysis of cytokine expression in liver tissue sections, I then sought, with the support of Miss Katherine Hampton, to investigate the behaviour of macrophages in response to dead cell stimulus in vitro. Dead cell clearance is essential for the removal of pro-inflammatory stimulus, recovery from cytotoxic injury and initiation of proper liver regeneration [127].

The accumulation of cytotoxic bile acids during cholestatic disease causes profuse cell death within the liver, the debris from which is a pro-inflammatory stimulus and the clearance of which is conducted by phagocytic innate immune cells- neutrophils and macrophages [219]. To investigate the phagocytic capacity of macrophages towards dead cell stimulus and the effect of Bt on this pathway, mature macrophages were extracted from the bone marrow of untreated (BDL), antibiotic recovered (ABBDL) and antibiotic recovered Bt reconstituted (ABBtBDL) BDL mice and were exposed to dead cell stimulus. These dead cells were stained with pH rhodo – a stain which fluoresces when phagocytosed. The cells were exposed to this stimulus and then analysed by flow cytometry to analyse pH rhodo fluorescence.

This analysis demonstrated that antibiotic pretreatment significantly reduced the capacity of macrophages to phagocytose dead cell stimulus post-BDL, which was restored when Bt was reconstituted prior to BDL (Fig 4.13).

This data demonstrates that Bt plays a significant role in promoting the clearance of dead cell stimulus by macrophages during cholestatic liver disease and that reconstitution of said following antibiotic pretreatment, restores the dead cell clearance capacity of macrophages.

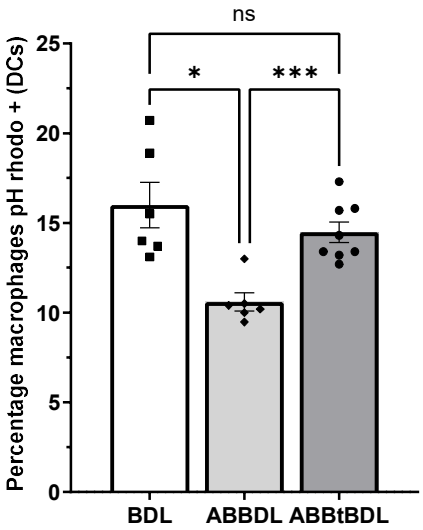


Figure 4.12 Dead cell phagocytic capacity of mature macrophages extracted from bone marrow. *Percentage of pH rhodo fluorescent macrophages (F4/80+) analysed by flow cytometry. Data indicates that, post-BDL, macrophages from antibiotic pretreated mice display a reduced dead cell phagocytic capacity when compared to untreated mice, whereas Bt reconstitution restores untreated phagocytic capacity. Statistical analysis conducted by Brown-Forsyth and Welch ANOVA, * = P<0.05 *** = P<0.001. This analysis was conducted once.*

4.4 Discussion

This chapter sought to investigate the effect of *Bacteroides thetaiotaomicron* reconstitution following antibiotic induced microbial population change and its impact upon the progression of cholestatic liver disease.

We observed in the previous chapter that *Bacteroides* was heavily depleted as a genus following antibiotic recovery. *Bacteroides* is generally considered to be a beneficial genus within the microbiome, being highly functional and thereby generating various beneficial metabolites [152].

My overall hypothesis for this chapter was that, following the results of the previous chapter, reconstitution of Bt would have an ameliorating effect upon the progression of cholestatic liver disease in antibiotic pretreated mice. Given the studies showing that Bt has an anti-inflammatory effect upon tissues distant from the gut [170,171,218], I hypothesised that this effect would centre on reductions in pro-inflammatory responses to cholestatic disease and alterations to innate immune cell activation and infiltration.

The initial investigation of this chapter sought to confirm that Bt gavage achieved recolonisation following antibiotic recovery and the effects of this recolonisation upon

intestinal microbiota and liver metabolite production. Analyses confirmed that Bt recolonisation was successful and that this recolonisation may produce increased faecal propionate content after one week of colonisation, though the confounding factor of cholestasis must be considered. However, considering in the previous chapter that BDL further reduced faecal propionate we can more confidently say that Bt is the driving factor here. It is also well documented that Bt is a producer of acetate and propionate in the human microbiome [152]. While we see no differences in acetate content following Bt colonisation, propionate content is significantly increased.

As was expanded upon in the previous chapter, propionate has been shown to have anti-fibrotic effects in atherosclerosis and to have modulatory effects on cholesterol and lipid metabolism [209–211]. Furthermore, propionate supplementation has been shown to limit macrophage infiltration to atherosclerotic lesions [209] suggesting that the increase of propionate content shown following Bt reconstitution may play a role in the reduction of fibrosis and macrophage infiltration.

The following analyses demonstrated that fibrosis is reduced by Bt reconstitution independently of levels of necrotic damage or the extent of cholestasis. To date, Bt has not been shown to impact upon fibrosis in liver diseases directly, though in NAFLD it has been shown to reduce the level of fibrosis by slowing steatosis and disease progression [170]. In order to determine the impact of Bt upon cholestatic disease therefore, I then investigated macrophage behaviour. Macrophages are potent drivers of liver fibrosis in cholestatic liver disease and are impacted by Bt colonisation [124,171,184,218], furthermore, in the previous chapter, macrophages had been shown to be driven to a pro-inflammatory phenotype following antibiotic recovery in vitro.

Data in this chapter demonstrated that, whole-liver cytokine expression is unaffected both following antibiotic recovery and Bt reconstitution, however macrophage infiltration and periductular and perivenular clustering is restored by Bt reconstitution. The clustering of macrophages in this compartment has been shown to be associated with the extent of ductular fibrosis, with anti-inflammatory, pro-resolution macrophage presence associated with reduced fibrosis and pro-inflammatory macrophage clustering with worsened progression [186]. Bt derived OMVs have been shown in a recent study to promote anti-inflammatory macrophage responses, driven by epigenetic changes to Nf-κB pathway activation. Without isolating macrophages from Bt reconstituted mice, we cannot determine if this effect is produced in our model, ameliorating the pro-

inflammatory macrophage polarisation produced following antibiotic recovery shown in vitro. However, we do observe that the level of macrophage infiltration and localisation to the peribiliary compartment in Bt reconstituted mice (ABBtBDL) appears more comparable to that of untreated BDL mice than antibiotic pretreated mice (ABBDL). Furthermore, there is no increase in fibrosis and cholestatic damage in Bt reconstituted mice. Therefore, this would suggest a restoration of 'antibiotic naïve' macrophage behaviour when Bt is reconstituted.

Subsequently, I analysed the expression of fibrosis related genes- TIMP3, MMP2 and MMP9 in the liver. Generally speaking, MMPs are responsible for the degradation of extracellular matrix and tissue remodelling, while TIMPs are responsible for the inhibition of their activity [220]. MMPs and TIMPs also have secondary functions especially in the activation of cytokines by cleavage and inhibition of this activity. MMP9 and MMP2 (gelatinases 1 and 2 respectively) were selected for their noted roles in fibrogenesis and are matrix metalloproteinases (MMPs). The primary role of MMPs is in the context of tissue remodelling, whereby they are expressed in response to tissue damage [220]. Tissue damage initiates an immune response whereby phagocytic cells are recruited to the area of damage in order to initiate the clearance of cellular debris, these innate immune cells- neutrophils and macrophages- then initiate tissue remodelling [221]. Matrix deposition is a key component of this reparative process which, under normal conditions, results in cellular proliferation and restoration of normal function [127,221]. However in the context of chronic or extensive damage, optimal tissue remodelling cannot occur, instead extensive fibrosis occurs, resulting in loss of tissue function [127,221]. MMP9 and MMP2 both play roles in fibrogenesis. They are expressed in response to fibrosis to degrade the deposited collagen, limiting fibrogenesis and promoting appropriate tissue remodelling, hence broadly speaking, they have an antifibrotic effect [127]. MMPs also have a pro-inflammatory effect by cleaving inactive cytokine precursors into bioactive signalling molecules, for example MMP9 cleaves both pro-TNFα and pro-Il1β [220].

TIMP3 is inhibitory toward the activity of MMP9. In the liver context MMP9 activity has been noted to be less impactful upon the degree of fibrosis, having a greater impact on the level of tissue inflammation [127]. While MMP2 expression has been linked to limited fibrogenesis in chronic models of liver fibrosis, both in the cholestatic context and in other chronic fibrosis models such as the carbon tetrachloride (CCl_4) model [127,132].

My results demonstrated that TIMP3 expression showed limited changes, with no significant differences seen between the livers of any treatment group. Although, it is interesting to note that MMP9 expression appears reduced, however not to a statistically significant degree, in Bt reconstituted BDL mice. Given the role of MMP9 in the cleavage of pro-inflammatory cytokines into active signalling molecules such as in the cases of pro-TNFα, pro-Il1β, pro-TGFβ (Transforming growth factor beta) etc. [220]. This effect would suggest an anti-inflammatory and anti-fibrogenic (in the case of TGFβ [127,184]) effect independent of direct cytokine expression.

MMP2 expression, on the other hand, shows significant reduction in expression in antibiotic recovered BDL mice in comparison to untreated mice, whereas in mice where Bt is reconstituted, MMP2 expression is restored to near untreated levels. MMP2 has a complex role in a broader network of fibrogenic and fibrolytic enzymes driving liver fibrosis during chronic injury. Increased expression of MMP2 is linked to increased fibrosis [132] as it is expressed in response to fibrosis. However, in long-term cholestatic disease sustained increase in MMP2 expression is linked to reduced fibrosis progression, delaying extensive fibrosis and cirrhosis [222]. Furthermore, depletion of MMP2 in the CCL4 murine model of liver fibrosis results in accelerated fibrosis progression [223]. These studies demonstrate a mechanism by which fibrosis may be increased following antibiotic recovery in BDL and ameliorated by Bt reconstitution. Interestingly, in the context of liver disease, MMP2 expression appears to originate primarily in hepatic stellate cells (HSCs), suggesting that this effect is driven by alterations to HSC activity [127,132,184]. However, in diseases in other tissues, macrophages are noted as producers of MMP2 independently of other tissues such as fibroblasts [224]. It remains unclear, therefore, whether alterations to macrophage or HSC activity are responsible for the alterations to MMP2 expression.

The analysis of dead cell phagocytosis by bone marrow isolated macrophages showed a reduction in phagocytic capacity following antibiotic recovery and BDL restored by Bt reconstitution. This assay was used as a model of necrotic debris clearance and this effect would therefore be expected to limit the clearance of pro-inflammatory, pro-fibrogenic dead cell stimulus following cholestatic damage.

Taken together, the results of this chapter demonstrate that *Bacteroides thetaiotaomicron* is restored 24 hours after gavage. This Bt reconstitution has limited impact upon the generation of SCFAs in the gut, though it does significantly increase

faecal propionate content after one week of colonisation. The reconstitution of Bt prior to BDL attenuates the increased fibrosis arising from cholestatic disease generated by antibiotic pretreatment. F4/80 immunohistochemistry demonstrated that macrophage density and clustering appear to be restored by Bt reconstitution. Furthermore, qPCR of cytokine and MMP expression and macrophage phagocytic activity showed that whilst this seemingly has no impact upon organ wide cytokine expression, both MMP2 expression and macrophage dead cell phagocytic capacity are restored when Bt is reconstituted prior to BDL.

4.5 Future work

The results presented in this chapter demonstrate that Bt reconstitution alleviates the increase in fibrosis following BDL induced by antibiotic pretreatment. The mechanism for this restorative effect appears to centre around alterations to macrophage infiltration and dead cell clearance, in combination with increased liver MMP2 expression.

The first objective for future work would be to determine the mode by which Bt alters macrophage activity and liver MMP2 expression. To investigate this, one would need to determine how macrophages respond to pro-inflammatory and pro-fibrotic stimulus in isolated macrophages from antibiotic pretreated mice versus in those from Bt reconstituted mice. The experiment would likely use a methodology similar to the BMDM experiment in the previous chapter. BMDMs would be isolated by cell sorting from antibiotic recovered and Bt reconstituted animals and these macrophages could be stimulated with dead cell and LPS stimulus and measure the cytokine and MMP responses. This would inform the investigator of the specific alterations to the responses of infiltrating macrophages achieved by Bt reconstitution and whether the restoration of MMP expression in the liver is owing to changes in macrophage behaviour. It would also be revealing to investigate the behaviour of BMDMs from antibiotic recovered and Bt reconstituted animals to investigate if the effects generated by Bt reconstitution are independent of direct Bt, or Bt generated metabolite, exposure and whether the alterations are persistent and generated prior to differentiation.

The second objective would be to determine the mechanism by which Bt generates these alterations. In order to investigate this, an initial, similar experiment may be used where macrophages from antibiotic pretreated and untreated mice were isolated or

differentiated from bone marrow. These macrophages would then be exposed to whole Bt bacteria, bacterial homogenate or to OMVs. OMVs, as mentioned previously, have been shown to mediate anti-inflammatory effects in tissues remote from the gut, especially in the liver [171,202,217]. RNA seq would likely be used to investigate the cellular transcriptome and determine the overall changes to macrophage gene expression, providing the essential information to inform a mechanistic study. Following this, once the pathways impacted by direct exposure to Bt stimulus had been determined more in-depth analyses could be conducted to fully illuminate the mechanism of Bt mediated anti-fibrotic, pro-phagocytic macrophage behaviour, such as the receptors involved and the route by which gene expression is altered.

These further experiments would be expected to highlight the mechanism by which Bt reconstitution influences macrophage behaviour and alleviates cholestatic fibrosis following antibiotic recovery. In addition, they may further highlight the pathways in macrophages impacted by Bt which generates this alleviation in increased fibrosis generated by antibiotic pretreatment.

4.6 Conclusion

Overall, the results presented in this chapter demonstrate that Bt reconstitution is restorative to the pathophysiology of cholestasis following antibiotic treatment and recovery. The alleviation of fibrosis appears to be mediated, at least in part, by restorations in MMP2 expression, macrophage infiltration and dead cell phagocytic capacity. These effects appear to be mostly independent of changes to gut microbial metabolite production, though increases to propionate content may play a role.

To date, Bt has not been shown to affect hepatic fibrosis apart from indirectly in the context of NAFLD by reducing hepatic steatosis thereby reducing disease progression [170]. However, it appears evident in this case that Bt reconstitution ameliorates the antibiotic pretreatment generated increase to hepatic fibrosis in cholestatic disease. Furthermore, it appears that this effect is driven by a recovery of macrophage population in the cholestatic liver, a recovery of MMP2 expression and a restoration of macrophage dead cell phagocytic capacity. The restored macrophage population in the cholestatic liver, in concert with their restored ability for dead cell clearance would assist in their ability for tissue remodelling and recovery from cytotoxic damage. In addition, the restoration of MMP2 expression will limit fibrotic deposition in response to necrosis and aid in slowing

cholestatic disease progression. Therefore, the main object of investigation in any future work is to further clarify the mechanism by which Bt generates the reduction in fibrosis, the pathways influenced by Bt in macrophages and the centrality of these effects to alleviated fibrosis.

Chapter Five.
Analysis of the effect of age upon the microbiome and cholestatic disease progression following antibiotic recovery.

5.1 Introduction

In chapter 3 we explored the effect of antibiotic induced microbial population change upon cholestatic disease. The results demonstrated that the antibiotic recovered microbiome has a significantly altered composition, with a significant depletion in the genus Bacteroides. In the previous chapter we saw Bacteroides depletion to be key in the exacerbation of fibrosis following BDL. Furthermore, there was a significant alteration in intestinal SCFA and bile acid content, with hepatic bile acid content also perturbed.

These effects were observed in mice from 8-10 weeks of age, i.e. in youth. However, studies have shown that the recovery from antibiotic treatment is highly variable, depending on many factors, such as antibiotic treatment, diet, initial microbiome composition, length of the recovery period and perhaps most pertinently, age [20]. The effect of age upon the impact of antibiotic treatment and recovery cannot be understated. Age also impacts both diet and initial microbiome composition, as these both change as one ages. Additionally, it encompasses other effects such as changes to metabolism, inflammaging and gut health [20,225]. Throughout life, studies, have suggested that the microbiome is initially variable, though more able to recover to a close to pre-treatment composition following disruption such as antibiotic depletion. Although the impacts which are retained significantly impact health and disease in later life [20,26,31]. The microbiome then progresses from this initially variable period, becoming more compositionally stable in adulthood [20,136]. This stability is also seen in the response of the microbiome to disruption. Studies suggest that the microbiome is less impacted by factors such as antibiotic treatment, however, given this stability, the microbiome appears to have a reduced capacity to recover to its original composition [20].

Throughout life the capacity of the gut microbiota to generate metabolites, such as SCFAs, fluctuates as a function of age [19,136,139]. The early life microbiota possesses low population diversity, the composition is primarily comprised of maternally derived microbes with some environmental or diet derived microbes appearing as time progresses [19]. This microbiome has high functional homogeneity and therefore produces a less diverse pool of metabolites. As age increases however, the microbiome increases in diversity as more functional genera begin populating the gut, increasing the capacity for metabolite generation. This colonisation stabilises (excluding perturbation) at around

adulthood, with the microbiome being both relatively functionally and compositionally diverse [19,20]. As age progresses however, there are further changes in the microbiome, with alterations to population composition and functional diversity, most studies agree that this effect is deleterious to both functional and compositional diversity [58,136,139,226]. Hence, at different periods of progression, the microbiome possess distinct metabolite outputs, which regulate whole organism function.

Age related effects are reported both in bile acid and SCFA generation. As age progresses, the bile content of the gut, liver and serum has been shown to decrease [58], with interventions which restore youthful bile acid compositions and total bile content even observed to increase lifespan [58]. Furthermore, the expression of both FXR and TGR5 decreases throughout life, suggesting that bile acid autoregulation is impaired. This is corroborated by studies showing that exogenous TGR5 and FXR agonists limit the progression of age-related kidney deterioration and osteoarthritis [227]. These observations demonstrate that throughout life the regulation of bile acid levels and bile composition is altered and that homeostasis is impaired and therefore may be more susceptible to perturbation. Additionally, the capacity of the microbiome to generate SCFAs also decreases as a function of age. Analysis of the human microbiome throughout life demonstrates that in aged individuals there is a significant reduction in microbial genes involved in SCFA metabolic pathways [226].

Immune homeostasis, particularly that of the innate immune system, alters throughout life, this effect is commonly termed 'inflammaging' [226]. It is characterised by increased generalised, chronic, low-grade, inflammation which involves various systems and perhaps most pertinently, the gut microbiota [226]. Aging is a well characterised risk factor for all chronic diseases and it is noted that inflammatory markers are strong predictors of morbidity in chronic diseases and generalised mortality [228]. Interestingly, inflammaging is characterised by a generalised increase in immune activity in lieu of inflammation, which is to say that both pro and anti-inflammatory activity is increased [229]. The relationship between the microbiota and the process of inflammaging cannot be underestimated. In a recent study, it has been noted that as aging progresses, there is a rearrangement in the gut *Firmicutes* population, which gradually becomes dominated by pathobionts and pro-inflammatory stimulating bacteria, in lieu of anti-inflammatory stimulating bacteria, especially *F.prausnitzii* and related species [230]. Additionally, another recent study reported that regardless of age, the human microbiome is dominated by

three bacterial taxa; *Bacteroidaceae, Lachnospiraceae and Rumminococcaceae.* These decrease in abundance throughout life, being replaced by bacteria typical of other bodily niches such as the periodontal environment, this is especially true in extreme age [231]. It has been noted that retaining anti-inflammatory taxa associated with the youthful microbiota- particularly *Biffidobacteria* and *Akkermansia*- is associated with healthy aging, anti-inflammation and protects against impaired metabolic homeostasis in age [230].

The liver is heavily impacted by aging, with age being a potent risk factor for the development of chronic liver conditions [232,233], particularly so in the development of MASLD, MASH and subsequent hepatocellular carcinoma (HCC) [232,233]. The liver undergoes many morphological and functional changes throughout the ageing process. Aging is associated with increased hepatocyte size and binucleated cell number, reduced mitochondria, increases in visceral fat and pro-inflammatory cytokine secretion [232], changes which impact liver morphology, physiology and oxidative capacity. Pertinently to hepatic fibrosis arising from disease, the basal activation of hepatic stellate cells (HSCs) becomes more marked in age. Pro-fibrotic, HSC activation markers such as α smooth muscle actin (αSMA) and collagen 1α1/2 increase in expression in homeostatic conditions in aged rats [232,234]. This basal activation is progressive throughout age, correlated to increasing size and number of HSC cytoplasmic lipid droplets [184,235]. Additionally, liver progenitor cells become less functional and more quiescent in advanced age, limiting the capacity for regeneration following damage and promoting fibrosis, limiting the capacity for functional recovery [236]. Furthermore, Kupffer cells acquire a less phagocytic and more pro-inflammatory phenotype in age, which contributes to inflammation in chronic liver diseases and limits the clearance of dead cell stimulus [232].

Studies have demonstrated that, in the context of human liver disease, there is a contrast in the outcome of cholestatic liver disease in the two main cholestatic pathologies. In PBC it has been demonstrated that young patients experience worsened disease pathology and an increased risk of treatment failure [233]. Whereas, in the context of PSC it has been shown that ageing is a significant component of PSC mortality and increasing the risk of the development of cholangiocarcinoma arising from cholestasis [233].

Hence, the effect of age upon the microbiome is significant and generally results in a perturbation resistant, though plastic and more functionally diverse microbiota in

middle age. This declines in later life, when the microbiome is more unstable and less functional. However, it appears that inflammaging is a significant outcome of this maturation process and that this effect is likely progressive beginning early in development. Furthermore, it is evident that aging has a significant impact upon the response of the liver to cholestasis and the severity of chronic liver disease.

5.2 Hypothesis and aims

I hypothesise that the resistance of the aged microbiome to antibiotic induced microbial population change results in a lack of associated changes in microbial metabolite production. Furthermore, the lack of changes to bacterial populations and metabolites results in limited changes to cholestatic liver disease pathophysiology. Finally, I hypothesise that the extended recovery of the microbiome following antibiotic treatment in youth, to an equivalent age as aged mice, minimises the impact of antibiotic pretreatment upon cholestatic liver disease pathophysiology.

Therefore, the aim of the following chapter is to illustrate the role of age in influencing the impact of antibiotic induced gut microbiota population change upon cholestatic disease. Two durations of microbial recovery were investigated, along with two ages at which antibiotics were administered. Mice were administered with antibiotics at 8-10 weeks and the microbiome allowed to recover for 6 months, to investigate if the effects of antibiotic induced microbial population change in youth are retained following aging to 8-10 months of age (Termed herein as ABx6Mo). A second cohort at approximately 8-10 months of age were used to investigate the impact of antibiotic treatment and 3-week recovery upon cholestasis in middle age (Termed herein as aged). The several aims of this chapter are listed below:

1. To illustrate the compositional change elicited by antibiotic treatment and recovery in aged mice.
2. To characterise the faecal SCFA and faecal and hepatic bile acid pool throughout the administration of antibiotics and the progression of cholestatic disease in antibiotic treated mice in comparison to untreated mice.
3. To demonstrate whether the aforementioned metabolite changes are retained given a longer microbial recovery period.
4. To investigate the impact of antibiotic induced microbial population change, recovered over three weeks or six months, in aged mice upon the progression of cholestatic disease.

5. To analyse the effects of the aforementioned alterations to microbial population and metabolite output upon the immune response to BDL induced cholestatic disease in aged mice.

The structure of the mouse treatment and sampling is shown in the figure below to aid in data interpretation and general understanding (Fig 5.A).

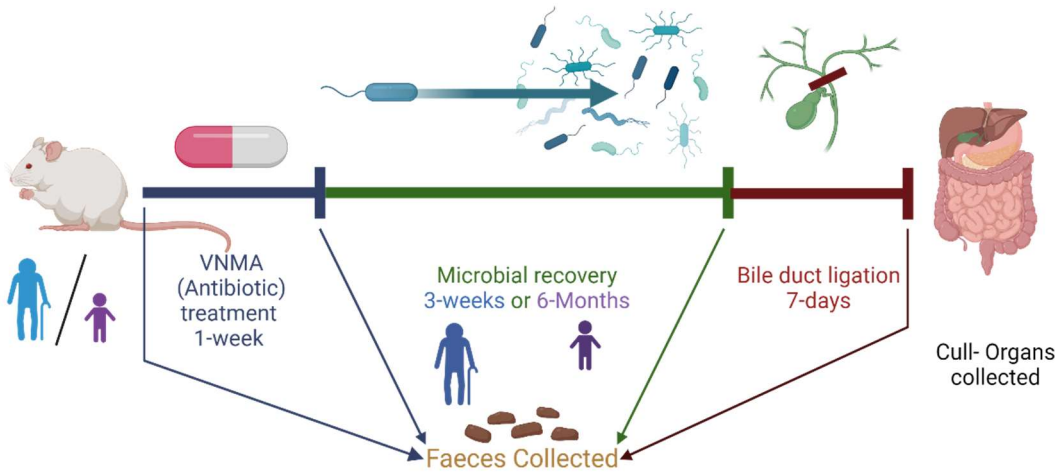


Figure 5.A. Experimental and sampling schematic; chapter five. *Antibiotics (VNMA) were administered for one week, to either 8–10-week-old or 8–10-month-old mice, following which the microbiome was allowed to recover for three weeks in aged mice or six months in young mice. Following the requisite recovery period BDL was then conduced and cholestatic disease progressed for a further week. Animals were culled at 1-week post-BDL, and samples collected terminally. Faeces was collected throughout this time course at the timepoints indicated. Aged and 6-month recovered cohorts have been analysed once.*

Created with BioRender.com

5.3 Results

5.3.1 In aged mice, antibiotic treatment depletes the faecal microbiota and whilst biomass recovers within three weeks, population composition is altered.

In order to investigate the impact of age upon antibiotic induced microbial population change, I first analysed the depletion of the gut microbial community and its subsequent recovery. As in previous chapters I extracted DNA from faecal pellets and performed universal 16S qPCR on this DNA. Data demonstrated that bacterial biomass is significantly depleted, by approximately 100x, following one week of antibiotic

treatment and recovered to pre-treatment levels following a three-week recovery period (Fig 5.1).

Although this data resembles what is observed in 3.1.1 (Fig 3.1), it is important to note that the depletion is 100 times lower in aged mice compared to young mice. This discrepancy may be due to the lower initial 16S content in aged pretreatment samples, which is roughly 10 times higher in younger mice. Taking this into account, the relative reduction is 10 times less in aged mice than in young mice.

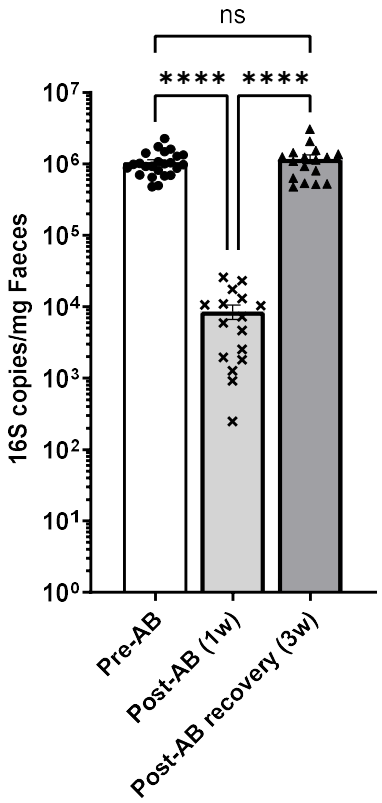
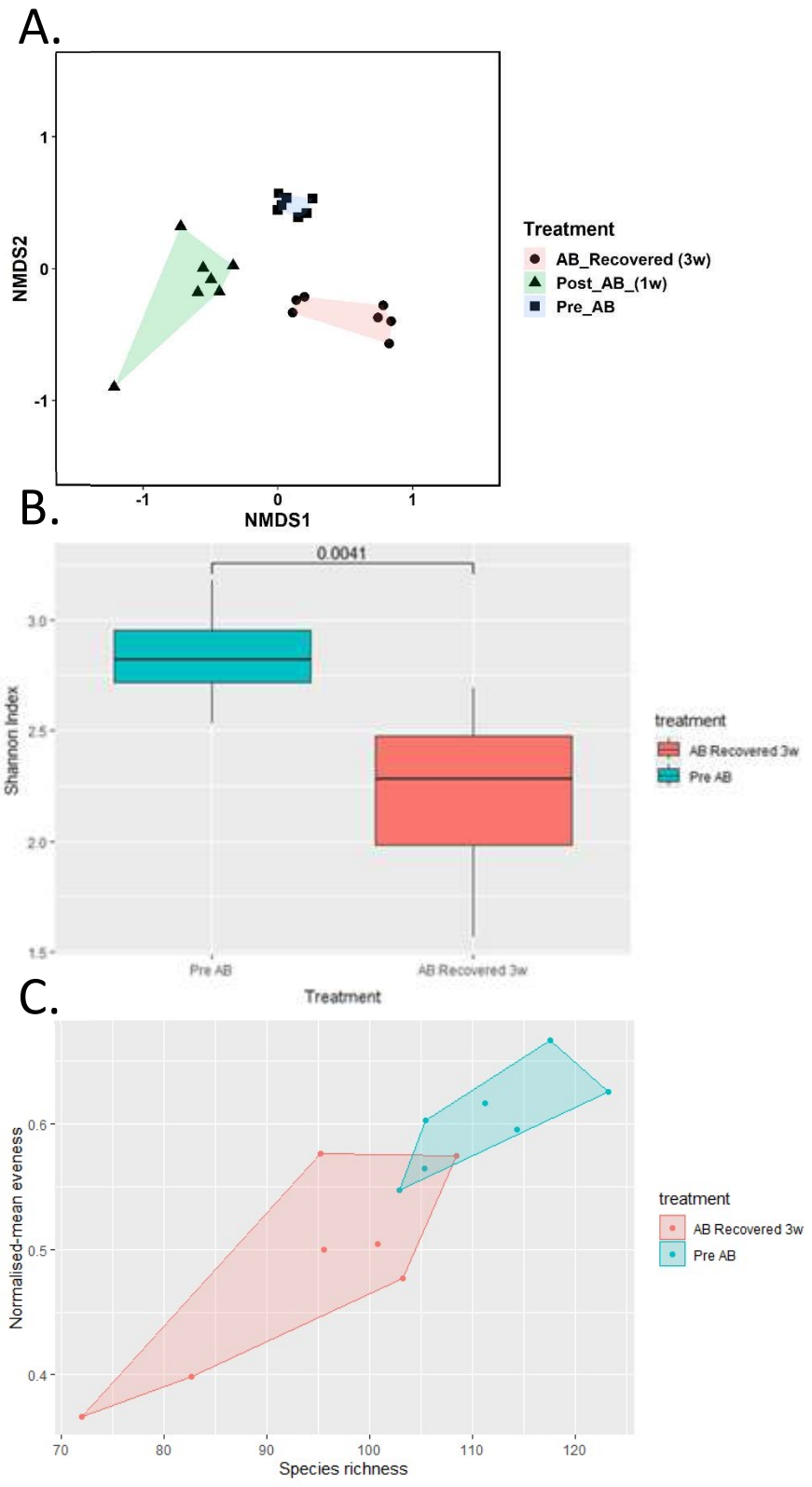


Figure 5.1 Faecal universal 16S qPCR showing depletion of bacterial biomass following antibiotic treatment and recovery after 3 weeks in aged mice. *Antibiotics significantly deplete bacterial biomass over 1 week and biomass recovers to approximately pre-treatment levels following 3 weeks recovery in aged mice. Ct was converted to copy number by reference to a standard curve. Data is represented on a log scale for ease of viewing. **** = P<0.0001 Brown-Forsyth and Welch ANOVA. This experiment has been conducted once.*

Having observed that VNMA treatment depletes the microbiome in aged mice, though to a lesser extent than in young mice and that 16S copy number recovers to approximately pretreatment levels, I then sought to investigate population composition. As previously, faecal sample DNA was sequenced by shotgun metagenomics, raw reads were quality filtered and trimmed, taxonomic assignment and metagenome reads conducted using Kraken 2 and Bracken was then used to estimate relative taxa

abundances. These species and genus abundance matrices were then analysed by alpha (Shannon) diversity (Fig 5.2a-b), beta (Bray-Curtiss dissimilarity) diversity between treatment groups (Fig 5.2 c) and LEfSe (Linear discriminant analysis of effect size) to show specific changes in genera (Fig 5.2 d) and species (Fig S4).



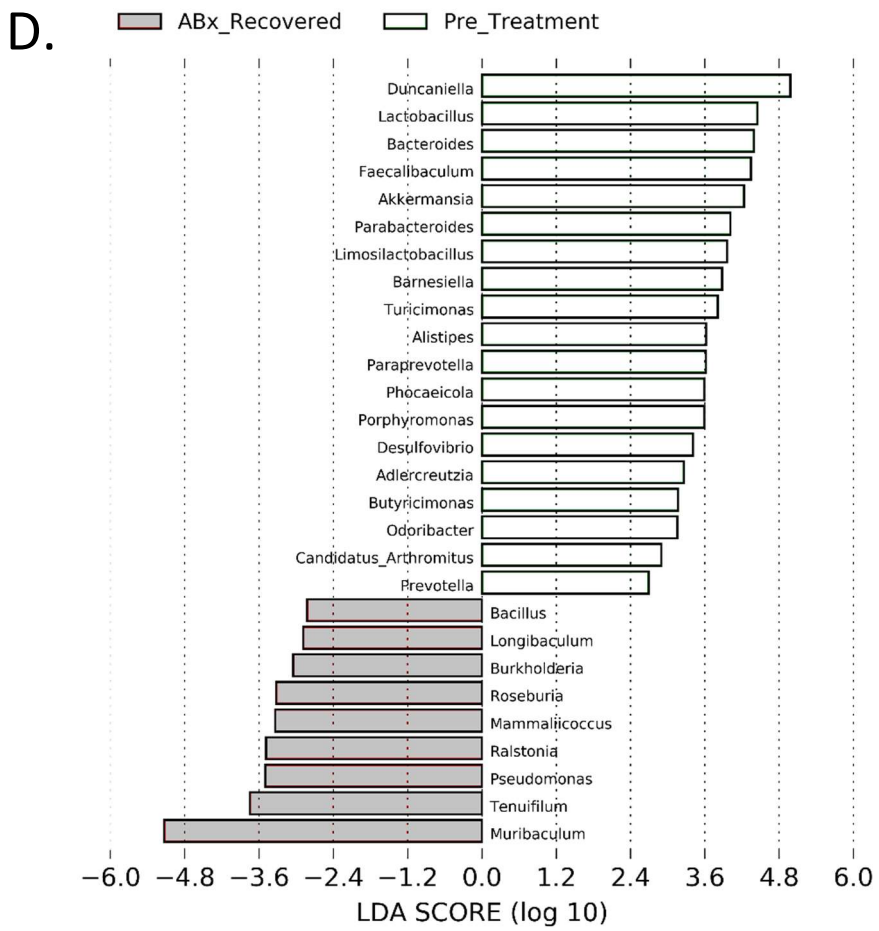


Figure 5.2 Comparative analysis of faecal bacterial population compositions throughout antibiotic treatment in aged mice and recovery analysed by shotgun metagenomics. *NMDS plot of β diversity (Bray-curtiss) demonstrates distinct clustering of pre-treatment, post-antibiotic and antibiotic recovered bacterial populations (A). Shannon index measurement showing significant reduction in population diversity following antibiotic recovery (P measured by Wilcoxson Rank-sum test) (B), with a reduction in both population evenness and richness (excluding one pre-treatment sample) (C). Linear discriminant analysis plot demonstrating differential enrichment of several key genera between pre-treatment and antibiotic recovered mice, significance value threshold P<0.01 (D). This analysis has been conducted once.*

Firstly, the analysis demonstrated that, following antibiotic treatment, there is a significant change to population composition which recovers to a composition distinct from both pretreatment and post-antibiotic (1w) compositions. This is shown by distinct clustering when analysed by Bray-Curtiss dissimilarity (Fig 5.2 A). It is also interesting to note that pretreatment aged microbiota compositions cluster tightly, suggesting a high degree of internal group similarity. The antibiotic recovered composition is also shown

to be significantly less diverse when measured by Shannon diversity, with a reduction of approximately 0.6, driven by both reduced population evenness and richness (Fig 5.2 B, C). Finally, LEfSe analysis displays a reduction in several functional genera following antibiotic recovery, the most notable being: *Bacteroides*, *Faecalibaculum*, *Akkermansia*, *Parabacteroides*, *Alistipes* and *Phocaecicola*. Each of these are highly functional genera being associated with reduced systemic inflammation and beneficial outcomes in liver diseases [166–175]. In terms of enriched genera, there are fewer, though *Bacillus* and *Pseudomonas* increase and each have opportunistic species noted to be pathogenic in cases of dysbiosis; *B.cereus* in the case of Bacillus and *P.aeruginosa* in the case of *Pseudomonas* [237]. Though no pathogenic *Bacillus* or *Pseudomonas* species are highlighted in the species level LEfSe analysis (Fig S4).

This data indicates that whilst bacterial biomass may recover to pretreatment levels, population composition is significantly impacted in aged mice. Though this is to a lesser extent than young mice, with different genera impacted.

5.3.2 Changes to faecal bile acid content are induced by microbial population change in aged mice and are somewhat maintained in 6-month recovered mice.

Given the noted changes in microbial population composition in aged mice following recovery from antibiotic treatment, I then sought to investigate changes to bile acid content in the gut. As previously, bile acids were extracted from faecal samples from both young mice treated with antibiotics and recovered for 6 months and aged mice treated at 8 months. The bile acid extracts were then analysed by LC-MS and content bile acid content quantified by percentage of total faecal bile acid pool.

Data demonstrated that, in aged mice, there was a significant increase in TMCA in concert with a significant reduction in TDCA, CA and DCA (Fig 5.3 A). This represents a significant increase in the primary FXR antagonist TMCA [79] and a significant decrease in two potent FXR agonists; CA and DCA [79]. However, there appears to be no significant alteration in total faecal biliary pool in aged mice (Fig 5.3 B).

When comparing 6-month antibiotic recovered faecal bile acid content to the content in pre-treatment faeces from the same mice at 8-10 weeks of age, it is evident that there is a limited alteration to faecal bile acid content. Between these two timepoints there is a significant increase in both TMCA and βMCA and a significant reduction in DCA content. However, when comparing 6-month recovered faeces to faeces from untreated mice of

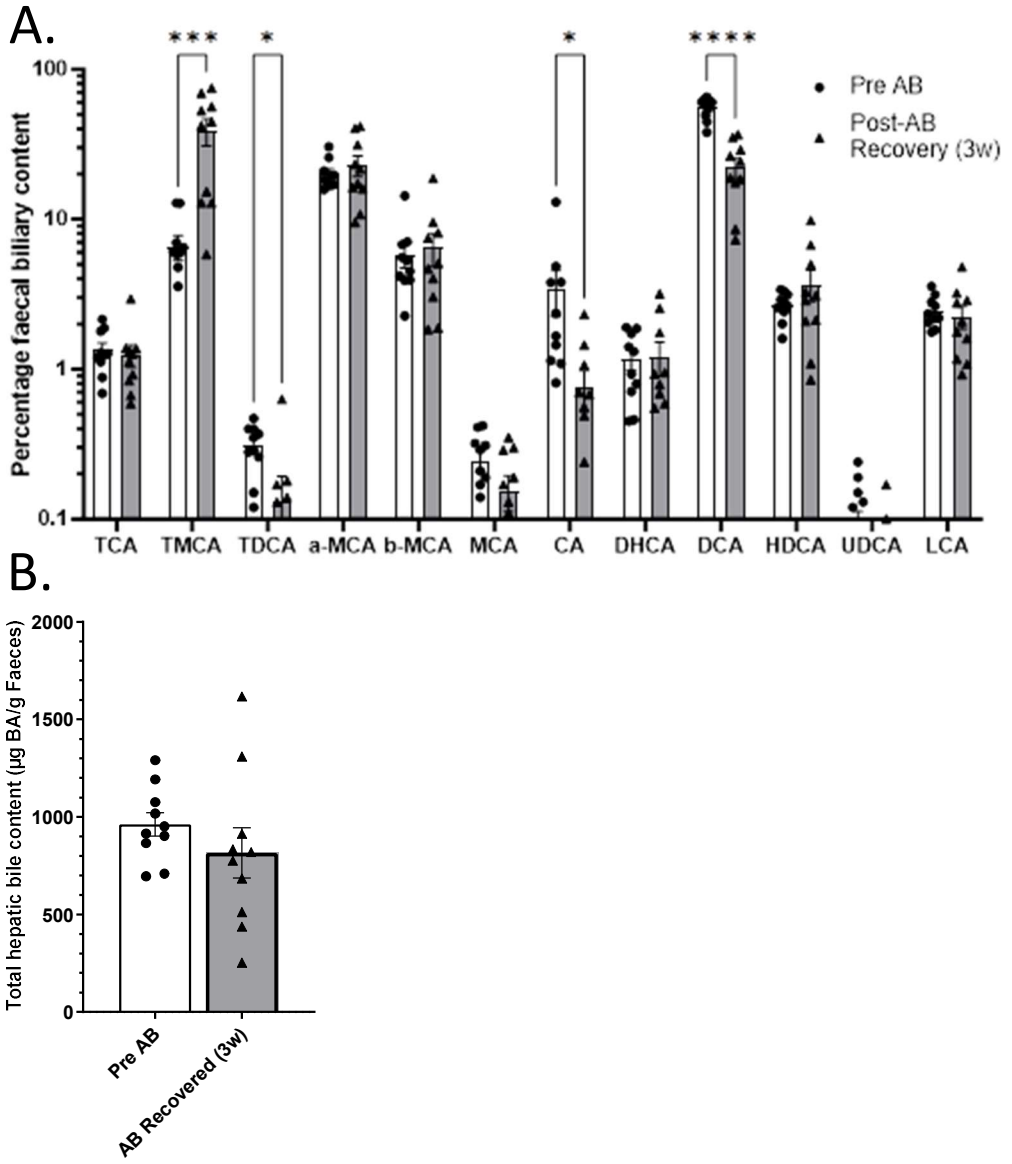


Figure 5.3 LC-MS quantification of faecal biliary content in pre-treatment and antibiotic recovered faeces from aged mice. *Graph showing bile acid content as a percentage of total faecal bile acid content (A) TMCA content is significantly increased following antibiotic recovery, while TDCA, CA and DCA are significantly reduced, data shown on a logarithmic scale for ease of viewing. Graph showing total bile acid content which remains unaffected following antibiotic recovery (B) * = P<0.05, *** = P<0.001, **** = P<0.0001. Welch T-test. This experiment has been conducted once.*

the same age, i.e. pre-AB (Aged), it is clear that there is a much larger alteration to faecal biliary content. This alteration is generated by significantly larger α and β MCA contents and lower DCA, HDCA and LCA contents in 6-Month antibiotic recovered faeces vs untreated aged faeces (Fig 5.4 A). Additionally, there is a noticeable reduction in total faecal biliary content. This decrease is observed both between untreated faeces from 8–10-week-old mice and the 6-month recovered faeces from the same mice, as well as

between untreated aged faeces and the 6-month recovered faeces. However, the reduction is more pronounced in the latter comparison (Fig 5.4 B).

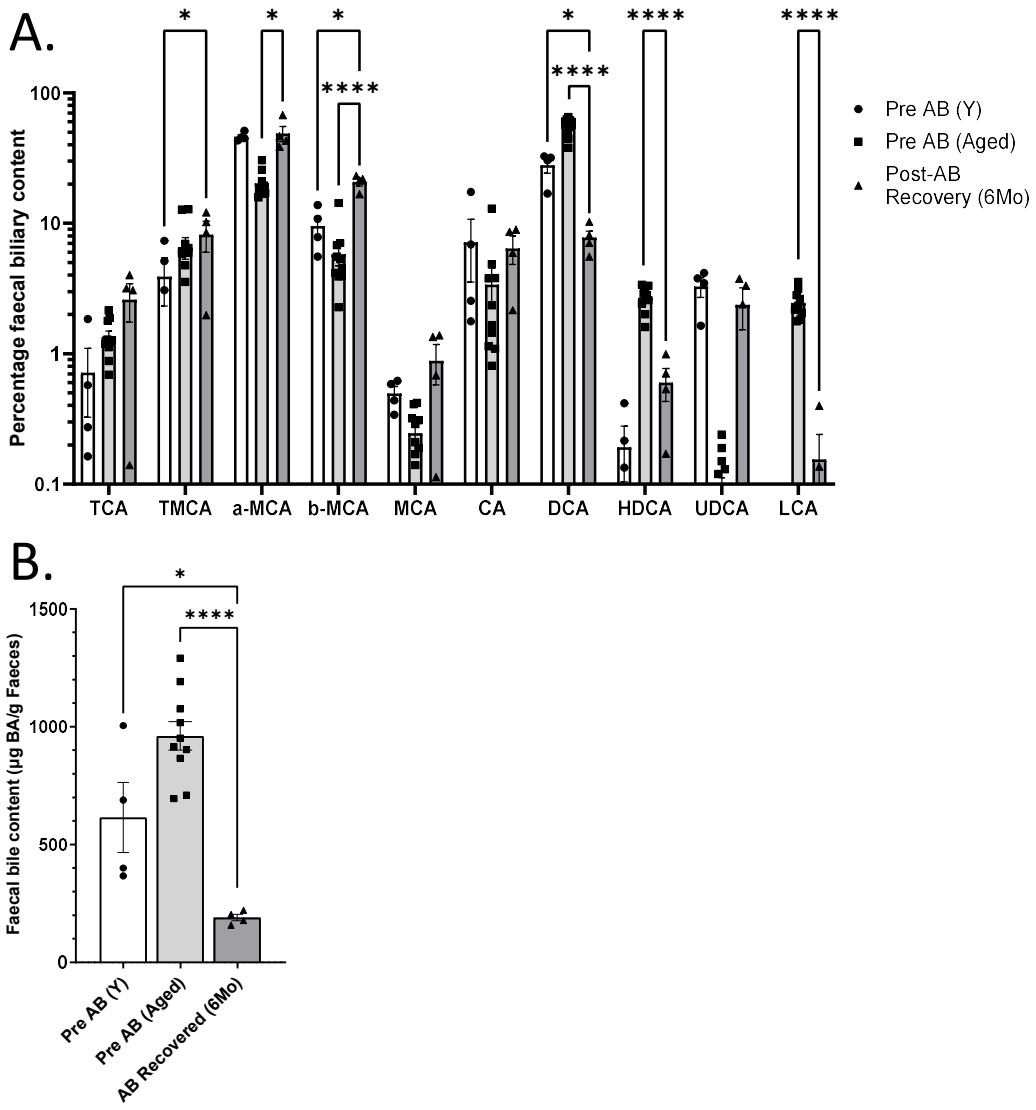


Figure 5.4 LC-MS quantification of faecal biliary content in pre-treatment and antibiotic recovered faeces from 6-month antibiotic recovered mice. *Graph showing bile acid content as a percentage of total faecal bile acid content (A) TMCA content is significantly increased following antibiotic recovery, while DCA is significantly reduced in comparison to pre-AB (Y). In comparison to pretreatment mice of the same age (Pre AB aged) there is a significant increase in both α and β-MCA data and significant reduction in DCA, HDCA and LCA. Data shown on a logarithmic scale for ease of viewing. Graph showing total bile acid content which is significantly reduced both from pretreatment in youth and from untreated mice of the same age (Pre-AB aged) (B). * = P<0.05, ****=P<0.0001 Paired T-test used for longitudinal comparisons between pre-AB Y and AB recovered 6Mo, unpaired Welch T-test used for Pre-AB aged and AB recovered 6Mo comparisons. This experiment has been conducted once*

Overall, this data demonstrates that the antibiotic induced microbial population change induced in aged mice produces a significant change in gut biliary content. This, as in young mice, generates an increased content of FXR antagonists and decreased FXR agonists [79]. In 6-month recovered mice there is a similar, though less substantial, increase in TMCA and reduction in DCA retained over a longer period of antibiotic recovery when compared to content of the same mice at a pre-treatment timepoint at 8-10 weeks of age. However, analysis of 6-month recovered faeces in comparison to untreated mice of the same age demonstrated that bile acid content of six-month recovered mice is significantly different. This could suggest that treatment in youth produces a more 'youthful' bile acid composition following antibiotic recovery. Furthermore, it appears that given a longer period of antibiotic recovery, alterations to gut biliary content and microbial population change generates a reduction in total gut bile acid content which is reduced to a greater degree in comparison to untreated aged mice.

5.3.3 Hepatic bile acid content is altered following microbial recovery in aged mice. In six month recovered mice there is a more limited effect upon hepatic bile acid content.

Having investigated the alterations to faecal bile acid content, I then sought to analyse the potential alterations to hepatic bile acid content. Bile acids were extracted from liver homogenate, analysed by LC-MS and bile acid quantities normalised to sample mass. Data demonstrated that in aged mice there was a significant increase in TMCA content coincident with a significant reduction in the content of TCA and TDCA (though TDCA was only present in trace amounts). In 6-month recovered mice the effects are more minimal, with a greater reduction in TDCA and an increase in CA, both of which are present in trace amounts (Fig 5.5 A). On the other hand, total hepatic bile acid content was unaffected in both aged and 6-month recovered antibiotic treated mice, with no significant differences observed between control and both 3-week and 6-month antibiotic recovered mice (Fig 5.5 B).

This data demonstrates that in aged mice there is an alteration to the content of primary bile acids, somewhat slighter in comparison to young mice, driven by an increase in the content of TMCA, which causes a coincident decrease in other primary bile acids. However, in 6-month antibiotic recovered mice there is a more limited alteration in

hepatic bile acid content, with a significant reduction in TDCA content and slight increase in CA content, though both are only present in trace amounts. This data shows that 6-month antibiotic recovery significantly limits the alteration to hepatic bile acid content seen in mice treated in age. Furthermore, as in young mice shown in 3.3.2, there is no alteration to total hepatic bile acid content regardless of the length of antibiotic recovery in aged mice.

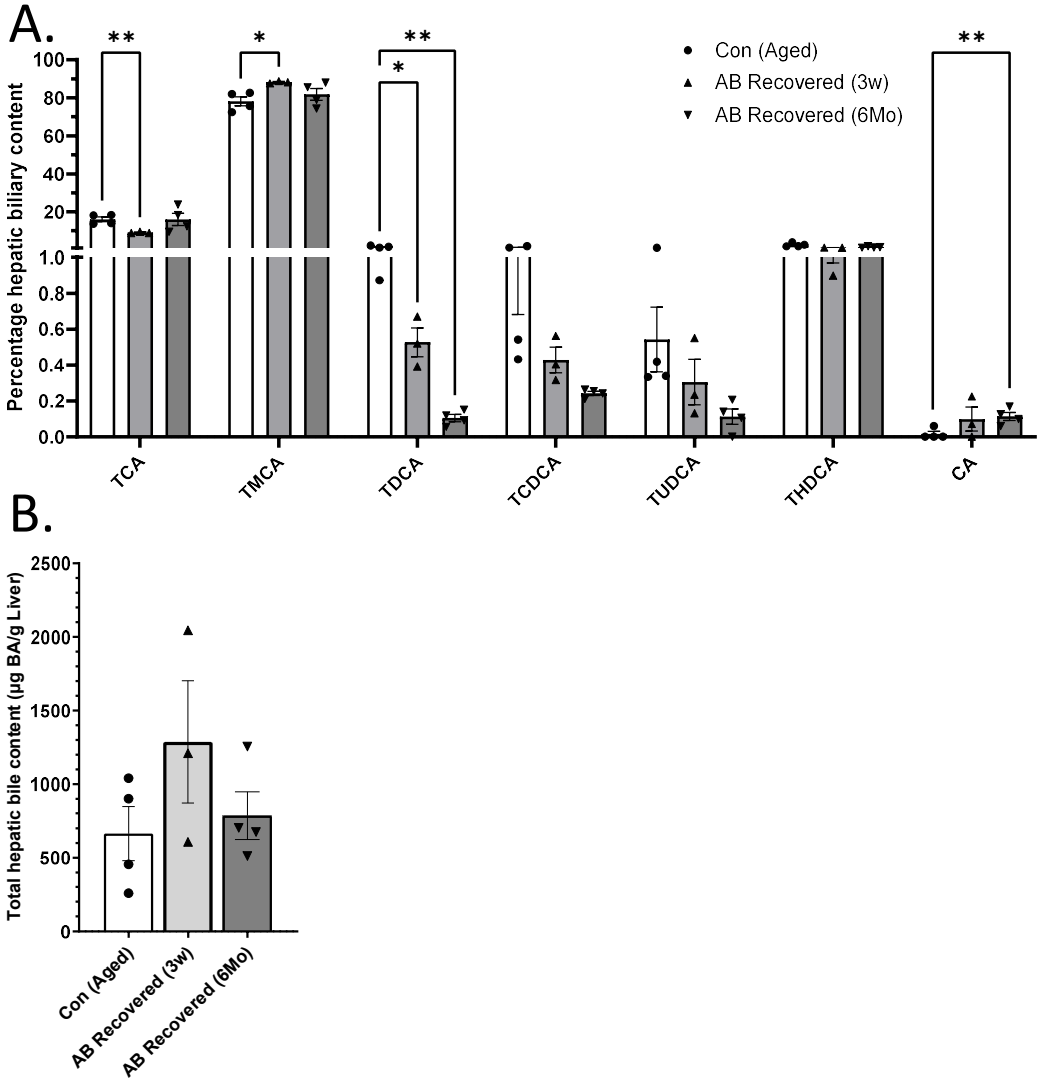


Figure 5.5 LC-MS quantification of hepatic biliary content in pre-treatment, aged antibiotic recovered, and 6-month recovered liver samples. *Graph showing bile acid content as a percentage of total hepatic biliary content (A). TMCA content is significantly increased following antibiotic recovery in aged mice, while TCA and TDCA are significantly reduced. In six-month recovered mice DCA is significantly increased and TDCA significantly decreased, though both are only present in trace quantities. data shown on a split scale (0-1%, 1-100%) for ease of viewing. Graph showing total bile acid content which remains unaffected following antibiotic recovery (B). * = P<0.05, **=P<0.01 Welch T-test. This experiment has been conducted once.*

5.3.4 Aged antibiotic recovered mice possess a slightly altered faecal SCFA pool, with long-term antibiotic recovery displaying a similarly slight alteration to faecal SCFAs.

In addition to faecal biliary analysis, faecal SCFA analysis was also performed to analyse alterations to the metabolism of these compounds produced following antibiotic recovery in aged mice. SCFAs were extracted from faecal samples and analysed by LC-MS.

Data demonstrated that the effects of antibiotic induced microbial population change in age are more limited than in young mice. Mice treated in age display a marginal increase in faecal butyrate, isobutyrate and isovalerate following antibiotic recovery, though both isobutyrate and isovalerate are only present in trace quantities (Fig 5.6 A). 6-Month antibiotic recovered mice show an increase in faecal isobutyrate from pretreatment- i.e young untreated control. In comparison to old untreated mice, there is a lower content of both faecal propionate and valerate (Fig 5.6 B). This data demonstrates that aged mice are more resilient to SCFA alterations resulting from antibiotic induced population change, in contrast to young mice, butyrate, isobutyrate and isovalerate content is increased following antibiotic recovery though the changes are limited. In 6-month antibiotic recovered mice, there is limited alteration from pretreatment faecal SCFA content in youth with a slight increase in isobutyrate content. On the other hand, this represents a larger degree of variance from aged untreated faecal SCFA content, with a reduced content of both propionate and valerate.

5.3.5 In aged mice, gut bacterial population is mostly unaffected by BDL, with antibiotic recovered BDL mice displaying an altered population composition.

In order to determine the impact of antibiotic induced microbial population change upon cholestatic disease in aged mice, it was necessary to determine the alterations to microbial population throughout antibiotic treatment and cholestatic disease. Therefore, having investigated the alterations to microbial population and metabolite production prior to the induction of cholestatic disease, I then investigated the effects of BDL upon the microbiota in aged mice to identify further changes to the microbiota occurring during the progression of cholestatic liver disease which may impact disease

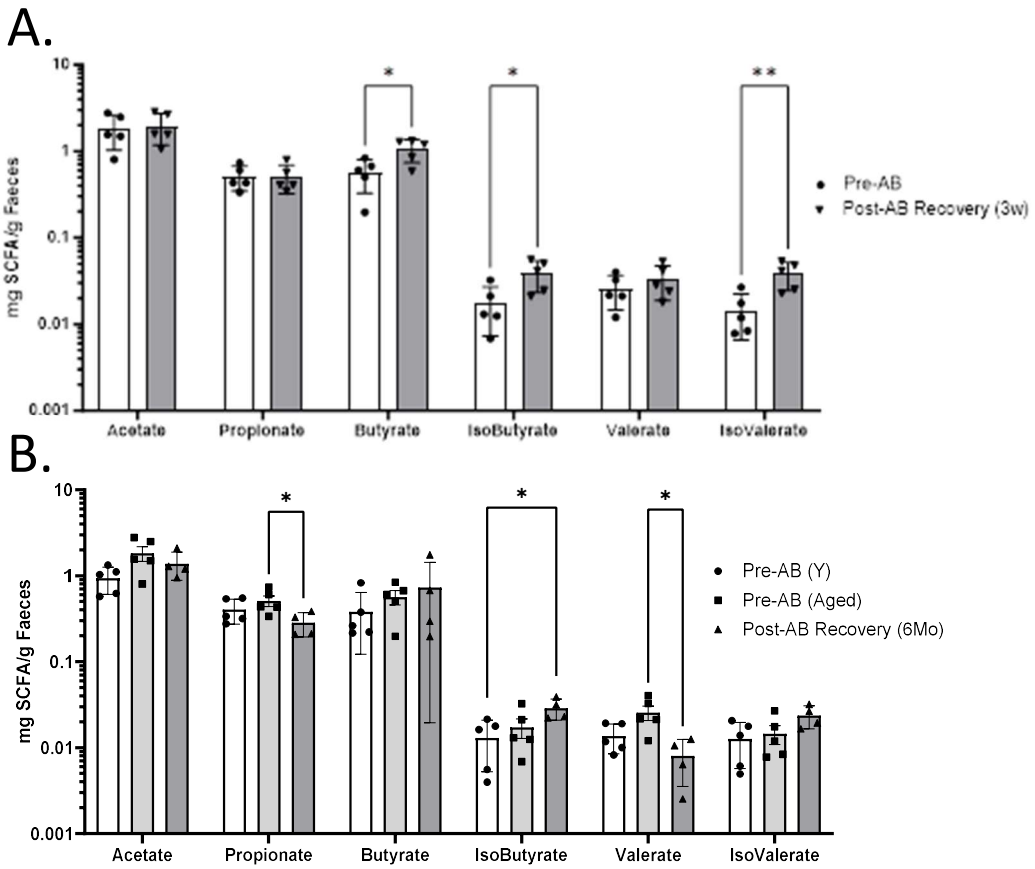


Figure 5.6 LC-MS quantification of the faecal SCFA content in pre-treatment and 3-week (A) and 6-month (B) antibiotic recovered aged mice. *Mice treated in age display slight increases in butyrate, isobutyrate and isovalerate content following 3-week microbial recovery (A). In six-month recovered mice there is an increase in isobutyrate content from pretreatment levels in youth (Pre-AB Y), in comparison to untreated mice of the same age (Pre-AB aged) there is a reduced content of both propionate and valerate (B). *=P<0.05, **= P<0.01, Welch T-test. This experiment has been conducted once.*

severity. 16S qPCR upon faecal DNA samples demonstrated that unlike in young mice, BDL had no impact upon gut bacterial biomass, either in untreated or antibiotic recovered mice (Fig 5.7).

Though bacterial biomass was unaffected following BDL, we sought to analyse the alterations to population composition. Bacterial DNA was analysed by shotgun metagenomics and changes to population composition analysed variously by Bray-Curtiss dissimilarity, Shannon alpha diversity and LEfSe. Analysis demonstrated that, following BDL, antibiotic pretreated mice display a distinct population composition from both untreated BDL mice and antibiotic recovered pre-BDL mice measured by Bray-Curtiss dissimilarity (Fig 5.7 A). What is evident however, is that following BDL antibiotic

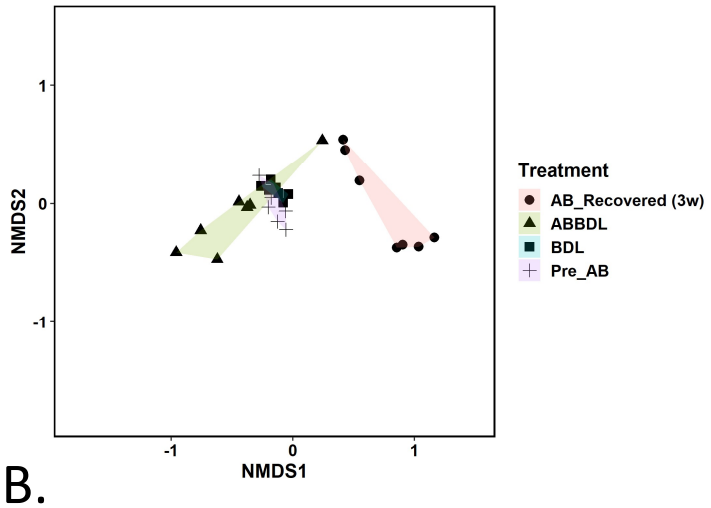
recovered BDL mice display less dissimilarity to both untreated BDL and pretreatment mice in comparison to antibiotic recovered pre-BDL mice. Additionally, there is no alteration to overall alpha diversity between untreated BDL and antibiotic pretreated BDL mice (Fig 5.7 B). Although, the gut microbiota of antibiotic pretreated BDL mice display approximately equal levels of population evenness when compared to untreated BDL mice, whilst richness appears reduced in the majority of samples (Fig 5.7 C). LEfSe analysis on the other hand, demonstrated that there were several enriched and depleted genera when comparing untreated BDL and antibiotic pretreated BDL mice. The most notable depleted genera following antibiotic recovery were Faecalibacterium and Pseudomonas and the most notable enriched genera were Escherichia and Parabacteroides (Fig 5.7 D).

Overall, this data demonstrates that following BDL there is no alteration in total bacterial biomass, though population composition is perturbed in antibiotic recovered mice. BDL alone does not seem to affect bacterial population composition. However, in antibiotic-recovered mice, the composition shifts from a pre-BDL antibiotic-recovered state to a post-BDL state. This post-BDL composition shows a greater similarity to untreated and BDL-only compositions. Furthermore, this change is driven primarily by depletions in genera such as *Pseudomonas* and *Faecalibacterium* and enrichments in genera such as *Parabacteroides*.

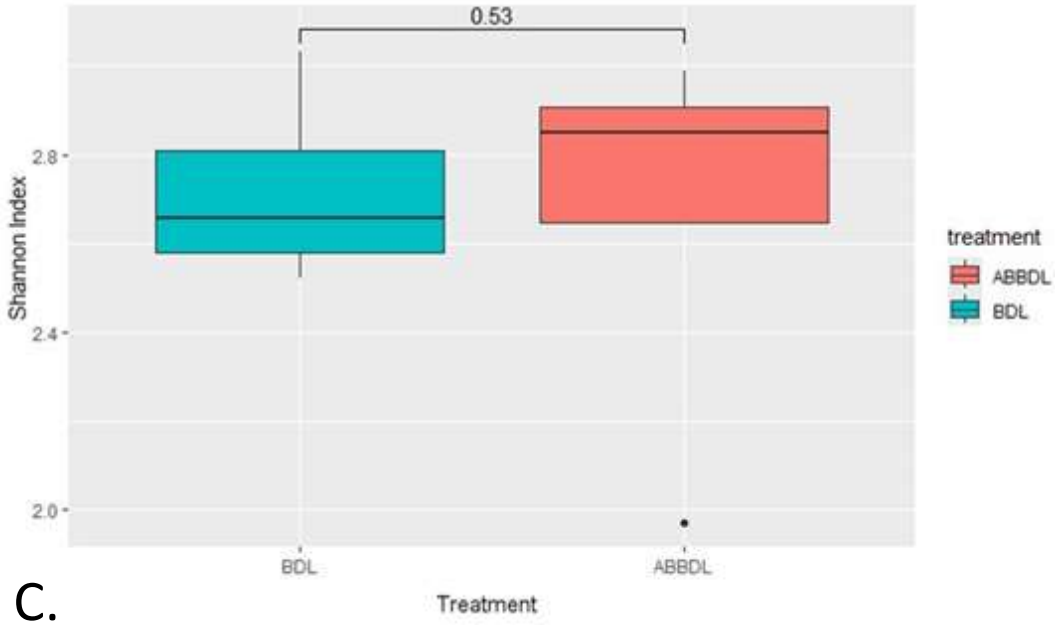
5.3.6 Antibiotic pretreatment has no effect upon hepatic bile acid content post-BDL in aged mice.

In order to investigate the effects of the noted bacterial population changes upon cholestatic disease I then investigated hepatic bile acid content in aged cholestatic mice. Bile acids extracted from liver homogenate was analysed by LCMS and analysed with quantity being normalised to total hepatic bile acid content. Data demonstrated that following bile duct ligation in aged mice there is a significant increase in TMCA content coupled with concurrent decreases in TCA, TDCA and THDCA when compared to untreated aged mice (Fig 5.8 A). However, antibiotic pretreatment does not appear to significantly alter hepatic bile acid content following BDL. In the case of mice antibiotic treated in age, there are no significant differences in hepatic bile acid content from untreated, aged BDL mice following 3 weeks of antibiotic recovery (Fig 5.8 A). The

A.


NMDS2
NMDS1
1
0
-1
-1
0
1
Treatment
AB_Recovered (3w)
ABBDL
BDL
Pre_AB

B.


0.53
Shannon Index
2.8
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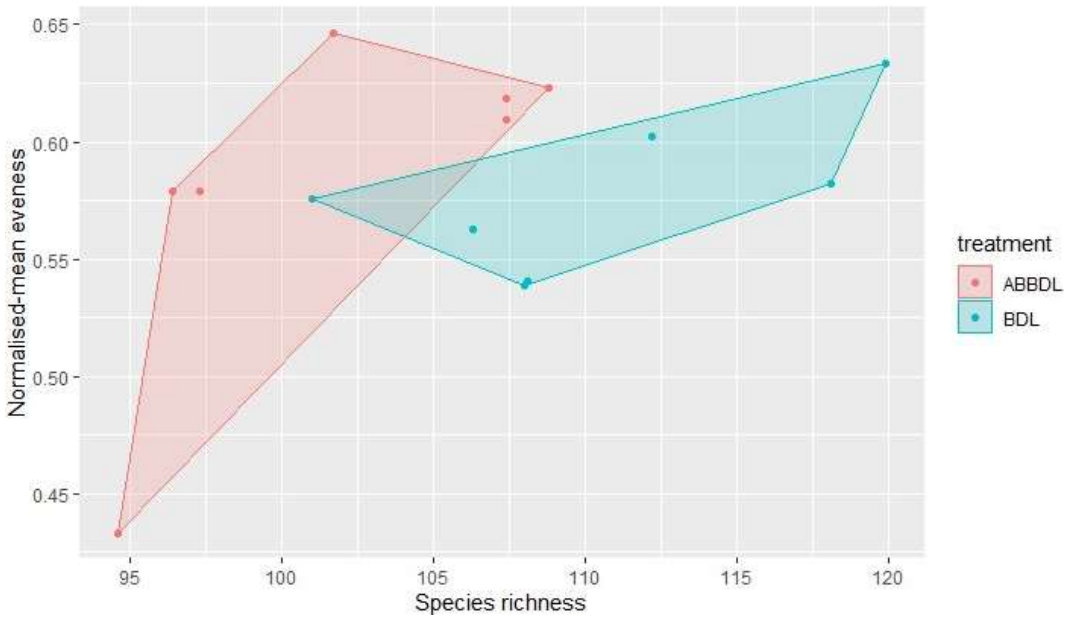
2.0
BDL
ABBDL
Treatment
treatment
ABBDL
BDL

C.


Normalised-mean eveness
0.65
0.60
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95
100
105
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Species richness
treatment
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BDL

D.

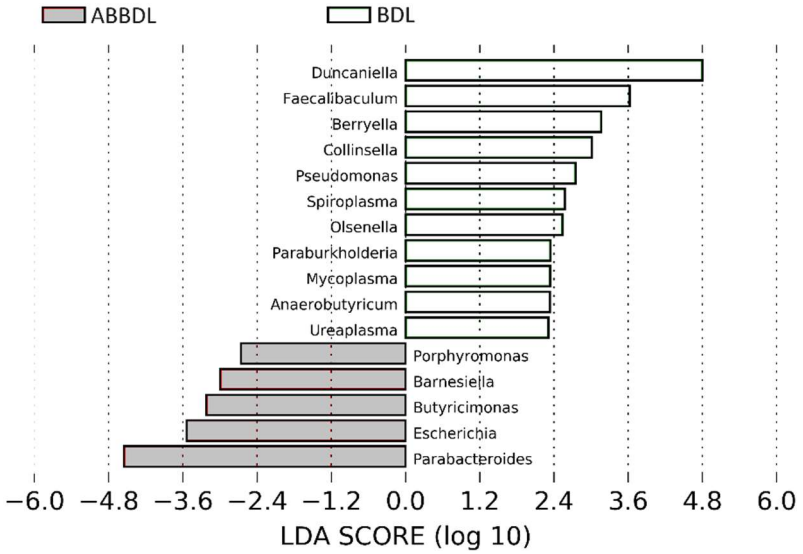

Figure 5.7 Comparative analysis of faecal bacterial population compositions between BDL and ABBDL aged mice analysed by shotgun metagenomics. *NMDS plot of β diversity (Bray-curtiss) demonstrates distinct clustering of Control, antibiotic recovered, BDL and antibiotic recovered BDL gut bacterial populations (A). Shannon index measurement showing no alteration in population diversity between BDL and antibiotic pre-treated BDL gut bacterial populations (P measured by Wilcoxson Rank-sum test) (B), with a general reduction in population richness (C). Linear discriminant analysis plot demonstrating differential enrichment of several key genera between BDL and antibiotic recovered BDL mice with a P value threshold of P>0.01 (D). This analysis has been conducted once.*

hepatic bile acid content of mice treated in youth is similarly unaffected following 6-month antibiotic recovery, though there is a significant decrease in TCDCA which is only present in trace quantities (Fig 5.8 A). Furthermore, in aged mice there is a significant increase in bile acid content following BDL, though antibiotic pretreatment does not impact total bile acid content regardless of recovery time. No significant differences are observed between untreated BDL and antibiotic pretreated BDL total hepatic bile acid content (Fig 5.8 B). This data demonstrates that antibiotic induced microbial population change has a limited impact upon hepatic bile acid content following BDL in aged mice, with aged, antibiotic pretreated, BDL mice displaying few to no significant differences in bile acid content regardless of the length of antibiotic recovery.

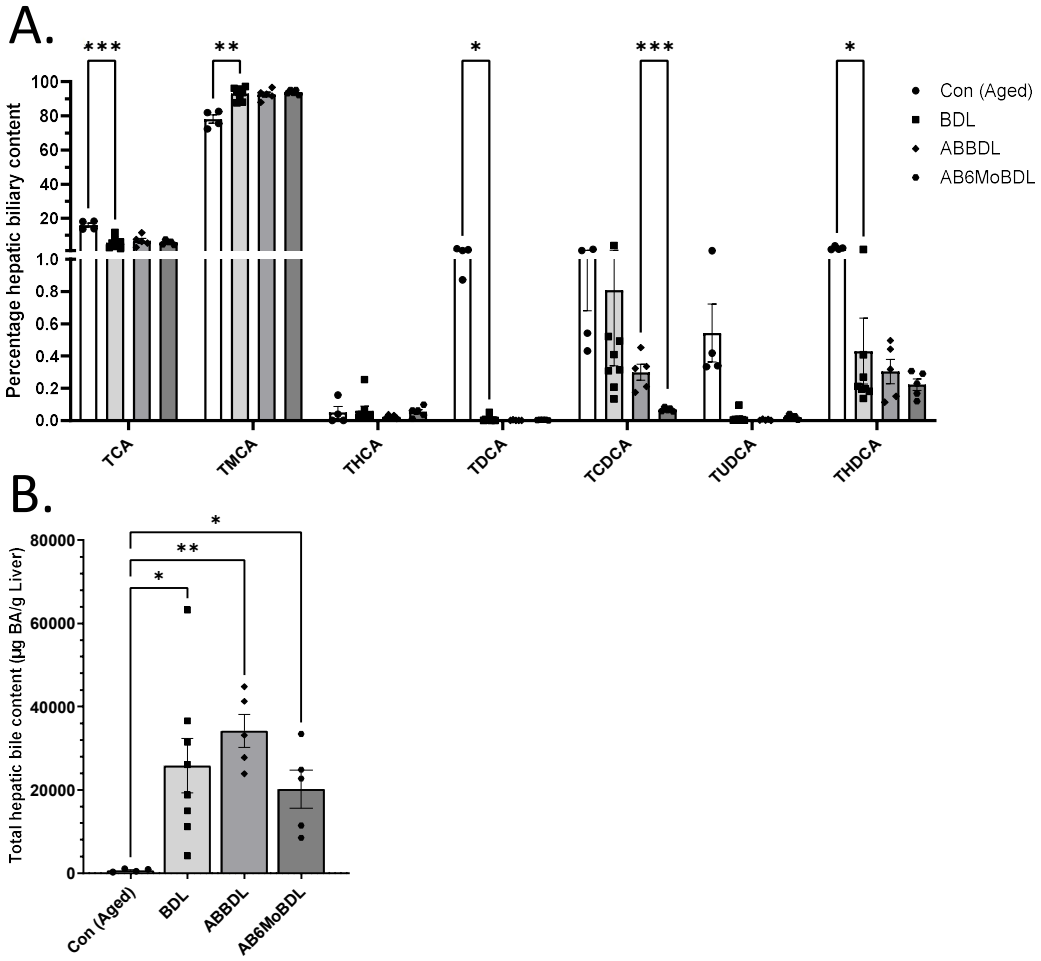

Figure 5.8 LC-MS quantification of hepatic biliary content in aged pre-treatment, BDL and (3-week and 6-month) antibiotic recovered BDL liver samples. *Graph showing bile acid content as a percentage of total hepatic biliary content (A). TMCA content is significantly increased following BDL, while TCA, TDCA and THDCA are significantly reduced (though they are only present in trace amounts), 3-week antibiotic recovered BDL (ABBDL) mice display no change to bile acid content from BDL only, while 6-month antibiotic recovered BDL (AB6MoBDL) mice display reduced TCDCA. Graph showing total hepatic bile acid content which is increased by BDL but unaffected by antibiotic pre-treatment. (B) * = P<0.05, ** = P<0.01, *** = P<0.001. Welch T-test. This experiment has been conducted once.*

5.3.7 BDL significantly impacts SCFA content with the effect of antibiotic pretreatment depending upon the length of microbial recovery time.

Following the analysis of hepatic bile acid content following BDL, I then sought to investigate the effect of cholestatic disease upon faecal SCFA content. SCFA content of faeces collected prior to antibiotic treatment and following the induction of cholestatic disease from untreated and antibiotic pretreated (3-week and 6-month recovered) aged

mice were analysed by LCMS. Analysis demonstrated that BDL alone significantly reduces the content of both acetate and propionate. Antibiotic pretreated, post-BDL faeces from 3-week recovered aged mice differs from untreated BDL mice only by a significant increase in isovalerate. On the other hand, faeces from 6-month recovered BDL mice displays a significant decrease in butyrate content when compared to ABBDL faeces and a large increase to isobutyrate in comparison to both untreated BDL and ABBDL faeces (Fig 5.9).

This data indicates that BDL produces some alterations to gut SCFA content in aged mice, with the effect of antibiotic induced microbial population change being dependent upon administration time and by extension, microbial recovery time. Mice treated in age display minor alterations to post-BDL SCFA content in the gut, whilst mice treated in youth post 6-months recovery display more significant alterations, particularly to butyrate content.

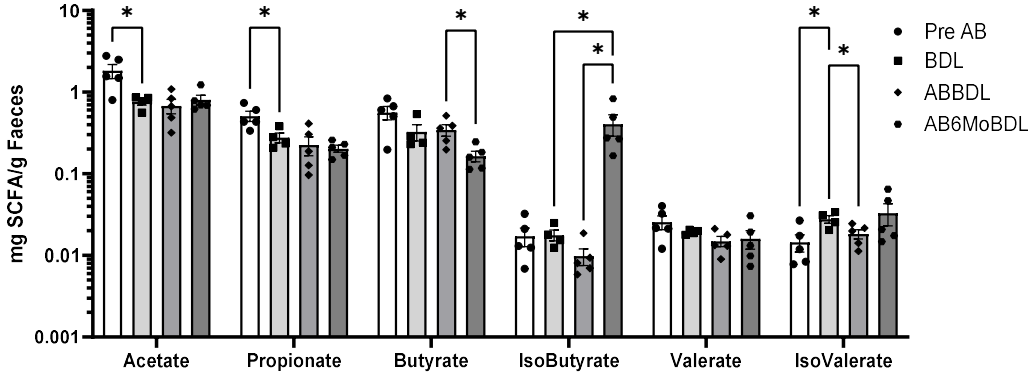

Figure 5.9 LC-MS quantification of the faecal SCFA content of aged pre-treatment, BDL and (3-week and 6-month) antibiotic recovered BDL mice. *Faecal acetate and propionate are reduced in BDL mice in comparison to pre-treatment mice. 3-Week antibiotic recovery prior to BDL (ABBDL) produces only a minor increase in isovalerate in comparison to BDL only. 6-Month antibiotic recovery prior to BDL (AB6MoBDL) produces a significant decrease in butyrate in comparison to ABBDL and a large and significant increase in isobutyrate in comparison to both BDL and ABBDL faeces . * = P<0.05, ** = P<0.01. Welch T-test. This experiment has been conducted once.*

5.3.8 Antibiotic pretreatment does not impact the progression of cholestatic disease in aged mice, regardless of the timespan of microbial recovery.

As we have seen in chapter 3, antibiotic pretreatment significantly impacts the progression of cholestatic disease in youth, exacerbating cholestatic fibrosis, whilst necrosis and the ductular reaction remained unaffected. Given the differences in the impact of antibiotic pretreatment in aged mice, I then sought to determine how these effects might impact the progression of cholestatic disease in aged mice. To investigate how cholestasis is impacted I first sought to determine the extent of cholestatic necrosis in untreated, 3-week and 6-month antibiotic recovered aged cholestatic mice. As previously, paraffin embedded liver samples were sectioned and H&E stained, the slides were then imaged, and necrotic areas manually quantified. Analysis revealed that antibiotic pretreatment had no effect upon the extent of cholestatic liver necrosis, regardless of the length of the microbial recovery period and age at which antibiotics were administered. There were no statistically significant differences observed between BDL, ABBDL or AB6MoBDL necrotic area (Fig 5.10).

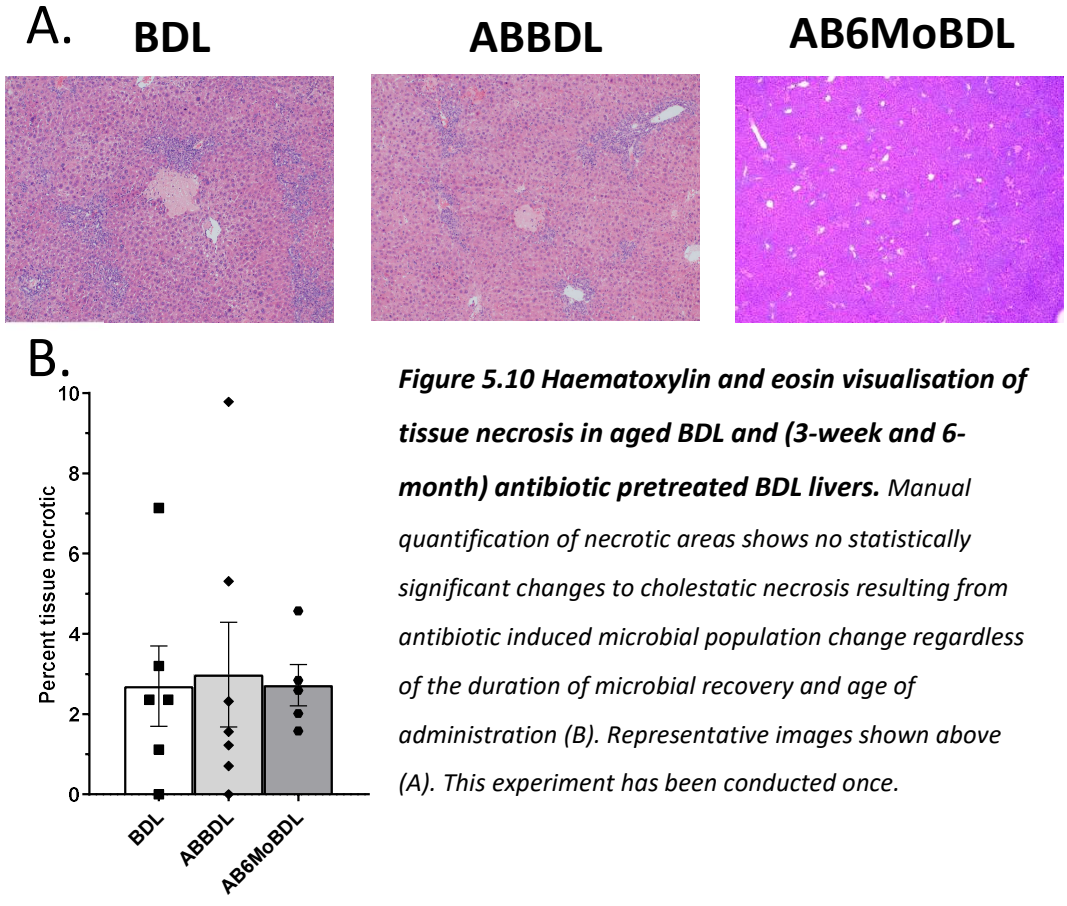

Figure 5.10 Haematoxylin and eosin visualisation of tissue necrosis in aged BDL and (3-week and 6-month) antibiotic pretreated BDL livers. *Manual quantification of necrotic areas shows no statistically significant changes to cholestatic necrosis resulting from antibiotic induced microbial population change regardless of the duration of microbial recovery and age of administration (B). Representative images shown above (A). This experiment has been conducted once.*

As a further measure of hepatic damage arising from cholestasis serum was isolated from whole blood and transaminase content measured using an automated serum chemistry analyser. Analysis demonstrated that ALT and AST were not significantly different, though in the case of 6-month antibiotic recovered BDL mice there was a notable increase in the content of both ALT and AST though neither were statistically significant (Fig 5.11).

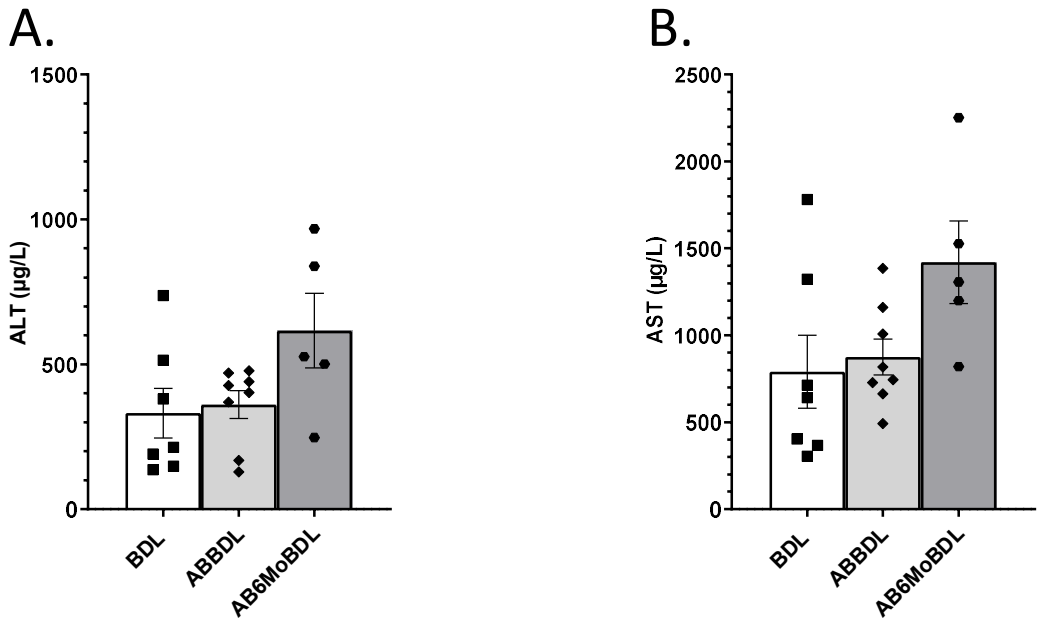

Figure 5.11 Serum transaminase quantification (Alanine transaminase and Aspartate transaminase) by RANDOX Daytona+ in aged BDL and (3-week and 6-Month) antibiotic recovered BDL mice. *There are no statistically significant differences in ALT levels (A) and AST levels (B) in aged BDL mice, though there are notable increases in ALT and AST in AB6MoBDL mice. Statistical significance measured by Welch T-test. This experiment has been conducted once.*

Having confirmed that hepatic damage was unaffected by antibiotic pretreatment in aged mice, I then sought to investigate the impact upon the ductular reaction, which as mentioned previously, is a proximal measure of the extent of cholestasis progression [150]. Paraffin embedded liver tissues were sectioned and immunohistochemically stained for CK19, imaged by brightfield microscopy and CK19 staining manually quantified. Analysis revealed that in aged mice antibiotic pretreatment had no effect upon the extent of the ductular reaction, regardless of the age of administration or length of microbial recovery time. No significant differences were observed between BDL and ABBDL or AB6MoBDL mice (Fig 5.12),however, there was a notable, though not statistically significant, reduction in CK19 staining in 6-month recovered samples when compared to both BDL only and ABBDL (3-week recovered) mice.

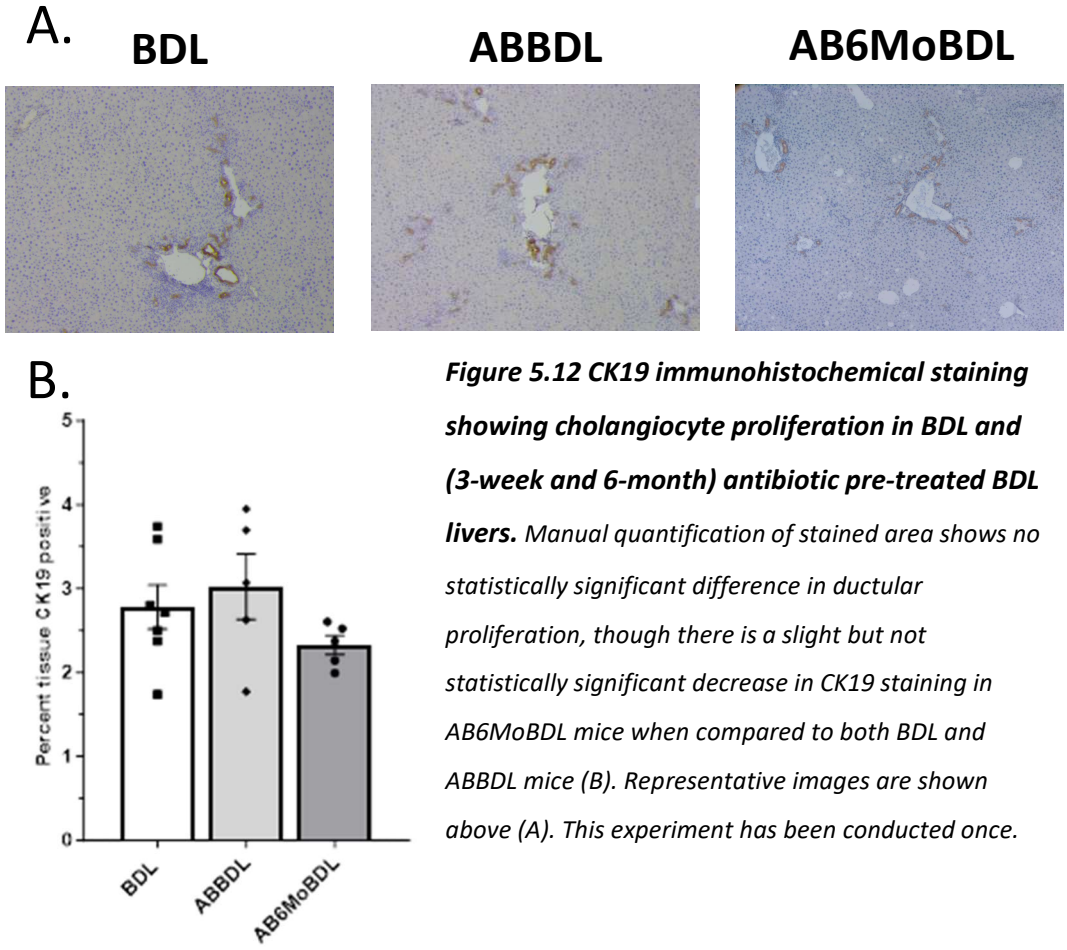

Figure 5.12 CK19 immunohistochemical staining showing cholangiocyte proliferation in BDL and (3-week and 6-month) antibiotic pre-treated BDL livers. *Manual quantification of stained area shows no statistically significant difference in ductular proliferation, though there is a slight but not statistically significant decrease in CK19 staining in AB6MoBDL mice when compared to both BDL and ABBDL mice (B). Representative images are shown above (A). This experiment has been conducted once.*

As a further analysis of the extent and severity of cholestasis in aged, antibiotic pretreated mice, I then analysed serum alkaline phosphatase and total bilirubin content. Serum alkaline phosphatase quantification being a routine measurement method of the extent and severity of cholestatic disease. Furthermore, total serum bilirubin indicates the extent of cholestasis by the leakage of bile into the bloodstream following buildup in the bile duct obstructed liver. Data showed that in the case of ALP, there are no significant differences in serum quantity, though there is a notable, statistically insignificant, increase in ALP content in 6-month antibiotic recovered animals (Fig 5.13 A). Total bilirubin is significantly increased in 6-month antibiotic recovered mice in comparison to BDL only, with a close to significant (P=0.0635) increase when compared to 3-week antibiotic recovered, aged, mice (Fig 5.13 B).

Having quantified the extent of both cholestatic necrosis and the ductular reaction I then analysed the effect of antibiotic pretreatment upon cholestatic fibrosis in aged

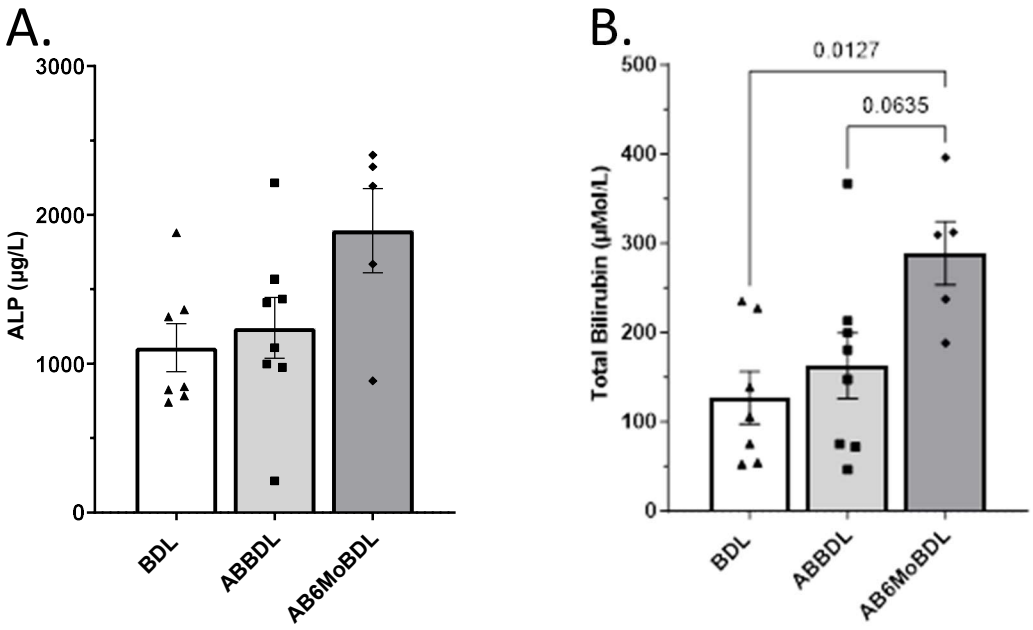

Figure 5.13 Serum Alkaline phosphatase and total bilirubin quantification. *ALP levels show no statistically significant differences between aged, untreated BDL and antibiotic recovered BDL mice though 6-month recovered mice display elevated serum ALP content, though not statistically significant. (A) Total bilirubin levels are significantly elevated in 6-month antibiotic recovered mice in comparison to BDL, with no significant difference observed between BDL and ABBDL mice (B). Significance measured by Brown-Forsyth and Welch ANOVA. This experiment has been conducted once.*

mice. Paraffin embedded liver tissue sections were sirius red stained as described previously and red stained fibrosis quantified manually by colour thresholding using ImageJ software. Analysis demonstrated that antibiotic pretreatment in aged mice does not affect the level of hepatic fibrosis arising from cholestatic disease, regardless of the age of antibiotic administration or the length of the microbial recovery period. There were no significant differences in fibrotic staining observed between BDL and either 3-week or 6-month antibiotic recovered mice (Fig 5.14).

Taken together, these data demonstrate that aged mice are more resilient to the exacerbations of cholestatic disease progression arising from antibiotic induced microbial population change observed in young mice previously in chapter 3. Furthermore, whilst the length of the microbial recovery period appears to somewhat affect necrosis, with a notable though not statistically significant increase from untreated BDL, and significantly increase total serum bilirubin, there is no alteration to cholestatic fibrosis or the ductular reaction. This suggests that the age of administration

and length of microbial recovery does not impact the effect of antibiotic induced microbial population change in aged mice.

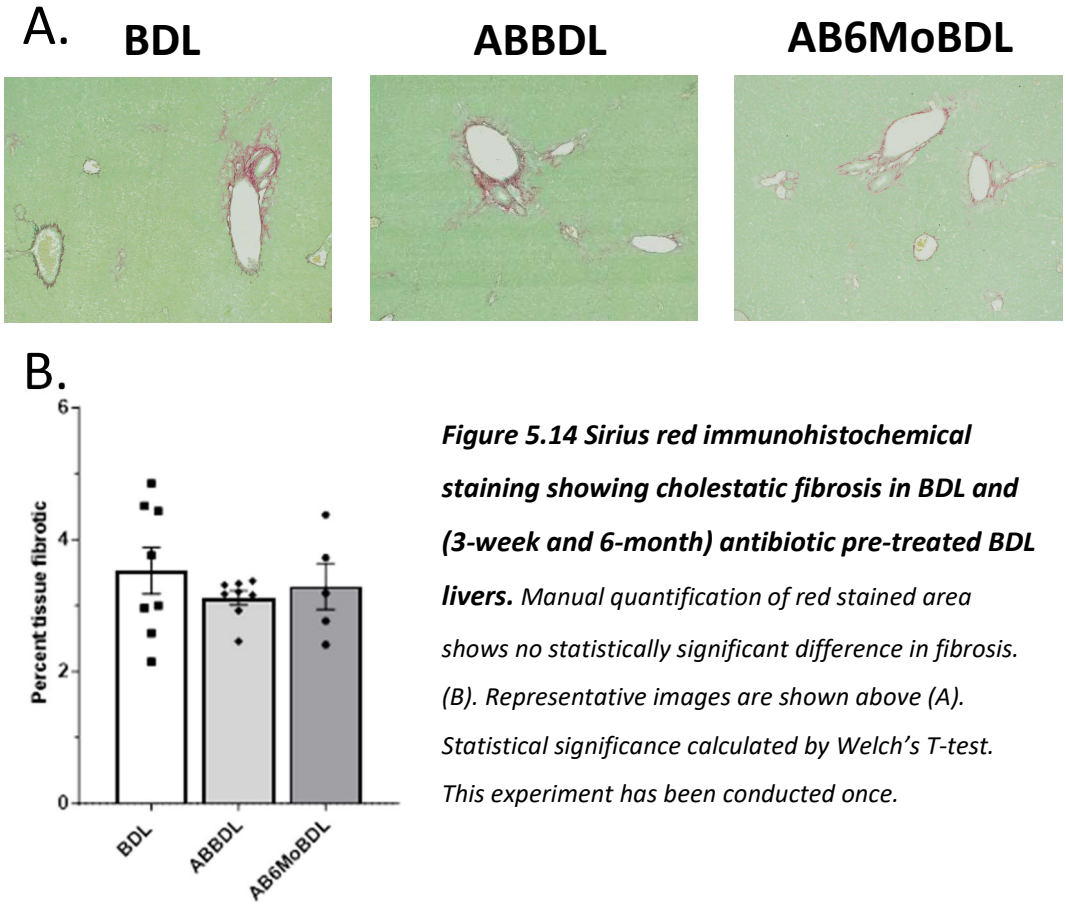

Figure 5.14 Sirius red immunohistochemical staining showing cholestatic fibrosis in BDL and (3-week and 6-month) antibiotic pre-treated BDL livers. *Manual quantification of red stained area shows no statistically significant difference in fibrosis. (B). Representative images are shown above (A). Statistical significance calculated by Welch's T-test. This experiment has been conducted once.*

5.3.9 Antibiotic pretreatment in aged mice reduces liver macrophage population prior to BDL.

In order to investigate the lack of worsened cholestatic disease progression in aged antibiotic pretreated mice, despite some antibiotic induced microbial population change, I then investigated the behaviour of macrophages post recovery from antibiotics. Liver immune cells were extracted, antibody stained and analysed by flow cytometry. Upon analysing macrophage populations prior to BDL it appeared that there was a slight, but not statistically significant, decrease in macrophage population in 3-week antibiotic recovered mice in comparison to untreated aged mice. On the other hand, 6-month antibiotic recovered mice display a significant reduction in macrophage population from control aged mice (Fig 5.15). This reduction was revealed to result from

a reduction in infiltrating monocytes, demonstrated by a significantly lower population of F480+, Ly6C & CD14 Hi cells in 6-month antibiotic recovered mice (Fig 5.16).

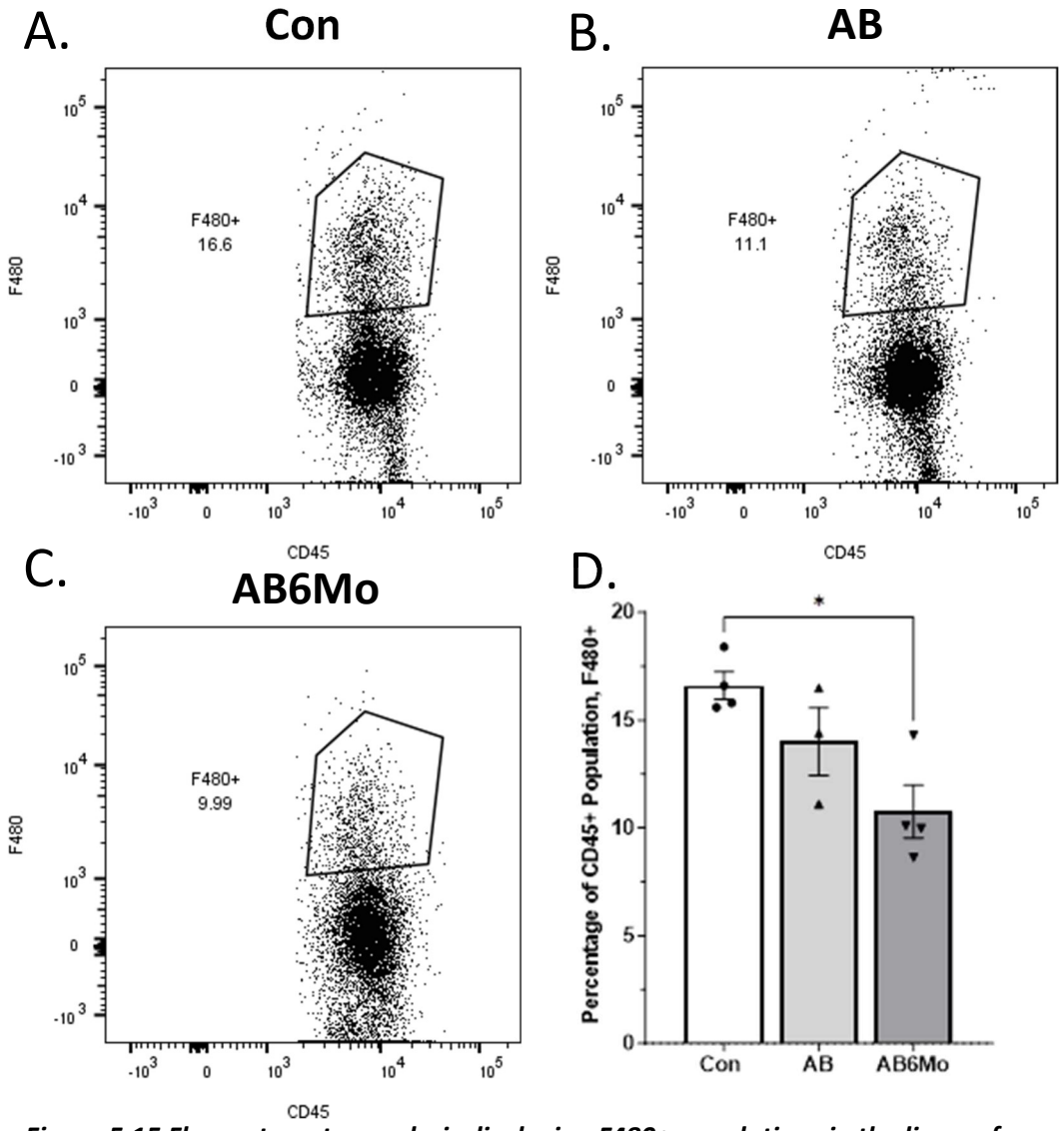

Figure 5.15 Flow cytometry analysis displaying F480+ populations in the livers of aged mice. *Flow cytometry plots showing F480+ cell populations in aged; Control (A), Antibiotic recovered (B) and 6-month antibiotic recovered (C) Livers. Graph showing F480+ cell population as a percentage of CD45+ cell population in the Liver (D). * = P<0.05 Brown-Forsyth & Welch ANOVA. Antibiotic pretreatment appears to reduce homeostatic liver macrophage populations in aged mice, whilst this reduction is not significant in 3-week recovered mice, there is a significant reduction shown in 6-month recovered mice. This experiment has been conducted once.*

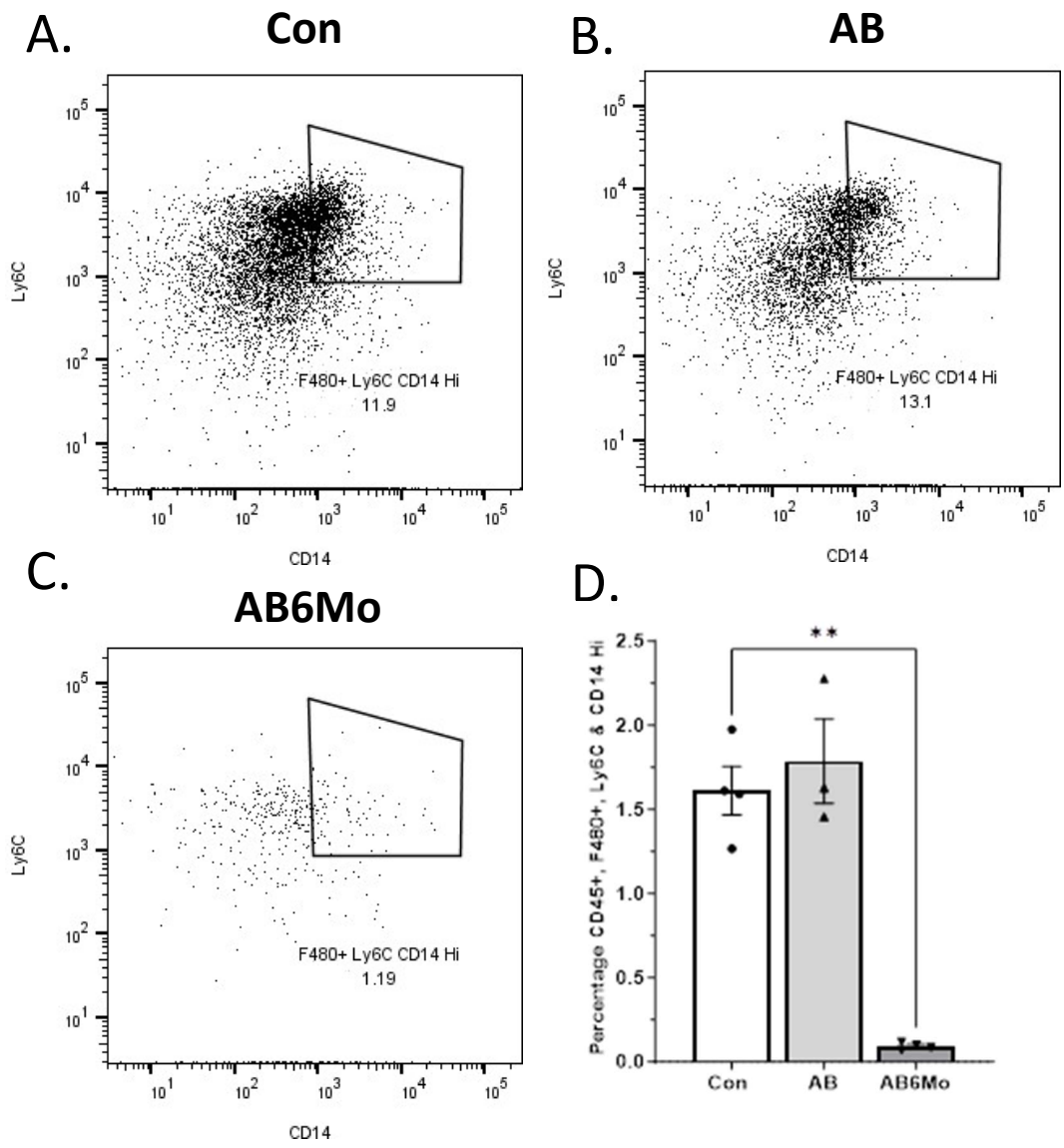

Figure 5.16 Flow cytometry analysis displaying F480+, CD14 & Ly6C Hi populations in the livers of aged mice. *Flow cytometry plots showing F480+, CD14 & Ly6C Hi cell populations in aged; Control (A), Antibiotic recovered (B) and 6-month antibiotic recovered (C) Livers. Graph showing F480+, CD14 & Ly6C Hi cell population as a percentage of CD45+ cell population in the Liver (D). ** = P<0.01 Brown-Forsyth & Welch ANOVA. Following 6-month antibiotic recovery, homeostatic infiltrating macrophage populations in aged mouse livers is reduced. This experiment has been conducted once.*

5.3.10 Antibiotic pretreatment has differential impacts upon macrophage populations dependent upon the duration of microbial recovery, post-BDL.

Given the alterations to immune cell populations prior to the induction of cholestasis in aged mice, I then investigated how these might be retained, or otherwise, following BDL.

Analysis of the flow cytometry data demonstrated that there is no significant difference in total F480+ cell population regardless of the duration of microbial recovery (Fig 5.17).

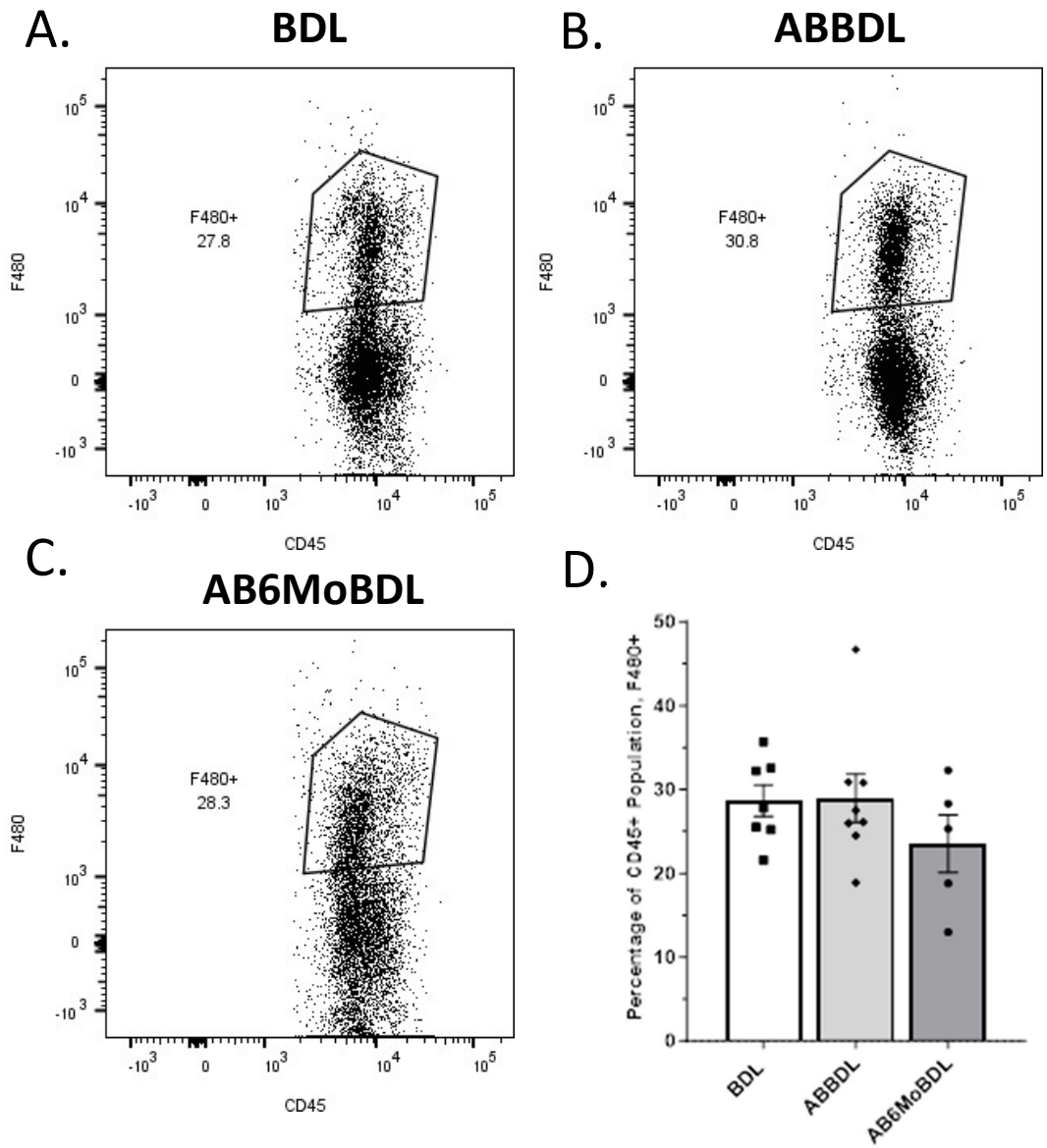

Figure 5.17 Flow cytometry analysis displaying F480+ populations in the livers of aged mice. Flow cytometry plots showing F480 populations in aged; untreated BDL (A), Antibiotic recovered (3w) BDL (B) and 6-month antibiotic recovered BDL (C) Livers. Graph showing F480+ cell population as a percentage of CD45+ cell population in the Liver (D). ** = P<0.01 Brown-Forsyth & Welch ANOVA. There is no significant change in hepatic F480+ population in aged mice following BDL given antibiotic pre-treatment, regardless of the duration of microbial recovery. This experiment has been conducted once.

However, there is a significantly reduced population of infiltrating macrophages (F480+, Ly6C & CD14 Hi cells) in 6-month antibiotic recovered BDL mice, suggesting an increased

resident macrophage population is responsible for the lack of change in F480+ cell population (Fig 5.18).

Overall, this flow cytometry data demonstrates that both in homeostasis and following BDL, the duration of microbial recovery determines the changes to innate immune cell populations. Furthermore, the lack of change in macrophage population following BDL demonstrates that aged mice are resistant to the reduction in macrophage infiltration wrought by antibiotic induced microbial population change in young mice following BDL. However, it must be noted that in 6-month antibiotic recovered mice there is a reduction in macrophage population derived from infiltrating monocytes, suggesting an increased liver resident macrophage population in 6-month antibiotic recovered mice.

5.3.11 Despite alterations to macrophage infiltration both pre and post-BDL in aged 6-month antibiotic pretreated mice, there is no change in the expression of key cytokines.

Having identified the changes in macrophage infiltration occurring in the antibiotic pretreated aged liver, I then investigated the alterations to cytokine expression in the liver. RNA was extracted from whole liver homogenate, cDNA was produced by reverse transcription and cytokine expression quantified by Rt-qPCR. Data indicated that in the cases of TNFα (Fig 5.19 A), IL1β (Fig 5.19 B) and IL6 (Fig 5.19 C) there is an increase in expression following BDL as would be expected, however, between untreated and antibiotic pretreated BDL aged mice, regardless of the duration of antibiotic recovery, there is no alteration to cytokine expression (Fig 5.19).

Combined with the previous flow cytometry data, this data demonstrates that the result of antibiotic induced microbial population change has a negligible impact upon liver immune function post BDL. This is despite a reduction in macrophage infiltration in 6-month antibiotic recovered BDL mice. This lack of altered immune function ultimately results in a lack of worsened cholestatic disease progression in antibiotic pretreated mice post-BDL, regardless of age at administration and the duration of microbial recovery from antibiotics.

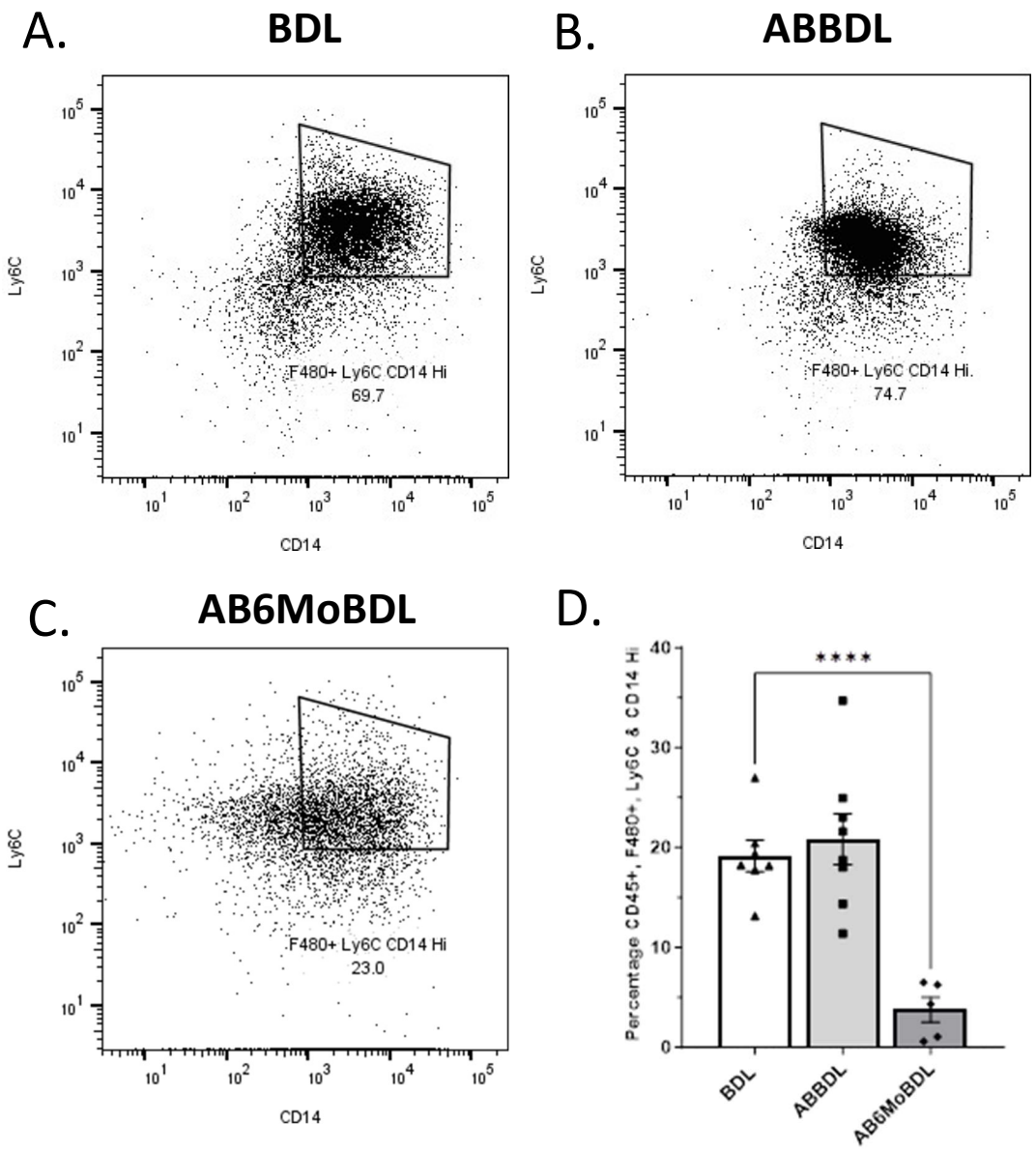

Figure 5.18 Flow cytometry analysis displaying F480+, CD14 & Ly6C Hi populations in the livers of aged mice. *Flow cytometry plots showing F480+, CD14 & Ly6C Hi populations in aged; untreated BDL (A), Antibiotic recovered (3w) BDL (B) and 6-month antibiotic recovered BDL (C) Livers. Graph showing F480+, CD14 & Ly6C Hi cell population as a percentage of CD45+ cell population in the Liver (D). **** = P<0.0001 Brown-Forsyth & Welch ANOVA. There is a no significant change in F480+, CD14 & Ly6C Hi cell population in 3-week antibiotic recovered aged BDL mice when compared to untreated BDL. In 6-Month antibiotic recovered BDL mice on the other hand, there is a significant reduction in macrophage infiltration. This experiment has been conducted once.*

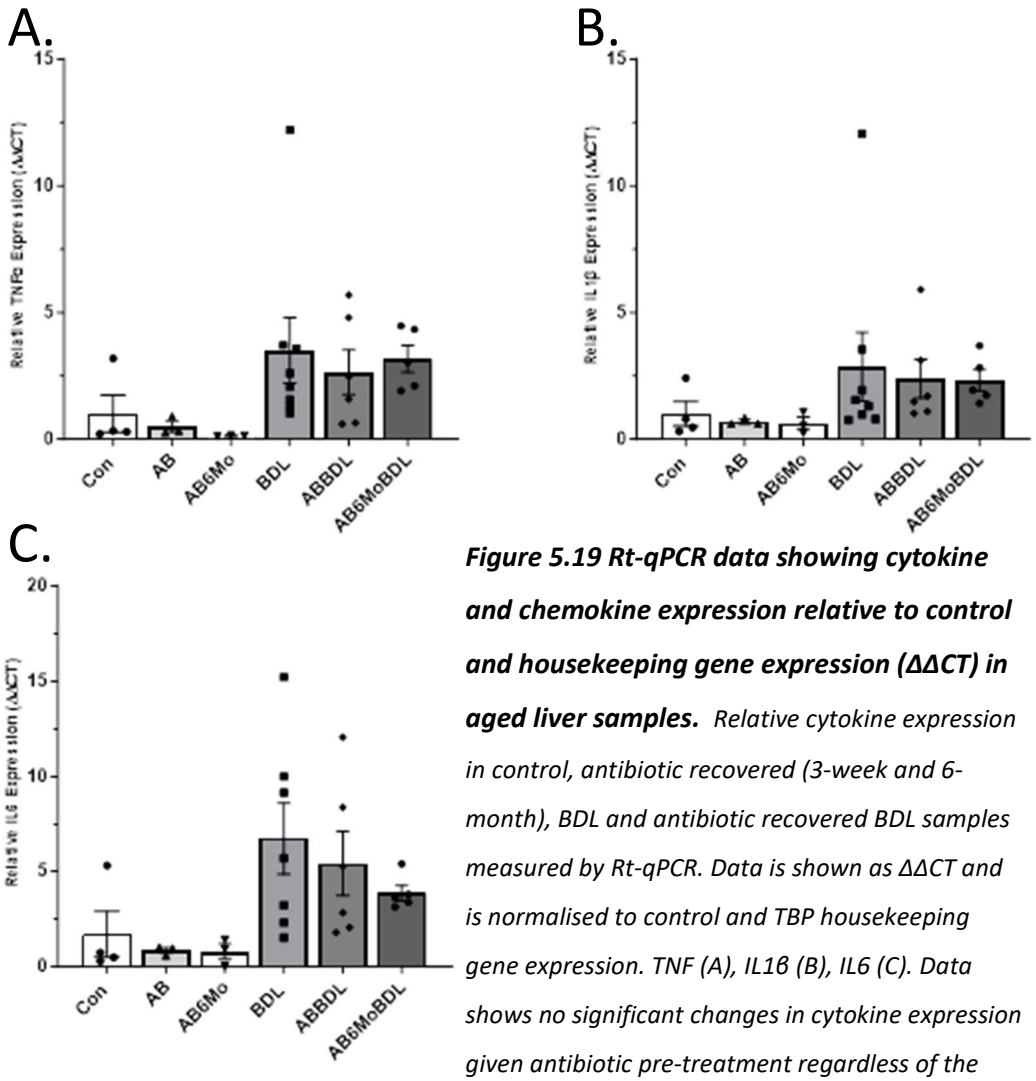

Figure 5.19 Rt-qPCR data showing cytokine and chemokine expression relative to control and housekeeping gene expression (ΔΔCT) in aged liver samples. *Relative cytokine expression in control, antibiotic recovered (3-week and 6-month), BDL and antibiotic recovered BDL samples measured by Rt-qPCR. Data is shown as ΔΔCT and is normalised to control and TBP housekeeping gene expression. TNF (A), IL1β (B), IL6 (C). Data shows no significant changes in cytokine expression given antibiotic pre-treatment regardless of the duration of microbial recovery. Significance measured by Brown-Forsyth and Welch ANOVA. This experiment has been conducted once.*

5.4 Discussion

This chapter sought to determine whether the microbiome of aged mice is sensitive to antibiotic induced microbial population change, the effect of this upon the progression of cholestasis and the mechanism by which any changes or otherwise might be driven by innate immune cell behaviour. We have observed in previous chapters that young mice are highly sensitive to antibiotic induced microbial population change, that the effects wrought by population change worsen cholestatic disease progression and drive increased pro-inflammatory macrophage activity and that these effects can be significantly ameliorated by reintroduction of *Bacteroides thetaiotaomicron*.

Given the observations of previous studies that the aged gut microbiota is more resilient to perturbation [20], I hypothesised that the antibiotic recovered microbiota of aged mice would be more similar to the pretreatment microbiota than in young mice. Furthermore, I reasoned that treatment in youth would generate perturbations which may be retained into age and generate effects upon cholestasis progression in age. Overall, I predicted that mice treated in age would be less sensitive to the effects of antibiotic induced microbial population change upon cholestatic disease and immune function.

The key observation of this chapter is that, in aged mice there is no alteration to cholestatic disease pathophysiology given antibiotic pretreatment regardless of the age of administration and duration of microbial recovery. There were no alterations to necrosis analysed by H&E staining quantification, the ductular reaction analysed by CK19 staining quantification of cholangiocyte proliferation, or fibrosis analysed by sirius red quantification. The effects upon the intestinal microbiota, metabolite production and innate immune cell behaviour which may contribute to the lack of worsened progression seen in young mice are explored below.

My initial investigation in this chapter was to determine the extent of antibiotic induced microbial population change in mice treated with antibiotics in age. Analysis demonstrated that, as with young mice, bacterial biomass returns to pretreatment levels, whilst population composition is significantly altered. As in young mice (discussed chapter 3), the genera *Bacteroides*, *Parabacteroides* and *Alistipes* are all significantly depleted following antibiotic recovery in aged mice. However, an additional noteworthy genus- *Akkermansia*- is depleted in aged mice.

Akkermansia's primary role in the gut microbiota is as a regulator of the thickness of the mucus layer [238]. *Akkermansia* degrades the mucus layer which stimulates increased mucus production [238]. The thickness of the mucus layer is correlated with the severity of various liver diseases [183]. *Akkermansia* abundance is increased in the faeces of patients suffering from alcohol induced steatohepatitis [239], though its abundance is negatively correlated with obesity and insulin insensitivity [240]. However, the role of mucus in alcoholic liver disease (ALD) is complex, as mucin deficiency (the primary glycoprotein component of mucus) is protective against Alcoholic induced hepatitis in mice [241], whilst increased mucus thickness results in improved liver function in ALD [242]. Increased mucus thickness is observed following BDL [243], though this has not yet been correlated with

worsened or improved disease. *Akkermansia* is a mucus degrading bacterium, the activity of which promotes increased mucus production, this role as a regulator suggests that depending upon abundance this bacterium could both negatively and positively correlate to mucus layer thickness. As such, disturbance to its abundance will have non-linear impacts upon mucus thickness and it is therefore difficult to predict the impact of antibiotic induced disturbance. In addition to its mucin degrading activity, *A.muciniphila* has also been shown to have anti-inflammatory and antifibrotic effects in the context of chemically induced liver fibrosis, reducing the expression of various pro-inflammatory cytokines and attenuating hepatic stellate cell activation [244]. Therefore, The depletion of this bacterium would be expected to have deleterious effects upon the progression of liver fibrosis. In addition to the depletion of *A.muciniphila* there was a significant expansion of the genus *Pseudomonas*. There is no mechanistic data on the role of *Pseudomonas* in cholestasis, though it has been noted to expand in both human PSC-IBD and PBC [245].

Following the investigation of pre-BDL microbial communities, I then investigated the post-BDL community which may impact upon the progression of cholestatic disease. However, many of the alterations to the composition of the intestinal microbiome seen pre-BDL are not seen between aged BDL and aged 3-week antibiotic recovered BDL mice. No differences in *Bacteroides*, *Akkermansia*, *Alistipes* or *Bacillus* were seen between untreated BDL and antibiotic pretreated BDL aged mice. In contrast, *Pseudomonas* population was reduced in antibiotic pretreated mice and *Parabacteroides* increased. Furthermore, the NMDS analysis demonstrated a decrease in dissimilarity between ABBDL and BDL mice, this data showed that, following BDL, the alterations to the microbiota in aged mice are limited in comparison to young mice. In addition, the increased *Parabacteroides* population seen in antibiotic recovered BDL mice is noteworthy as it is a noted beneficial genus as discussed in chapter 3. This may contribute to the lack of exacerbation of cholestatic disease in aged antibiotic pretreated BDL mice.

After having investigated the antibiotic induced microbial population change in aged mice, I then analysed the alterations to gut microbial metabolite production wrought from this population change. As with young mice, aged 3-week antibiotic recovered mice prior to BDL display an increased faecal content of the potent FXR antagonist TMCA and decreases in the content of both CA and DCA, which has no impact upon total faecal

bile acid content. On the other hand, 6-month antibiotic recovered mice show notable differences in the alteration to faecal bile acid content from previously observed differences wrought by antibiotic induced microbial population change. The faecal bile acid content of these mice displays a more moderate increase in TMCA from untreated mouse faecal bile acid content when compared to 3-week recovered aged mice. This increase appears to be moderated by concomitant increases in both α and βMCA, suggesting that in the 6-month recovery period there is an expansion of bacterial populations with bile salt hydrolase activity [51]. Furthermore, there is a greater reduction in DCA and a significant reduction in LCA and HDCA, each of which are potent FXR agonists as discussed in chapter 3 [79]. Unlike in 3-week recovered aged and young mice, these bile acid alterations are accompanied by a significant reduction in total faecal bile acid content.

This is more in-line with the expected outcome of reduced FXR activity as FXR is a repressor of bile acid synthesis which is remotely activated by intestinal FXR activity by FGF15 expression in the murine gut [204]. FGF15 is expressed in the murine gut in conditions of increased FXR activity and transported to the liver, where it binds with the membrane bound FGF receptor, the downstream effect of which is stimulation of FXR resulting in reduced bile acid synthesis [204]. This data suggests that, for disruption to bile acid synthesis to take place the alteration to the microbiome elicited by antibiotic induced microbial population change must be retained over a longer recovery period.

Whilst this is the case, the total hepatic bile acid content of the 6-month antibiotic recovered mice is unaffected. There are some alterations to specific bile acids in both 3-week and 6-month recovered mice, such as increased TMCA in 3-week recovered mice, decreased TDCA in both 3-week and 6-month antibiotic recovered mice and increased CA in 6-month recovered mice. These specific effects do not result in changes in total hepatic bile acid content, suggesting that biliary outflow in 6-month antibiotic recovered mice is in some way impeded, as decreased bile acid synthesis would result in total bile acid content in the liver also decreasing.

Following BDL, there are no significant changes in hepatic bile acid content between untreated and antibiotic pre-treated aged BDL mice. This is regardless of the duration of microbial recovery, excluding a decrease in TCDCA in 6-month recovered BDL mice which is only present in trace quantities.

In terms of SCFAs on the other hand, prior to BDL there are fewer and more limited changes both prior to and post-BDL in aged antibiotic treated mice in comparison to young mice. Aged, Pre-BDL, 3-week antibiotic recovered mice display slight though significant increases in both faecal butyrate and isobutyrate, while 6-month antibiotic recovered mice display reduced faecal propionate and valerate. Post-BDL, while 3-week antibiotic recovered BDL mice have only a minor decrease in isovalerate, 6-month antibiotic recovered BDL mice display a minor decrease in butyrate and a large and significant increase in isobutyrate.

Butyrate has a significant effect upon the progression of cholestasis [164,246] and is heavily impacted by antibiotic induced microbial population change in aged mice. It is a SCFA synthesised by many gut microbial residents by fermentation of indigestible dietary fibre as a metabolic byproduct [8,137]. It is utilised by the gut as an energy source [63]. Butyrate has many effects in various liver diseases, in NAFLD it is noted to restrict insulin insensitivity, inhibit cholesterol synthesis and fat accumulation and promote proper glucose homeostasis both in the liver and organism-wide [100]. In alcoholic liver disease it has a eubiotic effect in the gut, ameliorating the dysbiotic effect of alcohol consumption and in hepatocellular carcinoma it promotes the apoptosis of cancer cells and inhibits their proliferation [64]. In the context of cholestatic disease, butyrate restores intestinal epithelium integrity, has a eubiotic effect upon the gut microbiota and attenuates intestinal inflammation by reducing the expression of various pro-inflammatory cytokines, following BDL [246]. Additionally, in a model of FXR knock-out induced hepatitis, butyrate supplementation reduced the expression of pro-inflammatory cytokines, chemokines and profibrotic genes such as TIMPs [64]. Hence, increased butyrate and potentially isobutyrate content in aged antibiotic pretreated BDL mice may contribute to the attenuation of worsened cholestatic disease progression given antibiotic pretreatment in aged BDL mice as observed in young mice.

Finally, I investigated the infiltration of macrophages into the aged, antibiotic pretreated liver, both prior to and post-BDL. Macrophages are drivers of liver fibrosis, expressing many profibrotic cytokines and mediating the activity of liver specific myofibroblasts [126,127,133,184]. Unlike in young mice, where there was no overall alteration to the liver innate immune cell population prior to BDL and following antibiotic pretreatment (Figs 3.16 & 17), there were however, significant changes in the homeostatic liver population of macrophages in aged, antibiotic pretreated mice. Whilst aged, 3-week antibiotic

recovered mice display no significant alterations in macrophage population, 6-month antibiotic recovered mice display significantly reduced macrophage populations. This macrophage population decrease appears to be driven by reduced infiltration. The data suggests that following an extended period of antibiotic induced microbial population change there is an alteration to liver immune homeostasis, representing reduced macrophage infiltration. An effect which may represent a reduction in inflammatory stimulus within the liver.

Following BDL on the other hand there is no overall change in macrophage population, with untreated BDL, 3-week antibiotic and 6-month recovered BDL mouse livers showing no significant differences in F4/80+ cell populations. However, when investigating the origin of these cells we find that there is a significant reduction in Ly6C & CD14 Hi, F480+ cells in 6-month antibiotic recovered aged BDL mice. This suggests that macrophage infiltration occurs earlier in the disease time course in 6-month antibiotic recovered mice as the final macrophage population is equivalent to untreated BDL mice. This lack of alteration in macrophage infiltration results in no significant changes to inflammatory cytokine expression in aged BDL mice, regardless of the duration of microbial recovery.

5.5 Further work

The body of work presented in this chapter presents the case that aged mice are resistant to the effects of antibiotic induced microbial population change upon the progression of cholestatic disease. Furthermore, the results presented demonstrate that this effect may be mediated by the expansion of beneficial microbial genera following antibiotic recovery, demonstrated in 3-week recovered mice. This is combined with a lack of significant depletions in beneficial genera observed between antibiotic pretreated BDL and untreated BDL aged mice and an increase in butyrate content following antibiotic pretreatment.

Further work would initially focus upon the completion of the microbiota analysis in 6-month antibiotic recovered mice. By ascertaining the change in microbial composition that is retained over 6-months of microbial recovery, one may inform further study of extended microbial recovery periods prior to the induction of cholestasis.

Next, investigation would likely focus upon the effects of further ageing upon antibiotic induced microbial population change and subsequent cholestasis. Many of the negative changes associated with ageing affecting the stability and composition of the

microbiome occur in later life and cholestasis can present in humans at near any stage of development. As such, a study which investigated the effects of antibiotic induced microbial population change on superannuated mice may produce significant effects upon cholestatic disease progression. These may be comparable or in excess of those seen in young mice. To date, we have conducted one animal experiment investigating this in mice of one year of age and seen negligible effects upon the extent of cholestatic fibrosis (data not shown). The analysis of these samples is still ongoing, however depending upon the observed microbiome changes and other pathophysiological observations, study could progress to later ages. Additionally, the effects of extended microbial recovery durations could be explored, along with the age of administration. These exploratory studies would be used observe the retention, or otherwise, of the impacts of antibiotic induced microbial depletion into age.

Finally, a comparative analysis of the impact of antibiotic induced microbial depletion in young and aged mice could be undertaken. This would be used to compare the factors in aged mice which prevent the increased fibrosis resulting from antibiotic induced microbial population change, to those in young mice.

5.6 Conclusion

Overall, the results of this chapter demonstrate that the gut microbiota of aged, 3-week antibiotic recovered mice is more resilient to antibiotic induced microbial population change than that of young mice. This is demonstrated by fewer significant differences in specific microbial genera following antibiotic recovery and the restoration of a close to pretreatment population composition following BDL seen by Bray-Curtis dissimilarity. Furthermore, this results in more limited changes to both biliary and SCFA content between aged 3-week antibiotic recovered and untreated mice both pre and post-BDL. On-the other hand, 6-month antibiotic recovered mice display reduced total faecal bile acid content, suggesting that, over long periods of dysbiosis, greater changes are wrought to bile acid homeostasis, resulting in reduced bile acid outflow from the liver. Despite this, no changes in the pathophysiology of cholestasis are seen in either 3-week or 6-month antibiotic recovered mice, suggesting that the overall impact of antibiotic induced microbial population change is either negligible or are cumulatively equal in relation to cholestatic disease. To fully understand the effect of ageing upon antibiotic induced population change in BDL an understanding of the longer-term microbial population changes would need to be undertaken (I.e. sequencing of the 6-month

antibiotic recovered microbiota). Investigating the effects upon mice of greater age and different administration times would complete the investigation upon the impact of age upon antibiotic induced microbial population change and its effects in cholestatic disease.

Chapter Six.
Discussion

6.1 Thesis summary

This thesis presents the case that the remote impact of antibiotics has a deleterious effect upon the progression of cholestatic disease and that this effect is mediated by microbial population change following the recovery of bacterial biomass, which alters the metabolite output of the microbiota. I first demonstrated in young mice, that antibiotic induced microbial population change has a detrimental impact upon cholestatic disease progression, centring upon increased fibrosis and altered macrophage gene expression and infiltration. I then established that by reconstituting a metabolically functional and anti-inflammatory bacterial species (namely Bt), the worsened hepatic fibrosis seen following antibiotic induced microbial population change in young mice was ameliorated (Fig 6.A). Furthermore, data demonstrated that in aged mice the microbiome is more resistant to antibiotic induced microbial population change. I further demonstrated that this translates to fewer alterations to intestinal metabolite production and consequently no overall change in cholestatic disease progression. Finally, in the case of mice antibiotic treated in youth, then recovering for 6-months until 8-10 months of age, it was evident that this extended recovery period

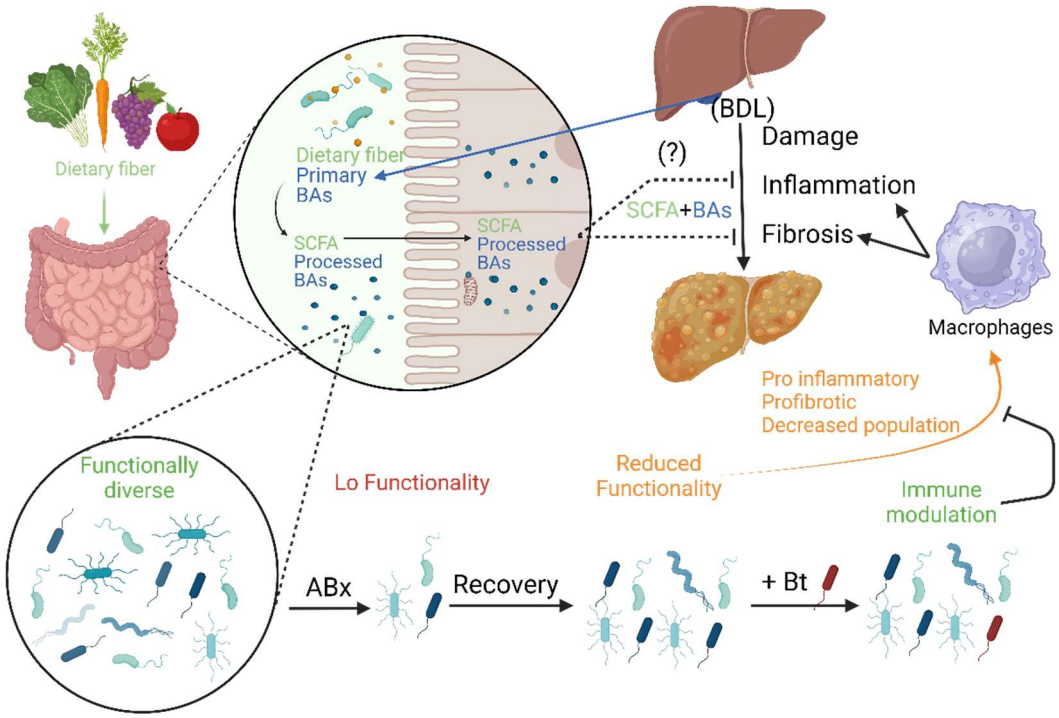

Figure 6.A. The Exacerbation of cholestatic liver injury mediated by antibiotic induced microbial population change in young mice and alleviated by Bt reconstitution.

resulted in greater changes in bile acid and immune homeostasis. This effect was likely produced by the extended retention of antibiotic induced microbial population change. However, these changes also did not result in changes to cholestatic liver disease progression in age.

6.2 How does this thesis relate to previously published literature?

Whilst, there has been no previous research into the long-term impact of antibiotic treatment upon cholestatic liver disease and very limited research in other liver diseases, the literature surrounding and adjacent to this research is extensive. The work presented here resembles the recent findings of both Schneider et al 2021 [103] and Awoniyi et al 2023 [164], who demonstrated that the immediate impacts of antibiotic induced microbial depletion worsened cholestatic disease progression. Schneider et al, demonstrated that impaired bile acid signalling and FXR agonism resulted in increased hepatic damage and bile acid cytotoxicity. Furthermore, they showed this was mediated by leakage of the increased hepatic bile acid volume from the peribiliary space into the parenchyma [103]. While Awoniyi et al showed that antibiotic treatment promoted intestinal dysbiosis, which contributed to increased intestinal permeability, increased hepatic inflammation, and worsened cholestatic disease progression. Furthermore, they demonstrated that in humans *Lachnospiraceae* population correlated to alleviated cholestatic disease progression, having a eubiotic effect upon the microbiome in cholestasis. They also showed that SCFA treatment alleviated the increased hepatic fibrosis of cholestatic disease resulting from antibiotic treatment when administered alongside vancomycin treatment [164]. Our work, whilst having a somewhat different focus to these studies- investigating antibiotic induced microbial population change, in lieu of the direct impact of antibiotics per se- demonstrates that some of the immediate impacts of antibiotic treatment upon disease progression are retained following the recovery of microbial biomass.

It must be noted however, that both of the previously mentioned studies used a transgenic model of murine cholestasis- namely the MDR 2-/- model, which is a spontaneously progressive model of cholestatic liver disease [181,247]. This model is used extensively as a cholestatic mouse model as the development and progression of disease closely resembles that in humans. It also allows for the organism-wide alterations to homeostasis in accordance with progressive cholestasis disease onset, such as intestinal microbiome changes, immunological responses and metabolic

disturbances [247]. It must be admitted that this is, perhaps, a more representative model of physiological disease progression than the BDL model, however, it limits the ability to specifically study long-term antibiotic induced alterations to the microbiome. This is because the gut microbial community remains in flux following antibiotic withdrawal, owing to the progressive nature of disease onset in the MDR2 -/- model. The BDL model has many additional benefits, the first being that cholestasis can be induced at a specified time following antibiotic treatment. The second being that the microbiota changes induced following BDL closely resemble those seen in human PSC [183]. And the third being that it rapidly induces a strong cholangiocyte proliferative, necrotic and profibrotic response following surgery, making it an effective and rapid model for investigating the damage and fibrosis arising from cholestasis [181].

Two recent studies have however, studied the immediate effects of antibiotic treatment in the BDL mouse model. The first of which, by Labiano et al 2022 [248], investigated the role of TREM2 in cholestatic liver disease induced by BDL. They do not conduct a direct comparison between wild type and antibiotic treated wild type BDL mice, however they demonstrate that antibiotic treatment abrogates the worsened inflammation and cholangiocyte proliferation elicited following BDL in TREM2 -/- mice [248]. The second of these studies, from Zhou et al 2023 [165], investigates the immediate impact of antibiotics in BDL mice. This study more closely resembles the results seen in MDR2 -/- mice, with increased serum transaminases and liver damage following BDL. Interestingly, they discover a negative correlation between *Bacteroidaceae* and serum ALT. Although they do not investigate cholestatic fibrosis, it is interesting that they identify a link between Bacteroides and cholestatic damage. As mentioned previously, the study identifies alterations to lipid metabolism which could bear relevance to future work conducted on the basis of the findings of this thesis (see chapter 3 discussion). Finally, they demonstrate that antibiotic induced microbial depletion exacerbates the expression of pro-inflammatory cytokines arising during cholestatic liver disease. Whilst we do not demonstrate this, the increased pro-inflammatory polarisation of antibiotic recovered macrophages suggests that some of these effects may be retained and localised to the peribiliary space. The data presented here demonstrates that macrophages accumulate in the antibiotic recovered cholestatic liver, despite overall reduced macrophage infiltration [165].

My work, whilst distinct from these studies, builds upon these findings by demonstrating that antibiotics administered at a time remote from the development of cholestatic liver

disease impacts upon the progression of the disease. And it furthermore demonstrates that this is driven by the lasting changes to the gut microbiota and homeostasis, in a similar fashion to that seen in other studies immediately after antibiotic treatment. Whilst we do not observe exacerbated hepatic damage, the worsened disease progression we observe is similar that shown by Awoniyi et al. Moreover, the correlations between specific microbial genera, metabolite depletion and cholestatic disease severity, that we see following an extended recovery period, demonstrate that the impact is more owing to microbial population change than to antibiotics per se.

Two previous studies have however, shown contradictory findings. The studies by Schrumpf et al 2017 [249] and Ma et al 2018 [250] both utilised transgenic mouse models of progressive biliary inflammation of autoimmunity, Schrumpf et al using the NOD.c3.c4 model of immune driven cholestasis, which develops disease with reduced fibrosis [251]. Ma et al using the dnTGFβRII model, a generalised autoimmunity model in mice, which develops progressive cholestasis resembling human PBC. Both studies agreed that the microbiota is heavily affected by disease onset (both show significant depletions in *Bacteroidetes*), that this dysbiosis combined with other inflammatory effects promotes intestinal permeability and that this permeability promoted increased gut-liver bacterial translocation, which exacerbated liver injury [249,250]. Each study then showed that treatment with antibiotics reduced cholestatic disease severity. The Schrumpf study showed reduced biliary infarcts and liver weight with ampicillin and neomycin administered until sacrifice [249], whilst the Ma study demonstrated reduced T and B cell mediated autoimmunity and infiltration following ampicillin, neomycin and metronidazole treatment throughout life[250].

These models of cholestatic disease are distinct from both the BDL and MDR2 -/- model of cholestasis. They involve immune-mediated induction of biliary atresia and degradation, more closely relating to PBC and are intermediated by alterations to immune cell behaviour [181]. Furthermore, they both cite reductions in the effects of bacterial translocation upon cholestatic liver disease as the mechanism by which disease severity is alleviated. These studies both deal with the direct impact of bacteria upon the severity of cholestatic disease, through translocation and the elicited inflammation and hepatic damage. Whereas the studies cited earlier, showing worsened cholestasis severity, investigate the impact of alterations to the network of interactions between the gut and the liver. This does not make these studies more or less valid in fact, in many ways, the autoimmune model may be a more accurate representation of the induction

of cholestasis in humans. However, what is demonstrated is that based both on disease aetiology, effect upon the microbiome and the regimen of antibiotic exposure, disparate disease outcomes can be observed. Furthermore, as I have displayed in this thesis, antibiotic induced microbial population change does not alter the degree of intestinal permeability arising from cholestasis and as such the observed alleviations to cholestasis in the two prior studies may not be applicable to this work.

There is currently some controversy in the field over the impact of antibiotics in human cholestatic liver disease [252] as the outcome of antibiotic treatment in human disease is somewhat disparate from those seen in the mouse model. In human patients, results are somewhat contradictory, but they have demonstrated that antibiotics can have a positive impact upon the progression of cholestatic liver disease. Several studies have indicated that vancomycin treatment alleviates serum liver damage markers and mayo risk score [162,163]. However, a recent retrospective study somewhat refuted these findings showing no overall change in disease severity given vancomycin treatment [253].

Studies have demonstrated that age plays a large role in determining susceptibility to and the progression of cholestatic liver disease [232,233]. However, this work demonstrates that due to the differential outcome of antibiotic induced microbial population change in aged mice, there is a lack of worsened disease progression given antibiotic pretreatment in these mice.

Overall, this thesis opens a new avenue of investigation in this field, previous work has focused upon how antibiotic treatment up to and including the time of disease onset, i.e. in a treatment setting, may impact upon disease progression. By demonstrating that antibiotics have the potential to impact cholestatic liver disease through long-term alterations to population composition, we have shed further light upon how preconditioning prior to disease onset impacts the course of cholestatic liver disease.

6.3 Implications and future work

The body of work I have presented bears an evident implication upon the treatment and progression of cholestatic liver disease. The observation that antibiotic induced dysbiosis impacts upon the progression of cholestatic liver disease at a time remote from administration prompts consideration from healthcare providers as to effective treatment. To date there is no curative treatment for cholestatic liver disease and the current treatments are used to reduce symptoms to prevent discomfort (such as

rifampicin to alleviate pruritus [109,112]), or to slow disease progression (such as in the case of exogenous UDCA [109,112]).

The foremost consideration may be in the context of familial cholestasis as the disease has shown some sign of heritability [219]. By considering the risk of developing cholestasis in later life, pro or prebiotics could be administered in an informed manner either coincident with, or post, antibiotic administration. Considering the observed benefits of the genus *Bacteroides* and particularly the species Bt for example, analysis could be conducted upon faecal samples post-antibiotic treatment and changes from the theoretical ideal population could be remedied by probiotic treatment. Furthermore, exogenous SCFAs- particularly propionate (whose loss is highlighted consistently in this thesis and may correlate to worsened cholestatic liver disease)- or FXR agonists could be administered to compensate for the loss of the bacteria from which they are sourced in the intestinal microbiota. This treatment may be especially pertinent in spontaneously arising disease, where antibiotics will be administered to prevent bacterial sepsis.

Once the future work outlined in the chapter specific discussion has been completed, the studies which may arise from the work outlined in this thesis would first focus upon broadening the number of models used to simulate cholestatic disease. As has been mentioned previously, the BDL model is primarily used to investigate the hepatic pathophysiology arising from cholestatic liver disease as it is a non-progressive, surgical model of disease. By diversifying the number of models used (the most evident and relevant likely being the MDR2 -/- model) we can investigate the effects in a progressive model, more representative of the development of human disease. This would allow for the changes in the microbiome associated with the early, sub-clinical stages of disease progression to be investigated alongside antibiotic induced dysbiosis. Replicating the results we have observed here would bolster the credibility of the results and make them more translatable to the context of human disease. Focussing on the transgenic, autoimmune models of cholestasis outlined above, the alterations to immune function seen to result from antibiotic pretreatment in this thesis become more relevant. These effects may have a significant bearing on the outcome of the disease in these models and upon PBC, which they primarily model.

Following this, additional work would likely then move to informed use of individual antibiotics which deplete the beneficial genera or species of bacteria seen in this thesis and future studies. This is more relevant to clinical antibiotic use and again makes the

results more relevant to human disease. For example, in the context of *Bacteroides* or Bt, antibiotics targeting gram negative bacteria would have a more severe impact and would be more likely to impact cholestatic disease.

Finally, though the study would be highly intensive given the low incidence of cholestatic liver disease, a human cohort analysis could be conducted whereby human patients could be grouped based on their most recent antibiotic treatment- i.e. duration from antibiotic administration to disease presentation and class administered- and the severity of disease analysed. Given the large number of confounding factors inherent in such a study (such as disease aetiology, age, sex, diet, geographic location etc.) a very large cohort would be required. Hence, alternatively, a faecal microbiota study could be conducted, informed by antibiotic treatment history, and a bidirectional correlation analysis performed. This would aim to correlate gut microbial population composition to disease severity on the one hand and to antibiotic treatment on the other. Results may then be used to imply a connection between antibiotic treatment and cholestatic disease severity in humans.

6.4 Conclusion

The work presented in this thesis demonstrates that antibiotic induced microbial population change has the potential to exacerbate cholestatic liver disease. This exacerbation appears to be mediated by depletions of specific metabolically active and anti-inflammatory taxa. These depletions may then impact upon macrophage responses to cholestatic liver disease, through gut-microbial metabolite changes and promote fibrosis. Reconstitution of *Bacteroides thetaiotaomicron*, as one such metabolically active, anti-inflammatory species, rescues the worsened pathophysiology of cholestasis in young mice. Furthermore, my results demonstrate that aged mice are more resilient to antibiotic induced microbial population change and its effects upon cholestatic liver disease. They also show that, whilst a longer term of microbial recovery has a larger impact upon homeostasis, it fails to significantly affect cholestatic pathophysiology. This work provides a new approach to investigating the impact of antibiotics upon liver disease and suggests many avenues of future study. Finally, this study presents many considerations for administering antibiotics in an informed manner to retain beneficial taxa and essential metabolites, particularly in the context of familial disease.

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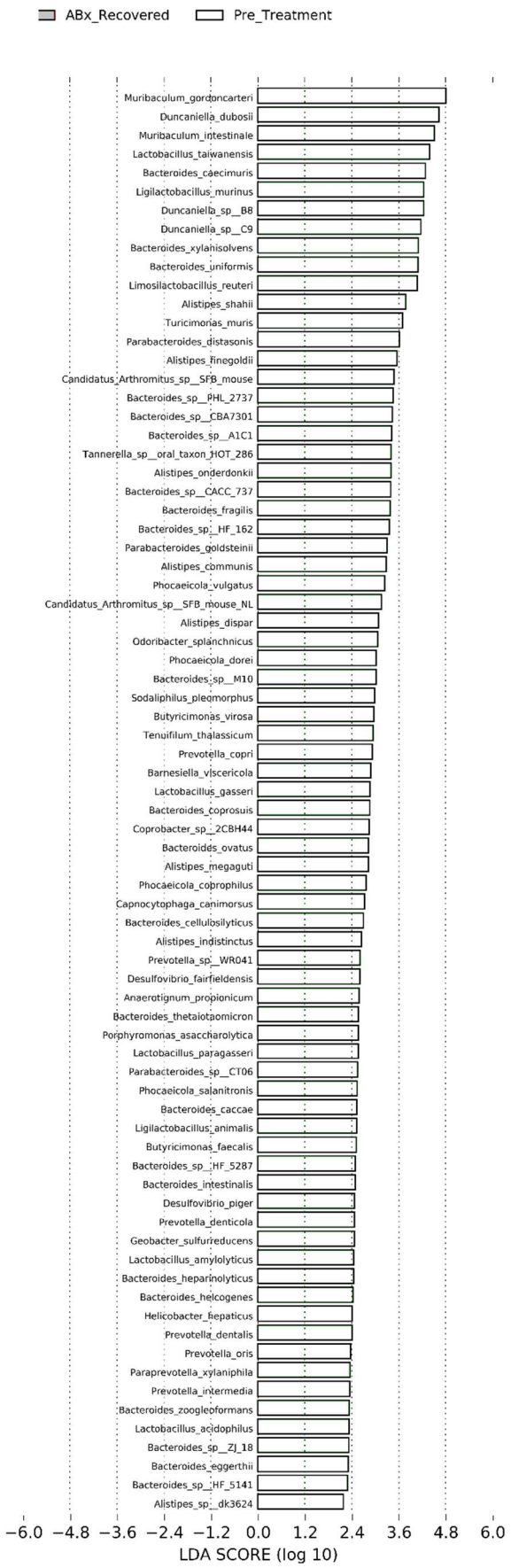
Supplemental figures



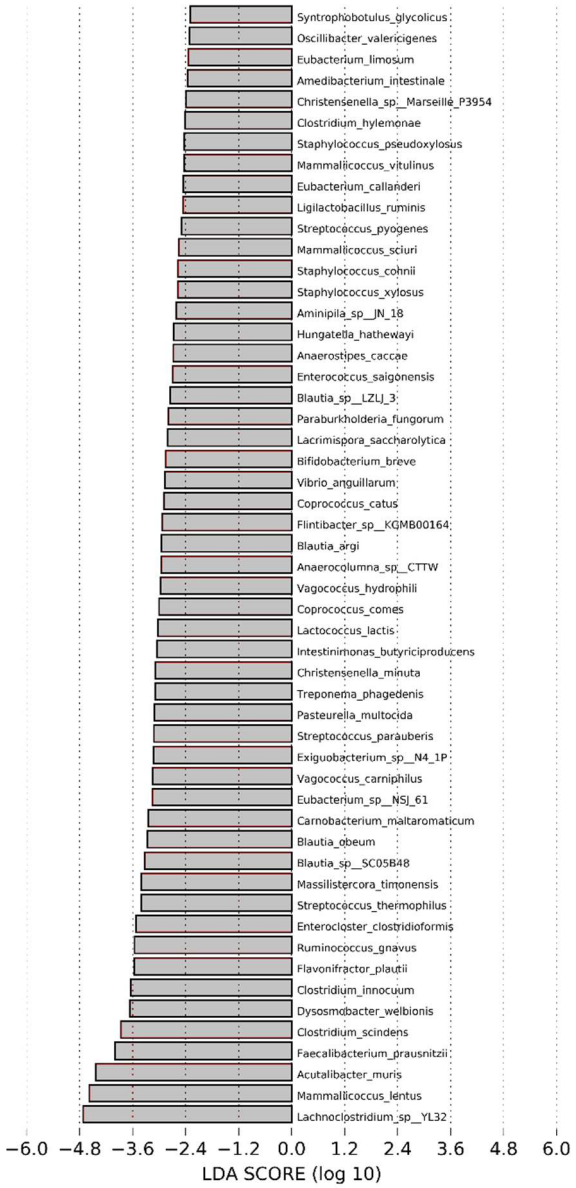


Figure S.1 Comparative analysis of faecal bacterial population compositions by species throughout antibiotic treatment and recovery in young mice analysed by LEfSe . *Linear discriminant analysis plot demonstrating differential enrichment of several key species between pre-treatment and antibiotic recovered mice, significance value threshold P<0.01.*

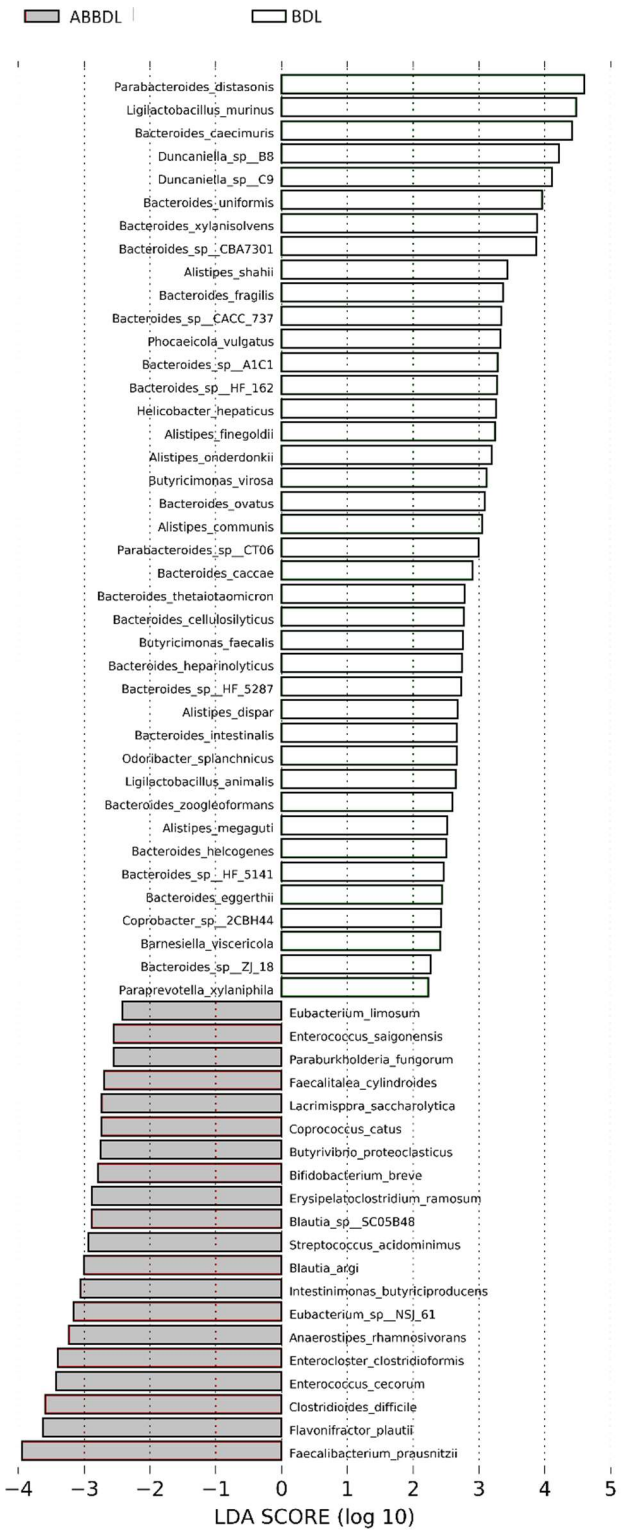


Figure S.2 Comparative analysis of faecal bacterial population compositions by species Between BDL and ABBDL young mice analysed by LEfSe . *Linear discriminant analysis plot demonstrating differential enrichment of several key species between BDL and antibiotic recovered BDL (ABBDL) young mice, significance value threshold P<0.01.*

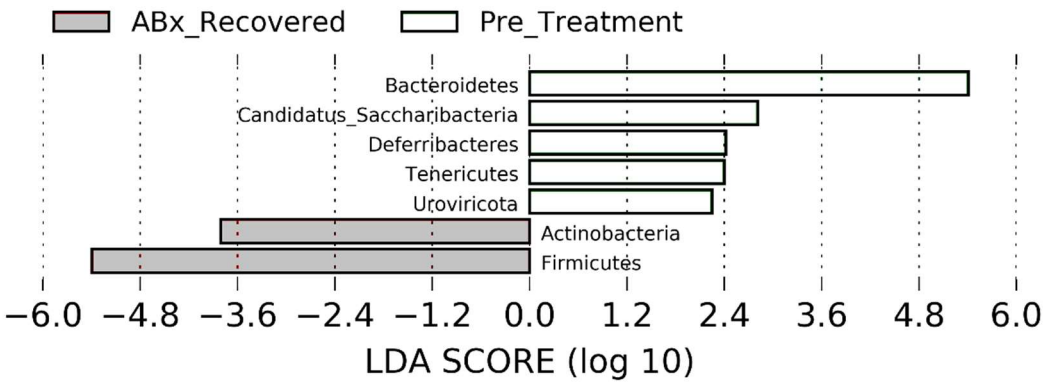


Figure S.3 Comparative analysis of faecal bacterial population compositions by Phylum throughout antibiotic treatment and recovery in young mice analysed by LEfSe. *Linear discriminant analysis plot demonstrating differential enrichment of several key phyla between BDL and antibiotic recovered BDL (ABBDL) young mice, significance value threshold P<0.01.*

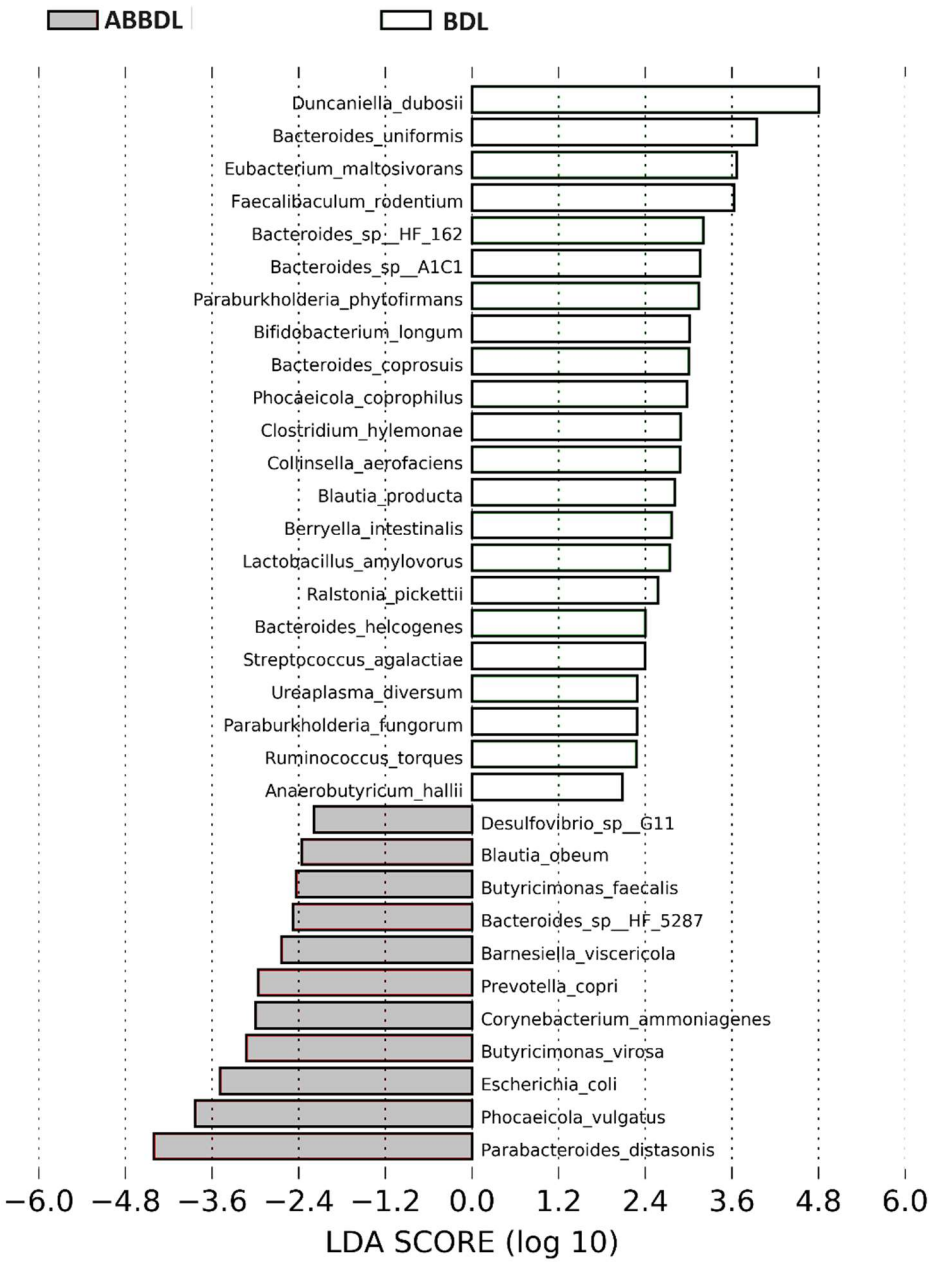


Figure S.4 Comparative analysis of faecal bacterial population compositions by genus throughout antibiotic treatment and recovery in aged mice analysed by LEfSe. *Linear discriminant analysis plot demonstrating differential enrichment of several key phyla between BDL and antibiotic recovered BDL (ABBDL) aged mice, significance value threshold P<0.01.*