A microbiological assessment of the human biliary tract in health and disease

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Abstract

The gastrointestinal tract in humans is home to 100 trillion bacteria, collectively referred to as the gut microbiota. This 'bacterial organ' has a vital role to play in both health and disease. Conventional wisdom dictates that bile in normal biliary systems is sterile. However, the liver is continually exposed to gut bacteria and their metabolites via the portal vein. Recent studies have identified bacterial populations within the biliary system of symptomatic patients undergoing cholecystectomy or biliary intervention. In this study we identified that there is a complex biliary microbiota within a normal biliary tract.

Many bacterial species were isolated from the bile of patients undergoing hepatic resection or cholecystectomy and their identity established through sequencing their 16S ribosomal RNA gene. These included *Staphylococcus, Micrococcus, Enterococcus and Bacillus sp.* Isolated bacteria were examined for their resistance to bile salts and the results suggested that all the isolates were able to survive under physiologically relevant bile concentrations with some isolates expressing bile salt hydrolase activity. An in-depth analysis of the biliary microbiome using 16S-based metataxonomics was performed. Results suggested that human bile has a diverse and varied microbiota, a large proportion of which were unculturable. 34 different genera were identified with *Pseudomonas* being the most prevalent. Dysbiosis was noted between diseased (e.g. gallstone and biliary obstruction) and normal samples.

The gut microbiota of the two most common chronic biliary conditions, Primary Sclerosing Cholangits and Primary Biliary Cholangitis, were also examined to see if dysbiosis was present. There is an emerging dysbiosis in patients with chronic cholestatic liver disease, although these results were possibly restricted through patient treatment with ursodeoxycholic acid.

This study is the first to describe a complex biliary microbiota in normal human bile and in the future a detailed understanding of the function of this microbiota may provide a therapeutic target for biliary disease.

Declaration

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

An abstract for the oral presentation of Chapter 3 and 4 is published in Journal of Hepatology, supplement 1, vol 66, pg65

In line with the regulations for the degree of Doctor of Medicine I have submitted a thesis that has a word count, including footnotes and bibliography, but excluding appendices of 36 993 words

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Oral Presentations

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Abbreviations

BHI	Brain Heart Infusion media
BSH	Bile Salt Hydrolase
CA	Cholic Acid
CBD	Common Bile Duct
CD	Crohn's Disease
CDCA	Chenodeoxycholic Acid
CRC	Colorectal Cancer
DCA	Deoxycholic Acid
DNA	Deoxyribose Nucleic Acid
ERCP	Endoscopic Retrograde Cholangiopancreatography
FMT	Faecal Microbiota Transplantation
GB	Gall Bladder
GCDCA	Glycochenodeoxycholic Acis
GI	Gastrointestinal
GOJ	Gastro-oesophageal junction
GWAS	Genome Wide Association Scan
HCC	Hepatocellular carcinoma
HDL	High density lipoprotein
HRS	Hepatorenal Syndrome
IBD	Inflammatory Bowel Disease
IBS	Irritable Bowel syndrome
LB	Luria-Bertani Media
LCA	Lithocholic Acid
LPS	Lipopolysaccharide
MRS	de Man, Rogosa and Sharpe media
NASH	Non-alcoholic Steatohepatitis

NGS	Next Generation Sequencing
OTU	Operational Taxonomic Unit
OCA	Obeticholic acid
PBC	Primary Biliary Cholangitis
PBS	Phosphate Buffered Saline
PCOA	Principal Component Analysis
PCR	Polymerase Chain Reaction
PSC	Primary Sclerosing Cholangitis
QIIME	Quantitative insights into microbial ecology
RA	Rheumatoid Arthritis
RNA	Ribonucleic acid
rRNA	ribosomal ribonucleic acid
TBE	Tris Borate EDTA buffer
TLR	Toll Like Receptor
UC	Ulcerative Colitis
UDCA	Ursodeoxycholic acid
UK	United Kingdom
VLDL	Very low density lipoprotein

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1 Introduction

1.1 The gut microbiome in health and disease

1.1.1 Microbiome in health

Over the past decade, the number of publications relating to the human microbiota has exponentially risen (Figure 1-1). Early research was reliant on culture dependant techniques, which had limitations as many of the bacteria resident in the gut are unculturable. However recent developments in metagenomic techniques such as high throughput sequencing and 16S rRNA based micro-arrays have allowed us to have a full understanding of the vast range of gut bacteria. Developments in metabolomics and proteomic techniques have allowed us to gain insight into the way that gene expression and microbial proteins shape the way the human intestine works. (Ahmad and Akbar, 2016)
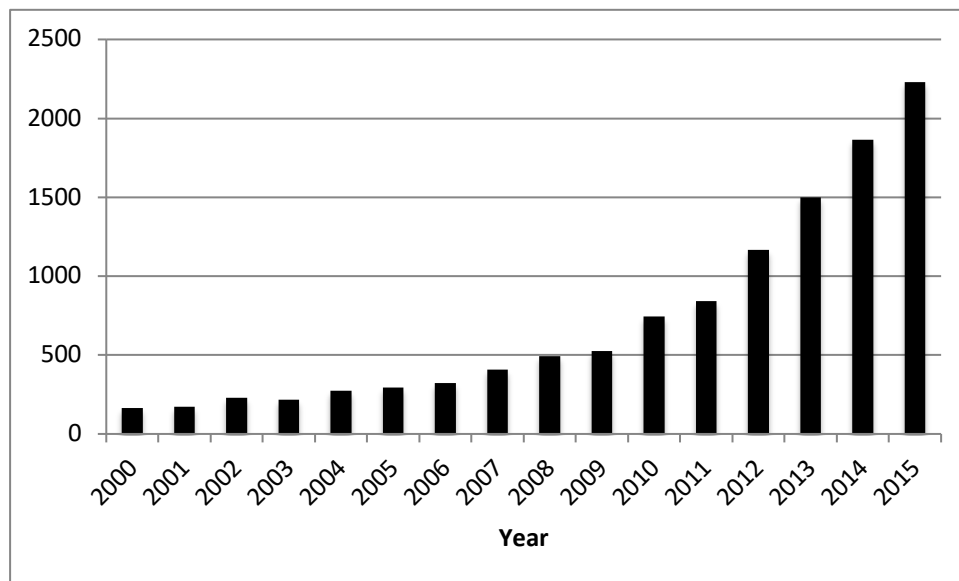
Figure 1-1 Number of publications relating to microbiota since 2000. Data obtained by searching PubMed by year with the following terms: intestinal microbiota, gut microbiota, intestinal flora, gut flora, intestinal microflora and gut microflora.

The gastrointestinal tract in humans is home to 100 trillion bacteria with at least 1000 different bacterial species (Shanahan, 2012). The number of bacterial cells increases throughout the GI tract, rising from 10^1 per gram of contents in the stomach to 10^{12} cells in the colon (Sekirov et al., 2010). These bacteria contribute 1.5 – 2Kg to total body weight (Tojo et al., 2014). The neonatal gut is thought to be sterile at birth. However, a microbiota similar to that of an adult is readily established throughout the first year of life. Factors that influence the colonization of the neonatal good include mode of delivery, diet and feeding, sanitation and exposure to antibiotics. Once established the microbiota remains static with changes due to environmental factors and medications often being transient. Indeed, there is some evidence that suggests alteration in the development of the microbiota in early life may have a vital role in disease expression in the future. The microbiota then remains stable until old age, where changes in diet and digestive physiology are thought to lead to changes in the microbiota composition.
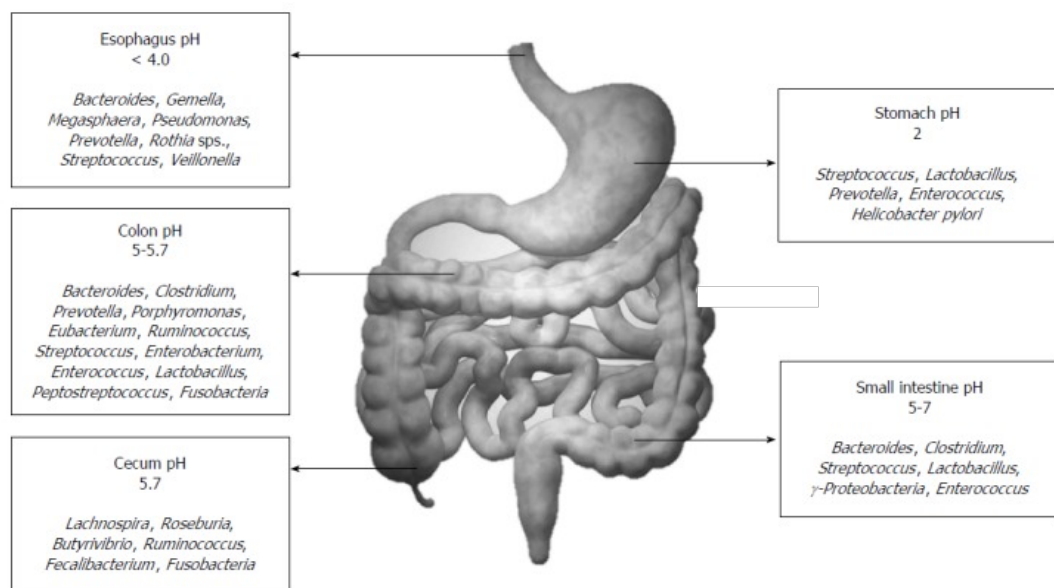
Figure 1-2 Distribution of the normal human gut flora. Taken from Jandhyala SM 2015

The majority of the microbiota is made up of bacteria with eukaryotes and viruses also contributing. Although there are more 1000 phylotypes at species-level (Tojo et al., 2014) there are 2 dominant phyla that make up 90% of all phylotypes in the human gut; Bacteriodetes and Firmicutes. However, there is variability in the prevalence of bacterial groups throughout the intestine (Sekirov et al., 2010) (See figure 1.2) and great variability at species level between individuals. As more is understood about the gut microbiota, three main clusters, or enterotypes, have emerged which span individuals and countries (see Table 1-1). Each of these

enterotypes have specific mechanisms in which to gain energy from the human colon. These enterotypes do not appear to be influenced by diet, age or BMI (Arumugam et al., 2011).

Enterotype	Predominant Genera	Energy Source
1	Bacteroides Parabacteroides	Carbohydrate and Protein degradation
2	Prevotella Desulfovibrio	Mucin desulfation and degradation
3	Ruminococcus Akkermansia	Mucin degradation and simple sugar uptake

Table 1-1 Main 3 enterotypes in the human colon

To date there has been limited study into the microbiota of the duodenum. This is in part due to the difficulty in obtaining samples in comparison to the colon (Wang et al. 2013). However, the duodenum is an important part of the gastro-intestinal tract with regards to this thesis as it has a close relationship with the biliary system with bile draining directly into it. It lies at a strategic crossroads between the acid-secreting stomach and nutrient absorbing jejunum/ileum with the duodenal microbiota being implicated in diseases such as small bowel bacterial overgrowth, coeliac disease and IBS (Li et al. 2015). Li et al discovered that the bacterial diversity of duodenal biopsies was as high as rectal biopsies with the predominant phyla being Proteobacteria and dominant genera being *Acinetobacter, Prevotella* and *Streptococcus* (Li et al. 2015).

The microbiome and host form a symbiotic relationship, with the gut providing a stable environment for proliferation with a plentiful supply of growth substrates through host diet. In return the microbiota provides energy sources through fermentation and production of essential vitamins and amino acids, breaks down indigestible food and provides a barrier against invasive pathogenic bacteria. (Le Gall et al., 2011; Liu et al., 2013).

Bacteria rely on undigested carbohydrates from the upper gastro-intestinal tract in order to survive. These complex polysaccharides are broken down by bacteria into short chain fatty acids including acetate, which other bacteria use as a nutrient source, butyrate and propionate, which the host can use as an energy source. (Rowland et al., 2017; Laparra and Sanz, 2010).

The gut commensals Bacteroidetes, *Propionibacterium,* Clostridia, Streptococci, Staphylococci and *Bacillus* have all been shown to have proteolytic properties in faecal samples, converting ingested dietary and endogenous protein into shorter peptides, amino acids and short chain fatty acids for use by the host (Macfarlane et al., 1986).

The essential vitamins that can be synthesised by the gut microbiota include vitamin K and the B group vitamins including biotin, cobalamin, folates, nicotinic acid, pantothenic acid, pyridoxine, riboflavin and thiamine (Hill, 1997). Many different bacteria are able to synthesise vitamins, with the vast majority of microbes from the phyla Bacteroidetes, Fusobacteria and Proteobacteria possessing the necessary pathways (Magnúsdóttir et al., 2015). Bacteroidetes appears to have the greatest range of vitamin production as well as having the strongest proteolytic properties (Rowland et al., 2017), which may explain why deficiencies in this phylum are most associated with disease.

The gut microbiota plays a role in protection against invasion by pathogenic bacteria. This is particularly evident in the small intestine where the mucus layer that protects the epithelium is discontinuous (Jandhyala et al, 2015). The inter-play between the microbiota and gut mucosal immune system is a complex one, as the gut needs to be tolerant of beneficial commensals whilst also being able to resist bacterial pathogens. The ability of gut to differentiate between pathogens and commensals is mediated through pattern recognition receptors, in particular toll-like receptors (TLR) (Valentini et al, 2014). TLR are activated by microbe-associated molecular patterns expressed by resident microbiota and result in the activation of signalling pathways resulting in production of cytokines, chemokine and transcription factors essential for mucosal barrier function and preventing infection (Valentini et al. 2014, Jandhyala et al. 2015). Two species that have been identified as having key roles in antimicrobial protection are *Bacteroides thetaiotaomicron* and *Lactobacillus innocua* (Hooper et al. 2003)*. B. thetaiotaomicron* is able to induce pathways which result in the cleavage of prodefensin to defensin, a cationic antimicrobial peptide which have broad spectrum antimicrobial activities against most pathogens (Xie et al. 2014). In contrast *Lactobacillus* species can directly aid the antimicrobial mechanisms of the host through production of lactic acid that aids the activity of the host lysozyme by disrupting the outer membrane of the bacterial cell wall of invading pathogens (Alakomi et el. 2000).

The microbiota clearly has a vital role in the development of the host immune system. This is demonstrated by mice studies where germ free mice have underdeveloped mucosal and systemic immune systems. Interestingly these deficiencies can be corrected through colonization with commensal bacteria (Liu et al., 2013).

1.1.2 Microbiome in gastrointestinal disease

Manipulation of the human microbiota in order to prevent or cure disease is not a new concept. *Clostridium difficile* was first discovered as an opportunistic pathogen following antibiotic therapy in the 1970's (George et al., 1978). 20 years later eradication of *Helicobacter pylori* was shown to have a long-term effect on the prevention of peptic ulcer disease (Forbes et al., 1994). It is clear that bacteria have an important role to play in the health of the human gastro-intestinal tract. Despite this it is only fairly recently that treatment aimed at restoring the normal microbiota, through faecal microbial transplantation (FMT), has become a recognised management strategy for relapse and remitting *Clostridium difficile* infection (CDI) (Borody et al., 2014). The success of FMT and the understanding that the human microbiota has a role to play in homeostasis and gut health has led to huge developments in the role of this "bacterial organ" and disease.

1.1.2.1 *Inflammatory Bowel Disease (IBD) and the microbiome*

Inflammatory bowel disease is a chronic idiopathic inflammatory condition with two main sub-types; Crohn's disease (CD) and ulcerative colitis. The exact pathogenesis of IBD remains unclear. It is clear that there is a definite genetic precipitant; the highest risk of development of IBD is seen in monozygotic twins and the discovery of NOD2 as a susceptibility gene for Crohn's disease in 2001 was just the first step in understanding the genetics of IBD with 163 high risk loci being identified through GWAS studies (Jostins et al., 2012). However, this alone does not explain the variance in incidence and disease prognosis. Environmental factors such as smoking, drugs and pollutants also clearly play a role but again are unable to explain the progression of disease. Evidence is beginning to mount that IBD results from an abnormal immune response to microbial stimulation in a genetically susceptible person. (Abegunde et al., 2016).

Changes to the commensal bacteria are well established in Crohn's disease with a reduced biodiversity described in both Western (Manichanh et al., 2006) and Eastern populations (Liu et al., 2012) characterised by a reduction in Firmicutes and Bacteroidetes and an abundance of Proteobacteria. The fact that these changes span different populations suggests that the microbiota probably play as important a role as environmental and genetic factors. Even when environmental factors such as smoking, alcohol intake, disease stage and treatments such as antibiotics and anti-inflammatories are excluded these changes appear to persist. *Kaakoush et al* (2012) studied the faecal microbiota of 19 newly diagnosed Crohn's patients prior to treatment versus 22 age-matched controls. Again, they demonstrated a reduction in Firmicutes*, in particular *Ruminococcaceae,* and a marked increase in *proteobacteriae*. They also demonstrated a non-significant increase in *Bacteroidetes.* As well as a reduction in biodiversity there is also a shift towards an inflammatory-promoting microbiome. Two examples that epitomise this are an abundance of sulphide generating *Desulfovibrio* species in UC (Rowan et al., 2010) and a reduction in the species *Faecalibacterium prausnitzii (Sokol et al., 2008*; *Sokol, Seksik et al., 2009)* that has been shown to have anti-inflammatory properties both *in vivo* and *in vitro* for colitis models. *Esherichia Coli* has repeatedly been reported in association with ileal CD (Tojo et al., 2014).

Surgery is a commonplace consequence *of* medical therapy in IBD with up to 80% of Crohn's patients requiring surgical resection (Ng and Kamm, 2008). Even before the current knowledge of microbiota, it has long been suspected that contact between the intestinal mucosa and faecal matter may have a role to play in disease recurrence post-operatively (D'Haens et al., 1998). It is now clear that surgery has a major impact on the intestinal microbiota and this may have an effect on prognosis and recurrence. Mice undergoing an ileo-colonic resection show a sustained and significant loss of microbial diversity in the colon. Interestingly, the bacterial populations between of luminal contents sampled from the jejunum and colon were nearly identical between individiuals 4 weeks post-surgery having been diverse prior.   The predominant effects seen are reduction in the phyla Bacteroidetes with expansion of the minor phyla Proteobacteria and Deferribacteres (Devine et al., 2013).

It is therefore possible that IBD is a result of invasive pathogen infection, subsequent poor clearance and recognition of said pathogen due to a dysregulated immune response, and subsequent chronic intracellular infection. Treatment may

lead to remission due to clearance of infection but the condition relapses upon reinfection.

More recently treatments looking to re-establish "normal" microbiota have been investigated. In particular, faecal microbial transplantation in UC has shown some promising results with statistically significant increases in remission rates and mucosal healing following FMT. However, in one of these studies responses were limited to stool from a single donor, with lesser effects seen from different donors implying the existence of "super donors" among human volunteers (Rossen et al., 2015; Moayyedi et al., 2015) This suggests that it is the composition of the microbiome, as well as dysbiosis in the host, that has an important role in mucosal health.

1.1.2.2 *Irritable Bowel Syndrome (IBS) and the microbiome*

Irritable bowel syndrome is a leading cause for referrals to the gastroenterology services in the UK. Up to 49% of lower gastro-intestinal endoscopies are performed for IBS related symptoms (Buono et al., 2017). It is categorized by a wide range of symptoms such as constipation, diarrhoea, abdominal pain and bloating. However endoscopic investigations are often normal. The aetiology and pathophysiology remains unclear and due to this, symptoms are often poorly controlled with medication. Hypotheses include visceral hypersensitivity, an abnormal brain-gut axis, gastrointestinal dysmotility and chronic low-grade inflammation (Ahmad and Akbar, 2016)

In the last few years it has become clear that the microbiota may have a role to play in this difficult and complex condition.

Following an episode of infectious gastroenteritis, the risk of developing IBS is increased six fold (Thabane et al., 2007) with up to 36% of patients developing IBS after an enteric infection (Spiller and Garsed, 2009). Thus, alterations in composition of microbiota as a result of infection may contribute to development of symptoms in those individuals who are susceptible.

Evidence has been produced to show an on-going alteration in microbiota composition of IBS patients, when compared to controls, although changes have been difficult to quantity and reproduce given the complexity of the condition, the wide range of symptoms experienced and the subjective nature of symptoms reported. There is a general decrease in bacterial species in IBS, with decreased

biodiversity, similar to that seen in UC (Noor et al., 2010). This seems particularly prevalent in diarrhoea predominant IBS where it has been shown that there is significant reduction in bacterial richness, in particular there are higher levels of *Enterobacteriae* with a loss of the *Faecalibacterium,* which is considered to be a beneficial microbe (Carroll et al., 2012). Furthermore, there are greater numbers of mucosa-associated bacteria per mm of rectal epithelium, predominantly *Bacteroides* and *Clostridia*, when comparing IBS patients to controls. However, the number of Bifidobacteria was reduced in the diarrhoea predominant subgroup, with the number of stools per day negatively correlating with the total number of mucosa associated Bifidobacteria and Lactobacilli (Parkes et al., 2012).

1.1.2.3   Colorectal cancer (CRC) and the microbiome

Like IBD, there is a strong genetic component in the development of colorectal cancer. However, this alone does not account for the development of cancer and many environmental factors have been described. The development of cancer has been associated with chronic intestinal inflammation. Indeed, patients with UC have an increased relative risk of developing CRC compared to the "normal" population depending on how much of the bowel is affected (14.8 whole colon, 2.8 left sided, 1.7 rectum) (Ekbom et al., 1990). It need not be pathogenic bacteria that are associated with the development of cancer. Mice models have confirmed that *Bacteroides fragilis* can promote release of pro-mitogenic cytokines through immune cell activation (Wu et al., 2009). It has long been established there is an association between *Streptococcus bovis* bacteraemia and advanced colonic carcinoma (Paritsky et al., 2015). Both of these bacteria are commensals of a "healthy" microbiota and this again highlights the importance of this complex system

Cancer in the colon develops through the adenoma – carcinoma pathway as highlighted in Figure 1-3.

Colonic Carcinoma

Large Adenoma

Small Adenoma

Normal Epithelium Hyperproliferative Epithelium

Mucosa

Submucosa

Muscularis Propria
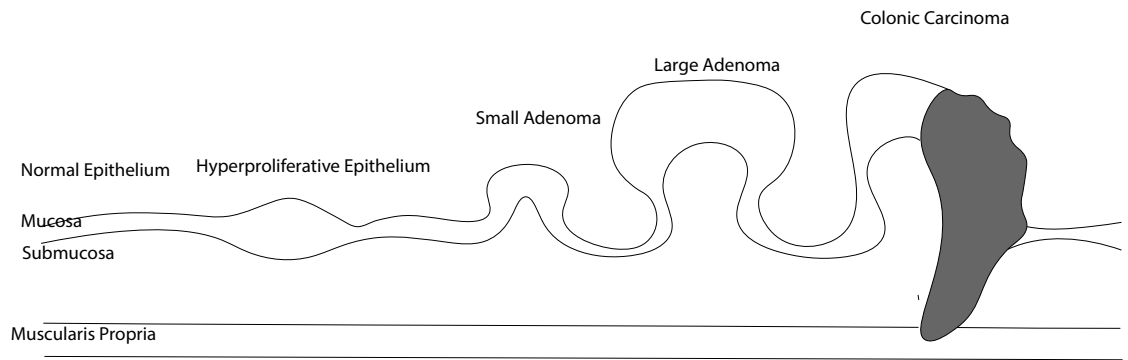
Figure 1-3 Polyp – Adenoma – Cancer Pathway for development of colonic carcinoma

It has been shown that the microbiota in stool samples from patients with distal and advanced adenomas is altered, with depletion of *Ruminococcaceae*, *Clostridiaceae*, and *Lachnospiraceae* families and enrichment in *Enterobacteriales*, *Actinomyces* and *Streptococcus* (Peters et al., 2016). There have also been changes described in the micro-environment with enrichment of *Bacteroidetes fragilis, Bacteroides vulgatus, Bifidobacterium longum, Clostridium butyricum, Mitosuokella multiacida, Escherichia coli, Enterococcus faecalis and Streptococcus bovis* in tumour samples and surrounding tissue. (Terzić et al., 2010). There is a well-established link between *Fusobacterium nucleatum,* an invasive pro-inflammatory anaerobe, and colorectal cancer with this bacterium being significantly more abundant in tumour biopsies when compared to matched healthy tissue (Castellarin et al., 2012). This bacterium has a direct link to tumorigenesis, increasing multiplicity and selectively recruiting tumour infiltrating myeloid cells (Kostic et al., 2013).

1.1.3 The colonic microbiota involvement in non-gastrointestinal tract disease

It is clear that the gut microbiota may have a role to play in auto-immune, inflammatory and functional disorders of the GI tract. However, evidence is beginning to emerge that suggests this effect is not only limited to the human intestine. How the microbiota may affect the liver and biliary system is covered in the next section, but there is some work that implicates the human microbiota in other conditions completely outside of the GI tract.

1.1.3.1 Gut-Brain Axis

Given that the microbiota plays a key role in signalling pathways in the development of the host immune system, it has been suggested that it may equally play a role in other feedback channels that are crucial for maintaining homeostasis. One such proposal was that of a microbiota – gut – brain axis. It is well documented that stress and emotion can trigger release of chemicals and hormones from the brain that have an effect on gut function such as motility, immunity, mucus production and permeability. Equally parasympathetic nerves in the gut send transmissions to the parts of the brain, in particular the pituitary gland, influencing production of these chemicals. (Carabotti et al., 2015) Therefore, factors influencing gut function, such as the microbiota, may have an influence on behaviour and possibly even psychiatric health. Mice studies have shown that infection with *Campylobacter jejuni* leads to anxiety-like behaviour with concomitant activation of the vagus nerve (Goehler et al., 2005) and transplanting the faecal microbiota can also lead to transfer of certain behaviours (Bercik et al., 2011). Whilst it is likely that effects on the host immune system may have a role to play in gut-brain interactions, it is also clear that gut bacteria may have a direct role to play through the production of neuroendocrine hormones (Foster et al., 2016) In particular it has been shown that dopamine and norepinephrine can be produced by gut microbiota in the caecum in quantities large enough to affect host neurophysiology (Asano et al., 2012). Two species commonly used as probiotics, *Bifidobacterium* and *Lactobacillus,* happen to be prolific producers of neurochemicals and have been shown to reduce anxiety and depression in mice (Bravo et al., 2011)

1.1.3.2 Endocrine

It has been recognised that many auto-immune conditions are likely to develop as a result of an abnormal host response to bacteria or viruses. More recently the gut microbiota has been suggested as the infectious trigger in many of these conditions. In type 1 diabetes it has been shown that in all stages of the disease, from pre-clinical and newly diagnosed to long term, there was increased intestinal permeability to the sugar lactulose, suggesting a damaged mucosal barrier (Bosi et al., 2006). In autoimmune thyroiditis, it has been shown that there is a

disproportionate incidence (40% vs 5% of the control group) of histological lymphocytic colitis, a condition associated with diarrhoea and increased intraepithelial lymphocytes. (Cindoruk et al., 2002). Equally in the small bowel there are differences in the structure of the microvilli of the small bowel in Type 1 diabetes and autoimmune thyroiditis (Sasso et al., 2004).

1.1.3.3 Asthma

There is good epidemiological evidence that early exposure to microorganisms is associated with decreased incidence of atopy and childhood asthma (Ege et al., 2011). The naso-pharynx and GI tract are closely linked with even small volumes entering through the nose ending up in the GI tract. Mice studies have shown that subjects whose microbiota is altered can develop allergic airways responses to allergens (Noverr and Huffnagle, 2005). Additionally, the human microbiota of patients with asthma have been shown to differ from normal subjects even prior to the development of atopy. (Lynch, 2016) As in Crohn's disease, the use of antibiotics in early life has been shown to be linked to the development of asthma. (Shreiner et al., 2008)

1.1.3.4 Rheumatoid Arthritis

Rheumatoid arthritis (RA) is an inflammatory autoimmune disease characterised by erosive synovitis affecting the small joints of hands, wrists and feet (Diamanti et al., 2016). In early rheumatoid arthritis, there are significant differences in the microbiome with a lower abundance of *Bifidobacteria* and *Bacteroides* when compared to controls with fibromyalgia (Vaahtovuo et al., 2008) again mimicking changes seen in IBD. In established RA, there is a decrease in gut microbial biodiversity that correlates with disease duration and antibody levels. This study also demonstrated expansion of the phyla Acintobacteria; predominantly due to increased abundance of the rare genera *Collinsella* and *Eggerthella (Chen et al., 2016). Collinsella* is positively correlated with serum cholesterol levels, has an association with insulin resistance and is known to have bile salt hydrolase activity (Lahti et al., 2013).

1.1.3.5 Obesity and the metabolic syndrome

Obesity is rapidly becoming a worldwide pandemic with up to 27% of people being obese in the UK currently, with a projected prevalence of 50% by 2050. (Public Health England, 2017). The metabolic syndrome is a collective medical term for the combination of increased blood pressure, increased blood glucose, central obesity and elevated cholesterol which occur together and increase an individual's risk of coronary artery disease, type 2 diabetes and stroke.

It is now clear that long -term diet can affect the microbiota, with high fat and protein diet being associated with enterotype 1 whereas diets high in carbohydrate are associated with enterotype 2 (Wu et al., 2011). Although this may suggest that diet is affecting microbiota, and obesity is a result of high calorie intake, it has been shown in mouse models that transferring a microbiota from obese mice fed a high fat diet to germ free mice also resulted in the transfer of an obese phenotype (Delzenne et al., 2013). This should not come as a surprise however, given that 10 years earlier Backhed et al (2004) showed that introducing a microbiota from the caecum of conventional mice to germ free mice resulted in a 60% increase in body weight. Furthermore, the same group showed that GF mice did not gain weight even when exposed to a high fat, high sugar diet. (Bäckhed et al., 2007).

The microbiota is thought to influence body weight through production of short chain fatty acids and fermentation of indigestible carbohydrates, which have both pro obesity and protective properties (Machado and Cortez-Pinto, 2016). Microbiota also influence expression of factors that can increase uptake of fatty acids in the adipose tissue and liver, promoting steatosis. Microbiota can also increase mucosal blood flow, thereby increasing nutrient absorption.

1.2 Liver disease

1.2.1 Liver and biliary system in health

The liver is the largest internal organ in the body. It receives 25% of the resting cardiac output of humans via the portal vein and hepatic artery. Its major roles are protein, carbohydrate and lipid metabolism, bile secretion, bile acid metabolism, drug and hormone inactivation, storage of essential nutrients and minerals and maintenance of host immunity.

The liver is responsible for the synthesis of the vast majority of circulating proteins in the human body. These include albumin, transport and carrier proteins, acute phase proteins and coagulation factors. It is also responsible for degradation of proteins.

The liver maintains blood glucose through the storage of glycogen and the release of glucose either through breakdown of glycogen or synthesizing glucose in the immediate fasting state.

Lipids are insoluble in water and therefore are transported in human plasma in protein-lipid complexes known as lipoproteins. The liver synthesises two types of lipoproteins – high-density lipoprotein (HDL) and very low-density lipoprotein (VLDL). It also forms triglycerides from free fatty acids and synthesizes cholesterol (Kumar and Clark, 2016).

The liver is the major site of drug metabolism. It is mediated by a group of enzymes comprising the cytochrome P450 system and cytochrome C-reductase. Metabolism occurs in 2 stages; firstly, the drug is inactivated through oxidation or demethylation (first pass metabolism) and then made water soluble through conjugation with glucuronide or sulphate (second pass metabolism). These drugs can then be excreted in the bile or urine (Tomankova et al., 2017).

The liver is also the target organ for many hormones such as insulin. The liver catabolises many hormones, such as glucagon, growth hormone, parathyroid hormone and glucocorticoids, making them inactive.
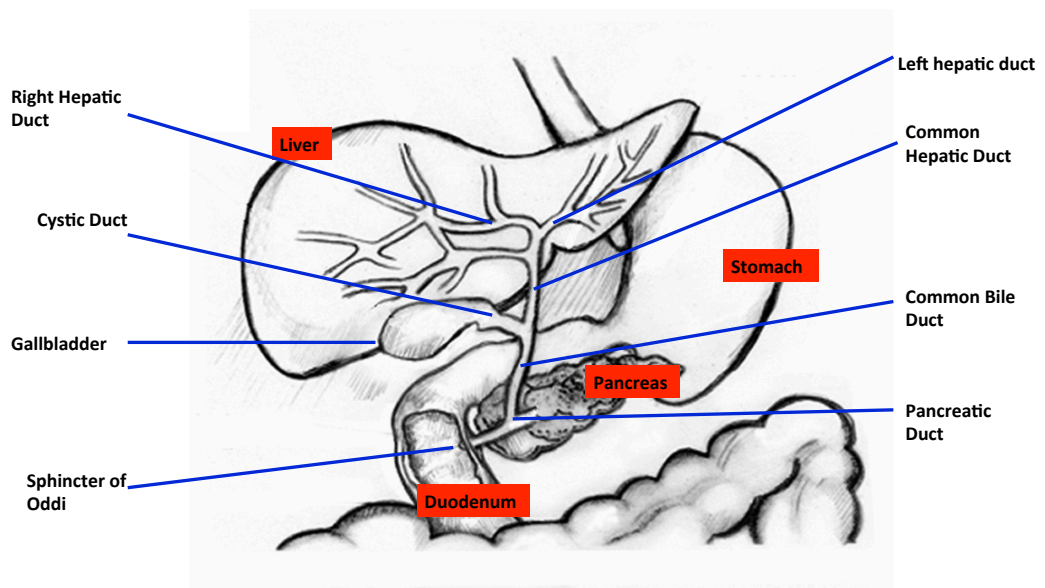
Figure 1-4 The human biliary system

The biliary system is a complex arrangement of tubes that carries bile through the liver and out to the small intestine. Bile produced by liver cells is secreted into microscopic canals known as canaliculi. These then drain into ductules and larger ducts within the portal tracts. These then combine to form the left and right hepatic ducts that drain each lobe of the liver. These ducts then join at the porta hepatis to form the common bile duct hepatic duct. The gallbladder lies just inferior to the right lobe of the liver and drains into the cystic duct. These ducts then combine to form the common bile duct, which drains into the duodenum. The gallbladder concentrates hepatic bile, which it then excretes when food passes into the duodenum in order to aid digestion (Kumar and Clark, 2016).

Bile is made up of water, bile acids, cholesterol, phospholipids and conjugated bilirubin. The liver produces approximately 600ml of bile each day. In the fasted state, approximately half of this is diverted to the gallbladder where up to 90% of the water is absorbed by the mucosa. When food containing fat reaches the duodenum the gallbladder releases this concentrated bile to aid digestion through emulsification of fat into smaller molecules which are more readily digested. This is predominantly done by bile acids. Bile acids are actively reabsorbed in the terminal ileum and recirculated through the enterohepatic circulation. The molecular aspects of bile acid synthesis/reabsorption are complex involving the farsenoid X receptor, the liver x receptor and the liver receptor homologue. These pathways are highlighted in figure 1.5. Approximately 20% are excreted in the faeces and replenished through hepatic synthesis (Berne and Levy, 1996). Bile

acids are synthesised in the liver from cholesterol. Initially the liver synthesizes the primary bile acids cholic acid and chenodeoxycholic acid, which are then 'conjugated' through the addition of either a glycine or taurine to a side chain of these bile acids.

Bile also has a role to play in excretion of waste products from the body, in particular bilirubin, phospholipids and cholesterol. Phospholipids, in the form of lecithin, and cholesterol are insoluble in water. However, they are able to dissolve in bile acid micelles and therefore can be excreted in the stool (Bowen R. 2017).
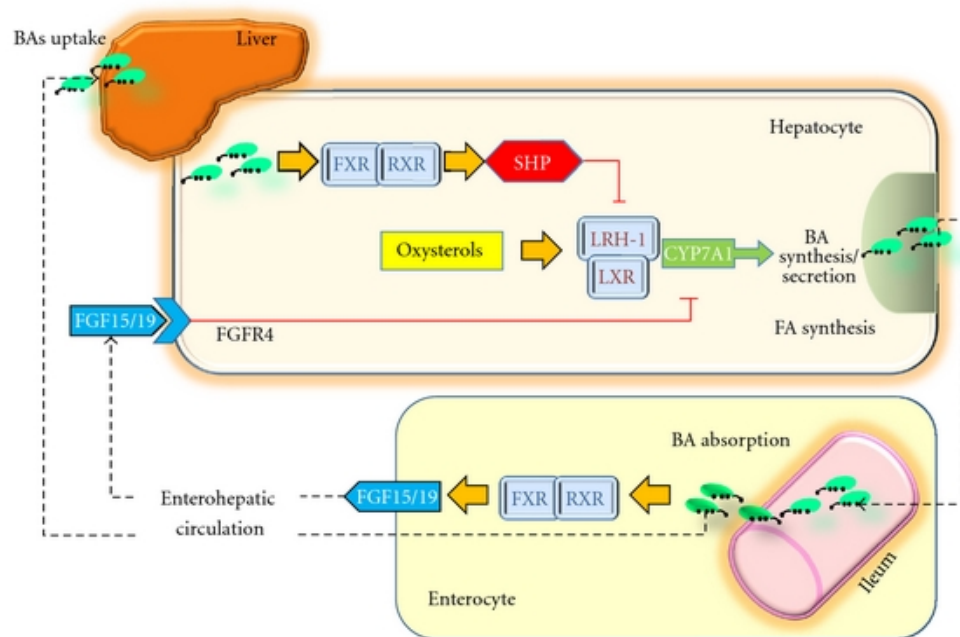
Figure 1-5 The molecular mechanisms of the enterohepatic circulation. Bile acids act as ligands for FXR, which regulates transcription by binding as a heterodimer with RXRs. This step results in increased SHP expression. SHP in turn inhibits LRH-1, preventing the activation of target genes that participate in bile acid and fatty acid synthesis. In the absence of bile acids, LRH-1 acts together with LXR to stimulate bile acid synthesis]. The important pathways in the intestine that contribute to modulation of bile acid synthesis are also depicted. There is a bile-acid-mediated activation of intestinal FXR and, as a result, the release of FGF15 in the small intestine. The secreted FGF15 by the intestine circulates to the liver, likely through the portal circulation or lymph flow and induces the activation of FGFR4 in the liver. The FGF15/FGFR4 pathway synergizes with SHP in vivo to repress CYP7A1 expression. Bas: bile acids; FGF: fibroblast growth factor; FGFR4: FGF receptor; FXR: farnesoid X receptor; LRH-1: liver receptor homologue-1; LXR: liver X receptor; RXR: retinoid X receptors; SHP: short heterodimer partner. Adapted from Garruti et al 2012.

1.2.2   Microbiota and Bile Salts

The human GI tract has several mechanisms by which it protects itself from enteric infections. One of these is the excretion of 3 bactericidal agents; gastric secretions, hydrochloric acid and bile. Gastric secretions and hydrochloric acid reduce the pH of the stomach to 3 (Merritt and Donaldson, 2009). The high acidity of gastric juice kills most ingested microorgansims (Berne and Levy, 1996) This is supported by the fact that mice with low gastric acid secretion are more prone to infections by *Yersinia*, *Citrobacter*, *Clostridium* and *Salmonella* (Tennant et al., 2008).

Bile, or more specifically bile salts, are thought to have bacteriostatic and bactericidal properties (Percy-Robb and Collee, 1972). The theory that bile protects against pathogenic bacteria stems from the fact that relatively speaking the small intestine, which contains a large amount of bile acids, typically contains very few bacteria. In conditions associated with impaired bile secretion, such as cirrhosis, small bowel bacterial overgrowth is a common feature (Lorenzo-Zúñiga et al., 2003).

There are multiple mechanisms through which bile salts are thought to exert an anti-bacterial affect. The primary effect is through disruption of cell membranes. At high concentrations, bile salts dissolve cell membranes causing leakage of cell contents and instantaneous cell death. At lower concentrations, they have more subtle effects on membrane permeability and fluidity. Unconjugated bile salts are also thought to have a more potent bacteriocidal effect. They are more readily able to pass through the bacterial lipid bilayer wall, as they are more able to dissolve in water (Begley et al., 2005). Additional effects of bile salts include induction of secondary structures in RNA, inducing DNA damage and activating enzymes involved in DNA repair, causing misfolding and denaturation of proteins, inducing oxidative stress and creating low intracellular iron and calcium through metal chelation (Kristoffersen et al., 2007; Merritt and Donaldson, 2009; Taranto et al., 2003).

Bile acids may also promote antimicrobial effects directly on the host further down the GI tract. Conjugated bile causes activation of the farsenoid X receptor in the terminal ileum. (Hofmann and Eckmann, 2006) As a result genes, involved in the biosynthesis of products associated with mucosal defence in the intestine are upregulated: iNOS, IL18 and Angiogenin (Inagaki et al., 2006). iNOS produces Nitric Oxide which has direct antimicrobial effects and also promotes mucus

secretion, vascular tone and epithelial barrier function. The cytokine IL18 is known to stimulate resistance to bacteria and has a role in the early acute phase of mucosal inflammation. Angiogenin is part of the acute phase response to infection and has direct antibacterial properties.

Despite the potent antibacterial effects of bile acids, the microbiota has adapted to utilise bile salts, which may have an important role in disease. It is now clear that the gut microbiota is solely responsible for production of the secondary bile salts deoxycholic acid and lithocholic acid through deconjugation, oxidation and subsequently dehydroxylation of primary bile salts (Hofmann, 1999). The mechanisms through which bacteria transform bile salts is discussed in section 1.3.6. The vast majority (around 95%) of primary bile salts are reabsorbed in the terminal ileum. Around 400-800 mg of primary bile salts pass through to the colon where a large proportion undergoes transformation by gut bacteria. This then allows these secondary bile acids to be passively reabsorbed and enter the enterohepatic circulation (Ridlon et al., 2006). These secondary bile acids have roles in both health and disease. Lithocholic acid is toxic to hepatocytes and has been linked to colon carcinogensis (Gérard, 2013). Deoxycholic acid has a role to play in the formation of the cholesterol gallstone and has been shown to promote secretion of pro-tumorigenic factors in hepatocellular carcinoma (Ridlon et al., 2014). Conversely the production of the secondary bile acid ursodexycholic acid is thought to be chemopreventive and is used in the treatment of gallstones and primary biliary cirrhosis (Gérard, 2013). Equally the dysbiosis seen in inflammatory bowel disease leads to decreased deconjugation and desulfation of the bile acid pool which may promote chronic inflammation (Gérard, 2013)

Therefore, the relationship between bile acids and microbiota is complex and any change in the homeostatic mechanisms may result in a shift from gut health to gut disease.

1.2.3 Chronic parenchymal liver disease

Chronic parenchymal liver disease relates to conditions that affect the actual liver cells as opposed to supporting structures. There are a wide range of conditions that can lead to chronic liver disease, be it infectious, autoimmune, metabolic or toxic (alcohol, drugs). Unlike other systems such as cardiac or respiratory, which are falling, the mortality rates in chronic liver disease are continuing to rise. The leading causes of chronic parenchymal liver disease are viral hepatitis (B and C), alcohol, and non-alcoholic fatty liver disease (Sanyal AJ, 2010).

Chronic liver disease is a progressive condition leading from normal liver architecture to fibrosis and subsequently cirrhosis (Garcia-Tsao et al, 2009). Cirrhosis can be defined as a liver diffusely affected by fibrosis and the formation of structurally abnormal parenchymal nodes. Fibrosis occurs through inflammation and necrosis activating Kupffer cells. These in turn activate stellate cells within the liver that reside in the subendothelial space of Diss between sinusoids and hepatocytes. The activation of stellate cells leads to increased production and deposition of fibrocollageous tissue, which causes irregular scarring. Damaged hepatocytes also attempt to regenerate, forming irregular nodules that have abnormal relationships with the surrounding vasculature and bile ductules. In the early stages of cirrhosis these hepatocytes are able to meet normal demands and synthetic function is near normal, but as cirrhosis progresses these demands are not met and the symptoms of decompensated liver disease manifest themselves.

There are 5 main complications of cirrhosis as summarised in Table 1-2. Once cirrhosis develops it is irreversible. However, the prognosis is still highly variable and is dependent on aetiology, presence of complications, and synthetic function. In general, 5-year survival is approximately 50%, but it can be as low as 3 months if synthetic function is impaired (Fortune & Cardenas, 2017).

Complications of Cirrhosis
Portal hypertension and variceal haemorrhage
Ascites
Encephalopathy
Hepatocellular Carcinoma
Renal Failure

Table 1-2 Complications of cirrhosis

The normal pressure within the portal vein is 5-8 mmHg. Portal hypertension develops when the portal pressure rises above 10 mmHg. In cirrhosis, this occurs as a result of increased vascular resistance due to mechanical distortion in liver architecture, increased production of the potent vasoconstrictor endothelin-1, decreased production of nitrous oxide in the liver and increased portal inflow as a result of splanchnic vasodilatation (Schwabl & Laleman, 2017). Portal hypertension is maintained through sodium retention and increased plasma volume (Fortune & Cardenas, 2017). The result of portal hypertension is the development of collateral blood vessels, known as varices, with the systemic venous circulation. Varices can develop at the gastro-oesophageal junction (GOJ), cardia of the stomach, anal canal, falciform ligament of the liver, abdominal wall and spleen (Garcia-Tsao et al., 2009). The most important of these are GOJ and gastric varices as they tend to be close to the mucosal surface and liable to rupture. 90% of patients with cirrhosis will develop varices over 12 years. Up to 50% of patients with varices will have an episode of bleeding, which is often life threatening. Despite improvements in emergency care, endoscopic therapy, radiological therapies and ITU care 6-week mortality from an index bleed is still around 20%.

Ascites is defined as the accumulation of free fluid in the peritoneal cavity. The leading cause of ascites is cirrhosis, accounting for 75% of cases. Ascites develops as a result of portal hypertension, hypoalbuminaemia and salt/water retention due to activation of the renin-angiotensin system which in turn occurs as a result of renal hypoperfusion following splanchnic pooling. The development of ascites is associated with 50% mortality within 2 years and is an indication for liver transplantation. Ascites can be complicated by spontaneous bacterial peritonitis. The condition is thought to occur due to bacterial translocation, which is discussed in section 1.3.1 (Cesaro et al., 2011). It is important as it has a mortality of 20% despite improving recognition and treatment. It occurs in 10 to 30% of patients with ascites and will re-occur in more than 2/3rds in the first year (Fortune & Cardenas, 2017).

Hepatorenal syndrome (HRS) is a diagnosis of exclusion and is defined as acute kidney injury in a patient with advanced liver disease without an identifiable cause. Type 1 HRS refers to a rapid deterioration in renal function, often in relationship to an acute decompensating liver injury. Type 2 HRS is a more slowly progressive kidney failure in the presence of ascites and sodium retention. The prognosis of HRS is exceptionally poor with a median survival of 3 months if treated. Like many complications of cirrhosis it occurs as a result of splanchnic vasoldilation, resulting in activation of the renin-angiotensin pathway promoting renal vascoconstriction. Reduced cardiac output and release of compounds known to affect renal circulation including endothelin-1 are also likely to play a role. (EASL Clinical Practice Guidelines, 2010)

Encephalopathy refers to a chronic neuropsychiatric condition characterised by disturbance in sleep – waking patterns, personality changes, confusion and impaired levels of consciousness. It is thought to occur as a result of shunting of portal blood to the systemic circulation, thereby bypassing the metabolic functions of the liver. This means the brain is exposed to nitrogen containing compounds, especially ammonia (see section 1.3.1). Encephalopathy is he second commonest cause for admission to hospital in patients with cirrhosis and has a huge economic burden (Piotrowski & Boroń-Kaczmarska, 2017). Encephalopathy is associated with a poor prognosis is an independent predictor of mortality in cirrhosis (Patidar and Bajaj, 2015).

Hepatocellular carcinoma is now the second leading cause of cancer death worldwide and its incidence is rapidly rising. Up to 90% of patients with HCC have a background of chronic liver disease, usually secondary to hepatitis B/C or alcohol, although cirrhosis due to any aetiology can increase the risk. (Sanyal et al., 2010)

1.2.4 Biliary diseases and complications

The most common disease of biliary tract are gallstones with a prevalence of 10-20% (Li et al., 2017). In the Western world, around 80% of these are cholesterol based. Risk factors include over-eating, low activity, obesity, metabolic syndrome and insulin resistance (Paumgartner, 2010). The vast majority of gallstones are asymptomatic (Li et al., 2017). However, they can give rise to pain and cholecystitis; inflammation and infection of the gallbladder, which can be acute or chronic (Rawls, 1971). If left untreated gallstones can migrate into the common bile duct and cause obstructive jaundice (Lee et al., 2016) or pass through to the intestine and cause gallstone ileus (Glenn, 1967). Other complications include cholecystoduodenal fistula (Aguilar-Espinosa et al., 2017) and pancreatitis, which can be fatal (Rawls, 1971). Treatment is generally surgical removal of the gallbladder or endoscopic removal of biliary stones.

Primary biliary cholangitis (PBC) is a slowly progressive chronic liver disease characterized by intrahepatic bile duct destruction, cholestatic liver biochemistry and positive auto-antibodies (Marchioni Beery et al., 2014). The prevalence of primary biliary cirrhosis has been increasing over the past 30 years and is now a significant cause of liver morbidity and mortality (Bowlus et al., 2016). The aetiology remains unclear although there is a clear auto-immune and genetic component as suggested by a weak association with HLA-B8, the discovery of 12 new susceptibility loci on genome-wide association studies (Mells et al., 2011), its association with extra hepatic autoimmune disease and a high concordance rate in monozygotic twins (Selmi et al., 2011).

Several infectious and environmental factors are thought to contribute to the onset of PBC, as evidenced by clustering of cases near toxic waste sites in New York, the significantly higher rate of urinary tract infections in patients with PBC (Varyani, West, & Card, 2011) and the demonstration of molecular mimicry between mitochondrial and nuclear auto antigens in PBC (Shimoda et al., 2003). Given that PBC is a chronic inflammatory disorder it is possible that exposure to bacteria in a genetically susceptible individual may precipitate the development of the condition. Both pathogenic and non-pathogenic Gram-negative bacteria have been proposed but as yet not substantiated (Selmi et al., 2011). Through this study, it may be possible to detect changes in the human microbiome in PBC patients which may

provide further evidence to an infectious cause. At present treatment options for PBC are limited.

Ursodeoxycholic acid (UDCA) is the only treatment that has approval, but studies have shown that although it improves liver biochemistry and histological progression, it may have no effect on mortality, progression to liver transplantation or symptoms (Rudic, Poropat, Krstic, Bjelakovic, & Gluud, 2012). If a link between the gut microbiota and primary biliary cirrhosis can be established then it may open doorways to novel treatment strategies which could prevent and possibly treat established disease.

Primary sclerosing cholangitis (PSC) is an autoimmune condition characterized by chronic inflammation, progressive fibrosis and stricturing of the intra and extra hepatic ducts (Marchioni Beery et al., 2014). It is a slowly progressive disease with a median time to liver transplant or death ranging between 12 and 18 years (Yimam and Bowlus, 2014). As well as progression to end stage liver disease, PSC is also associated with a greatly increased risk of cholangiocarcinoma (Ehlken et al., 2017). Like PBC the exact aetiology is unknown. It has a definite link with inflammatory bowel disease with up to 83% of PSC patients in Northern Europe having concurrent IBD (Folseraas et al., 2012). There is also a definite genetic susceptibility with a strong association with HLA haplotypes and serum auto-antibodies (Pollheimer et al., 2011). Up to 40 genetic loci being identified as high risk for PSC (Karlsen and Boberg, 2013). Equally 25% of patients have at least one other autoimmune condition outside of the colon and liver (Folseraas et al., 2012). There are no current effective treatments for PSC. UDCA can improve liver biochemistry but has no effect on morbidity and mortality (de Vries and Beuers, 2017). Antibiotics seem to be of benefit, improving liver biochemistry and symptoms in patients, especially those without cirrhosis. However, the long-term effects are unclear (Davies et al., 2008). It is therefore possible that bacteria may have a role to play in the development and progression of PSC.

1.3 Liver disease and microbiota

1.3.1 Complications of cirrhosis and barrier dysfunction

The liver receives 70% of its blood supply directly from the gastrointestinal tract via the portal vein. This results in continual exposure to gut bacteria and bacterial cell components and metabolites (Son et al., 2010). It is therefore conceivable that this complex gut microbiome has an important role in the development of chronic liver disease via this "gut-liver axis" and recent research has begun to explore this hypothesis.

Many complications of cirrhosis can be linked to bacterial translocation from the gut to mesenteric lymph nodes or other organs (Cesaro et al., 2011). Indeed it is felt that spontaneous bacterial peritonitis, high rate of infections in cirrhotics and hepatic encephalopathy occurs as a result of bacterial translocation, increased intestinal permeability and changes in faecal microbiota (Chen, Yang et al. 2011; Wiest, Krag et al. 2012; Tsiaoussis et al., 2015). Intestinal permeability in cirrhosis may develop as a result of microbiota alterations leading to increased endotoxin production and alterations in tight junction expression and integrity (Pijls et al., 2013). Equally intestinal permeability may develop as a direct result of portal hypertension with a significant correlation between portal pressure, intestinal permeability and plasma levels of inflammatory cytokines and LPS being reported (Reiberger et al., 2013). Bacterial translocation may also be promoted through small bowel bacterial overgrowth, which develops in cirrhosis as a result of prolonged gut transit and disturbances in small bowel manometry (Kalaitzakis, 2014).

One of the most disabling complications of cirrhosis is hepatic encephalopathy and small bowel bacterial overgrowth is associated with development of this condition (Zhang et al., 2016). Equally one of the most effective treatments for encephalopathy is the poorly absorbed antibiotic Rifaximin which has a broad spectrum of antibacterial activity against both aerobic and anaerobic Gram positive and Gram negative bacteria (Garcovich et al., 2012).

1.3.2 Non-alcoholic steatohepatits (NASH)

One of the complications of obesity and the metabolic syndrome is the development of non-alcoholic steatohepatitis (NASH). NASH refers to fatty liver disease with associated inflammation and fibrosis and in 2013 accounted for 10% of all liver transplants. It is projected to be the leading indication for liver transplant in the next few years. It has been shown that young patients with NASH have a distinct microbiome from healthy subjects, with a rise in *Bacteriodetes* and fall in *Firmicutes*. The same study also showed that although there were similarities between obese and NASH patients, the latter have statistically significant increases in the prevalence of *Escherichia*. Interestingly these are alcohol producing bacteria, which may provide a mechanism through which gut microbiota can cause liver inflammation (Zhu et al., 2013). As with a lot of microbiota studies these results tend to vary between studies with another group showing *Bacteroidetes* were reduced and *Firmicutes*, especially lactobacillus, were increased in obese patients with non-alcoholic fatty liver disease. They did not demonstrate an increase *Escherichia* or faecal ethanol. These patients however were not biopsied and so the degree of liver disease was not determined (Raman et al., 2013)

Although there is no consensus currently on the "typical" NASH microbiota, it is clear that changes in the composition of the bacteria described in different studies of NASH could potentially have an effect on hepatic inflammation. As well as *Escherichia sp*, several *lactobacillus* species and *Ruminococcus* are able to produce ethanol and other toxic products such acetate through fermentation. Ruminococcaceae produce the fatty acid butyrate, which can decrease gut permeability and regulate insulin resistance. A decrease in *Gammaproteobacteria* and increase in *Erysipelotrichi* has been shown to have a positive predictive value for development of fatty liver disease following a choline deficient diet (Spencer et al., 2011). Finally, an increase in *Bacteroides* may lead to an increase in deoxycholic acid, which can induce apoptosis in hepatocytes and is known to be increased in NASH.

1.3.3 Alcoholic Liver Disease

Alcohol is the leading cause of end stage liver disease in the Western world. However, only a small proportion of people who drink alcohol to excess develop cirrhosis (Tuomisto et al., 2014). It is possible that a combination of genetic susceptibility to bacterial products (Järveläinen et al., 2001), disruption of mucosal barrier by alcohol (Basuroy et al., 2005) and increased rates of bacterial translocation (Steffen et al., 1988) may lead to development of fibrosis and cirrhosis in alcoholic liver disease. A change in the composition of the gut microbiota so that more "harmful" bacteria are prevalent may also play a part. In this regard chronic alcohol abuse is associated with decreased numbers of *Clostridium* and *Bacteroidetes* and increased numbers of Proteobacteria (Mutlu et al., 2012). It has been shown that at autopsy there is an increased abundance of *Entereobactericaea* DNA in the liver of cirrhotic patients when compared to non-cirrhotic controls, although this did not reach statistical significance. However, expression of CD14, a bacterial recognition receptor, was associated with the total bacterial DNA (Tuomisto et al., 2014).

1.3.4 Other Parenchymal liver disease

Hepatitis B and C remain one of the leading causes of end stage liver disease and hepatocellular carcinoma (HCC). worldwide (Serigado et al., 2017). The degree of liver disease related to viral hepatitis may be influenced by the gut microbiota. Sandler *et al* (2011) demonstrated higher plasma levels of bacterial lipopolysaccharide (LPS) and soluble CD14 in patients with hepatitis B and C, with increasing circulating levels depending on severity of fibrosis. This suggests that the degree of liver disease in patients with Hepatitis B and C is associated with microbial translocation (Sandler et al., 2011). Conversely, the gut microbiota is also involved in immunity with mice studies showing that a gut microbiota is required to clear Hepatitis B virus (Chou et al., 2015).

Viral hepatitis and cirrhosis are the main risk factors for development of hepatocellular carcinoma. It is now emerging that the gut microbiota may also contribute to development of HCC. Bacterial translocation seems to have a major role to play with reduction in LPS through antibiotic treatment reversing dysbiosis, decreasing tumour growth and preventing tumour multiplicity in a mice model (Zhang et al., 2012). Further rat studies have shown that gut-sterilization in

advanced liver disease reduced tumour growth (Dapito et al., 2012). Therefore, it is possible that manipulation of the gut microbiota may prevent HCC and delay progression to advanced liver disease.

1.3.5 Biliary disease and gut microbiota

Conventional wisdom dictates that bile is sterile, partly because bile has bactericidal effects but also because traditional research has failed to grow bacteria from bile (Nielsen, 1976). However, improvements in metagenomics such as next generation sequencing and new surgical procedures now mean it is possible to perform microbiome analysis on human bile. As a result, there is now clear evidence that the diseased human biliary tract has a microbiota.

It has been established that cholesterol gallstones contain bacteria (Monstein et al., 2002) and bacteria including *H. pylori* has been detected within the mucosa of diseased gallbladders (Griniatsos et al., 2009) More recently studies have identified a diverse bacterial community within the bile of gallstone patients (Wu et al., 2013) and within diseased pancreatic ducts and biliary stents (Swidsinski et al., 2005). A large case series in Austria found 973 bacterial isolates from 249 patients undergoing ERCP for biliary disease. In total only 13% of patient samples were sterile (Hakalehto et al., 2010) To date however, no studies have been able to assess bile from patients not known to have biliary infection, biliary disease or biliary intervention.

Recently bacteria have been grown from the gallbladder bile and mucus layer of healthy pigs. Subsequent 16S rRNA gene analysis also confirmed a diverse microbiome consisting predominantly of *Proteobacteria*, *Firmicutes* and *Bacteroidetes* (Jimenez et al., 2014).

The pathogenesis of hepatobiliary diseases such as PBC and primary sclerosing cholangitis are thought to include an infectious trigger (Pollheimer, Halilbasic, Fickert, & Trauner, 2011) although studies have failed to show any significant bacteriaemia in mesenteric and peripheral blood samples (Weismuller et al, 2008). The immune response in PBC is restricted to the epithelial cells of the intrahepatic ducts (Selmi, Bowlus, Gershwin, & Coppel, 2011). Therefore, a direct interaction between bile duct and bacteria may be the trigger for auto-immune disease.

The human biliary tract appears to be programmed for the possible presence of bacteria in that biliary epithelial cells express a wide range of innate immune receptors that mediate the signalization pathways that initiate inflammatory responses, form a mucus layer through production of mucin and produce antimicrobial peptides such as *β-* defensins (Vernier et al. 2015). An example would be the recent discovery of mucosal associated invariant T cells around bile ducts, which have been shown to upregulate CD40 ligand, IL-12 and IL-18 when exposed to *E.Coli* (Jeffery et al. 2015).

1.3.6   Mechanisms of bile salt resistance

As mentioned previously bile acids have bacteriostatic and bactericidal properties. However, enteric bacteria have adapted to live in these adverse conditions and several pathogenic bacteria including *Listeria monocytogenes*, *Esherichia coli* and *Bacillus cereus* are able to invade the gallbladder (Merritt and Donaldson, 2009). Therefore it is clear that bacteria have evolved to become resistant to bile salts.

Several of these mechanisms for resistance occur at a gene level. In the presence of bile salts *Escherichia coli* and *Salmonella* upregulate genes that protect against oxidative stress (Merritt and Donaldson, 2009), allow cells to replicate in the presence of DNA damage (Foster, 2007) and repair bile salt induced DNA damage through mismatch repair and base-excision repair (Prieto et al., 2006; Cano et al., 2002). In the presence of bile salts *Salmonella* and *Bacillus cereus* upregulate a group of genes that are also known to confer multi-drug resistance as well as transcribing efflux pumps on the cell membrane to expel bile salts (Prouty et al., 2004).

Bacterial spores have a greater resistance to bile salts and therefore endospore formation may be mechanism by which bacteria are able to cause enteric infection. (Kristoffersen et al., 2007)

Perhaps the predominant mechanism of bile salt resistance however is through induction of bile salt hydrolase (BSH) and 7α dehydroxylase. Bile salt hydrolase is found in several bacterial species found in the human intestine including *Bifidobacteria, Clostridium, Listeria* and *Lactobacillus* (Jarocki et al., 2014). 7α dehydroxylase has been found throughout the *Clostridium* genus (Wells et al.,

2000). BSH deconjugates primary bile acids to produce unconjugated bile salts. This then allows further metabolism through 7α dehydroxylase to produce secondary and tertiary bile acids (Joyce et al., 2014). BSH are thought to facilitate incorporation of cholesterol into bacterial membranes thereby increasing the tensile stress of the membranes. It is also hypothesised that deconjugation may be a means of detoxification of bile salts, although this is currently debated (Begley et al., 2005). In addition to aiding resistance deconjugation is thought to confer a nutritional benefit to bacteria through provision of carbon, nitrogen and energy sources (Begley et al., 2005). The 7α dehydroxylation pathway is a means through which bacteria can acquire energy, with bile acids acting as electron acceptors. This production of secondary bile acids may also serve to eliminate bacteria sensitive to these compounds (Ridlon et al., 2006).

1.4   Aims and Objectives of the Study

The aim of this study was to undertake a microbiological assessment of the human biliary tract as this is pertinent in health and disease. The objectives within this were:

- To assess whether bile isolated from the normal biliary tract is truly sterile
- To see if there is a difference in microbial biodiversity between bile isolated from diseased gallbladders/biliary tracts containing gallstones, and normal gallbladders/biliary tracts
- To assess whether bacteria isolated from the biliary tract have bile resistant properties
- To investigate changes in microbial biodiversity between PBC and PSC when compared to healthy controls
- To see if treatment and stage of liver disease has an impact on faecal microbiota
- To assess the metabolic activity of the gut microbiota in PBC and PSC

1.5   Hypothesis

The human biliary tract is not sterile but has a diverse microbiota, which may play a role in the development of liver and biliary disease and that there is a progressive dysbiosis in patients with PSC and PBC

1.6   Novelty

There is emerging evidence that bacteria have a role to play in biliary and liver diseases and liver disease. However, studies to date have only examined patients with diseased biliary tracts. To my knowledge this is the first study looking to characterize the biliary microbiota in normal human bile and compare multiple sites within the biliary tree.

Although dysbiosis has been shown in cirrhosis, at the time of the study no publications had looked specifically at primary biliary cholangitis, although a study has been published in GUT recently (Tang et al,. 2017). I intend to examine changes in microbiota depending on stage of disease of primary biliary cirrhosis and cholangitis and compare this to healthy controls.

If it can be proven that there is a "normal" bililary microbiota this may lead to further studies examining what role these bacteria play in the development of disease and whether manipulation of the microbiota may lead to new treatment modalities.

1.7   Ethics

All aspects of this study received ethical approval initially by a Sub – Committee of the Faculty of Medicine and Health Sciences Research Ethics Committee, University of East Anglia.

The same body granted subsequent ethical approval for inclusion of patients undergoing bowel cancer screening colonoscopy at the Norfolk and Norwich University Hospital

Subsequent ethical approval was granted by the Research and Development Department, Cambridge University Hospitals NHS Foundation Trust to allow recruitment of patients and collections of biliary samples from Addenbrookes Hospital, Cambridge.

2 Composition of the biliary microbiota

2.1 Introduction

Conventional wisdom dictates that human bile is sterile. However, until recently it has been difficult to analyze human bile in any detail due to difficulty in obtaining normal human bile from healthy biliary systems and then providing a specific culturing environment to isolate bacteria. Many bacteria require specific conditions and media in order to grow and so most remained unculturable.

However, surgical techniques have improved so that minimally invasive procedures done as a day case have now replaced major open cases. As such several procedures can now be done laparoscopically in a single surgical session, increasing the possibility of obtaining biliary samples. Surgical techniques have also advanced so that procedures, such as liver resections for colorectal cancer metastases, are now possible. These techniques are summarized in Figure 2-1. Newer surgical techniques mean that we are now able to obtain 'sterile' biliary samples, which was not previously possible.

The difficulty in obtaining biliary samples is summarized below. In our study, we have attempted to obtain "normal" bile through sampling normal gallbladders removed at the time of hepatic resection for liver tumours, and sampling the common bile duct at the time of laparoscopic cholecystectomy. In order to sample bile as normal as possible patients were selected who had biliary colic but normal liver function tests, negative cholangiograms at the time of surgery, and no prescribed antibiotics.
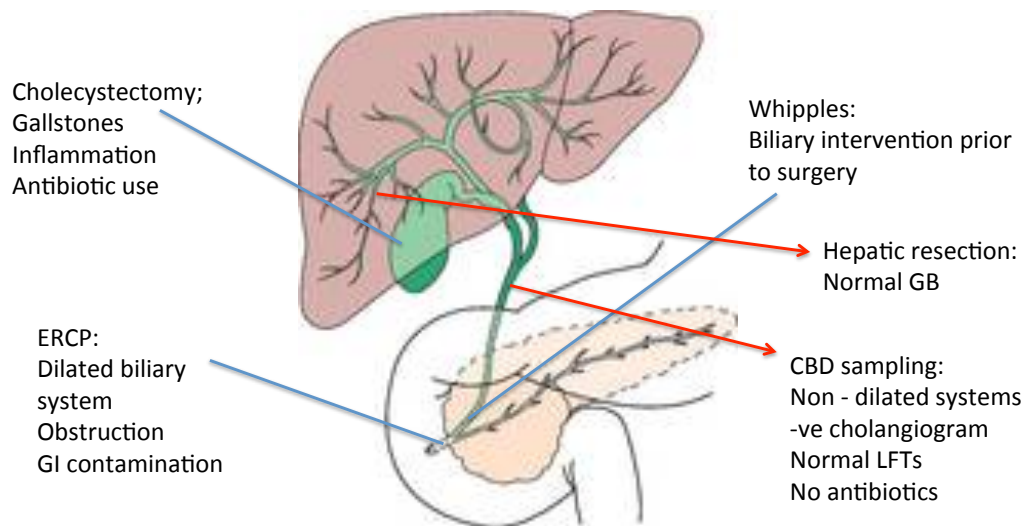
Figure 2-1 Difficulties in obtaining "sterile" biliary samples. Blue lines represent previous attempts to characterize the biliary microbiota and the associated issues. Red lines represent sampling methods for this study. ERCP = Endoscopic Retrograde Cholangiopancreatogram, GB = Gallbladder, CBD = Common bile duct.

The advent of Next Generation Sequencing (NGS) has revolutionized our ability to sequence a genome, improving accuracy, speed, cost and reducing manpower. As a result, we are now able to sequence large amounts of DNA at rapid speed. To put this into context the first human genome was sequenced by the Human Genome Project, and took 10 years at a cost of $3 billion. Today NGS can sequence a single human genome within a day for a cost of several hundred dollars. The result of this technological revolution means that we now have a much greater understanding of the structure of the human microbiome, and new discoveries are being made on an almost daily basis.

Initially the samples were sent for 454 pyrosequencing; a technique first launched by 454 life sciences in 2005. DNA is fragmented and generic adaptors are added to the ends of sequences, which are then annealed to beads. The fragments are then amplified by PCR prior to the beads being separated into wells. Thus, each well will contain a single bead covered in many copies of a single stranded PCR amplicon. The wells are then flooded with one of the four nucleotides (cytosine, thymine, adenine, guanine). If the nucleotide is complementing, it is bound to the DNA strand. If the nucleotide is repeated then more will be added. Each time a nucleotide is added a light signal is released, the more nucleotides that are added the stronger the light signal. The process is repeated for each nucleotide, and graphs are generated for each nucleotide wash. A computer reading the graph can then generate the DNA sequence.

16S rRNA amplicon

Fragmentation of 16S rRNA amplicon

Fragments immobilized on beads

Fragments amplified by emulsion PCR in an oil–aqueous solution

Amplified beads applied to a picotiter plate

Pyrogram reads chemoluminescent signal
Order of the nucleotide bases is determined and phylogenetic identification is possible

Chemoluminescence

G C C T G T A A C T G A
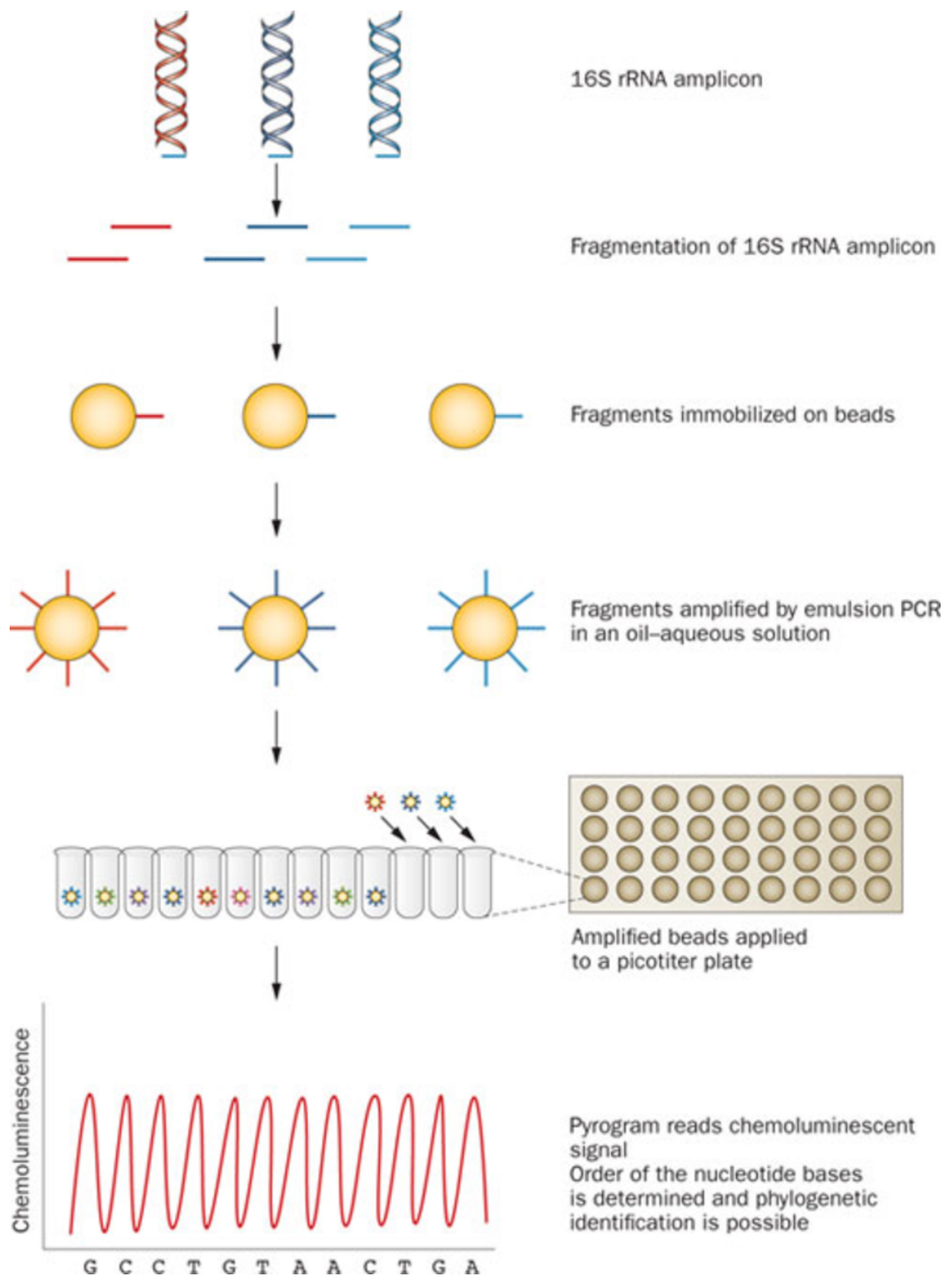
Figure 2-2 The 16S rRNA amplicon is fragmented and immobilized on beads (each bead crucially only attaches one DNA fragment). Fragments are amplified by emulsion PCR in an oil–aqueous solution and applied to a picotiter plate. The wells are then flooded with one of the 4 nucleotides. When a nucleotide base is incorporated a chemoluminescent signal is released, which is read into a pyrogram. Analysis of the pyrogram can, therefore, show the order of the nucleotide bases and thus phylogenetic identification is possible by comparison to databases. Adapted from Fraher et al 2012.

During the course of this project it became possible to have sequences analysed using the Illumina platform. Illumina is different to 454 in that it uses shorter fragments, which are annealed to a slide using adapters. PCR is performed to amplify each read, and the amplicons are then separated into single strands to be sequenced. The slide is flooded with fluorescently labeled nucleotides containing a terminator so only one base can be added at a time. An image is taken of the entire slide, the terminators are then removed and the process repeated. A computer reading the images then generates the DNA sequence.
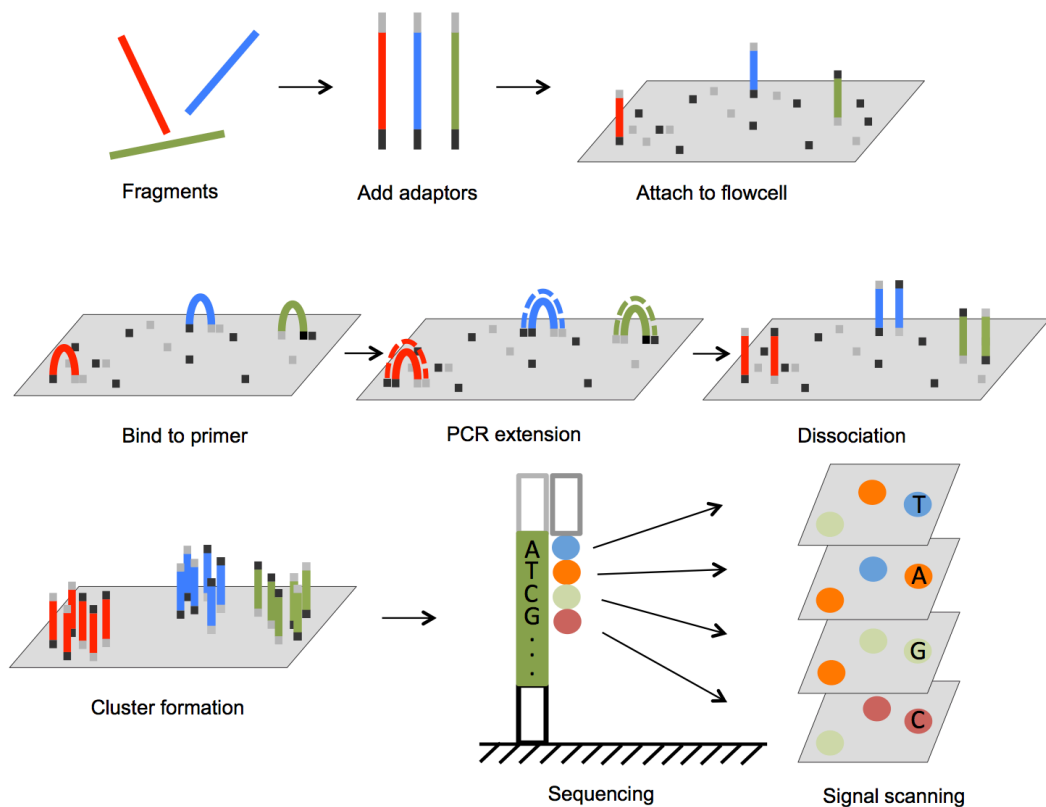
Figure 2-3 Outline of Illumina genome analyzer sequencing process. (1) Adaptors are annealed to the ends of sequence fragments. (2) Fragments bind to primer-loaded flow cell and bridge PCR reactions amplify each bound fragment to produce clusters of fragments. (3) During each sequencing cycle, one fluorophore attached nucleotide is added to the growing strands. Laser excites the fluorophores in all the fragments that are being sequenced and an optic scanner collects the signals from each fragment cluster. Then the sequencing terminator is removed and the next sequencing cycle starts. Adapted from Kulski J 2016

The advantages of Illumina over 454 sequencing are summarized below (Luo et al., 2012).

Advantages
More complete genes recovered due to higher sequencing error rates with 454
Greater depth of coverage
Cheaper in terms of sequences generated per pound
Ability to upgrade and improve sequencing method unlike 454
Improving technology means larger reads now possible – a previous weakness of illumina

Table 2-1 Advantages of Illumina sequencing over 454

It is due to this that Roche decided to abandon 454 sequencing technology in 2014 and phased out the technique over the following 18 months.

The aim of this study was to culture bacteria under aerobic and anaerobic conditions on several generalized media. However, given that many bacteria were likely to require specific growth conditions in order to grow, samples were also sent for NGS in order to identify those bacteria that were unculturable. The quantity of bile salts in each sample was also analysed to see if this had an affect on the growth of bacteria.

2.2   Methods and Materials

2.2.1   Patient selection

Patients undergoing liver resection, pancreatic surgery or laparoscopic cholecystectomy at the Norfolk and Norwich University hospital or Addenbrookes hospital were screened. Patients were excluded if they had active infection or had received antibiotics within the previous 6 weeks, if they had biliary intervention (ERCP, sphincterotomy, biliary stents), if they had previous biliary surgery, or had any episodes of jaundice preceding surgery.

2.2.2   Sample collection

Samples were collected at the time of surgery. For common bile duct samples, the CBD was identified and dissected. The gallbladder was clamped and either a sterile needle was inserted (during open resection surgery) or the duct was partially cut and a sterile catheter inserted. Up to 5 ml of bile was aspirated prior to cholangiography.

Bile samples were obtained from the gallbladder via a sterile needle once it had been removed from the patient.

Samples were then transferred to a sterile bijou which had been placed for 24 hours in an anaerobic cabinet.

2.2.3   Metataxonomics

2.2.3.1   Media Preparation

To try and maximize the potential growth of bacteria, 4 broad spectrum non-selective media were chosen. Luria - Bertani (LB) broth is rich in nutrients, and the most widely used medium for the growth of bacteria. De Man, Rogosa and Sharpe (MRS) agar is a medium developed primarily for the cultivation of lactobacilli, but is also used for the cultivation of the whole group of lactic acid bacteria. It is not selective at pH values greater than 5.7 (Progress in Industrial Microbiology Vol 37,

2003). Brain Heart Infusion (BHI) medium is a general-purpose nutrient medium recommended for the cultivation and isolation of a variety of micro-organisms. Blood agar is an enriched, bacterial growth medium that encourages the growth of fastidious organisms such as *Streptococci* and *Haemophilus.* The composition of each medium in shown in Table 2-2.

Media	Composition in 1L H2O	pH
LB agar	Tryptone 10g, Yeast extract 5g, Sodium Chloride 10g Agar 15g	
MRS agar	Peptone 10g, "Lab-Lemco" powder 8g, Yeast extract 4g, Glucose 20g, Sorbitol mono-oleate 1ml, Di-potassium hydrogen phosphate 2g, Sodium acetate H_2O 5g, Tri-ammonium citrate 2g, Magnesium Sulphate $7H_2$) 0.2g, Magnesium Sulphate $4H_2$) 0.05g, Agar 15g	6.2 +/- 0.2
BHI agar	Brain infusion solids 12.5g, Beef heart infusion solids 5g, Proteose peptone 10g, Sodium Chloride 5g, Glucose 2g, Disodium Phosphate 2.5g, Agar 15g	7.4 +/- 0.2
Blood agar	Pancreatic Digest of Casein 14.5g, Peptic digest of Soybean Meal 5g, Sodium Chloride 5g, Koenzyme Enrichments 1.5ml, Horse Blood 50ml, Agar 14g	7.4 +/- 0.2

Table 2-2 Compositions of bacterial culture media.

2.2.3.2   Sample preparation

Samples were transferred to the anaerobic cabinet and 100ul of each sample was diluted in 900ul of PBS to create dilutions of 1/10, 1/100, 1/000. 100ul of samples were then transferred to BHI, MRS and blood plates as well as liquid BHI. The diluted samples were then also plated aerobically on LB, BHI, MRS and blood plates. 100ul of undiluted samples was inoculated into liquid BHI. Plates were then left to grow at 37°C, and examined for growth at 24 and 48 hours. The remaining bile samples were then frozen at -20°C for use in metagenomics studies later.

Colonies were sampled using a sterile loop and replated to ensure a single colony was isolated. Total numbers of colonies were counted to calculate the number of colonies per ml of bile.

A single colony was inoculated into 10ml of BHI and incubated at 37°C for 24 hours. 500ul of sample was then transferred to a 2ml eppendorf containing 500ul 40% glycerol and frozen on dry ice before being transferred to -80°C freezer to form frozen glycerol stocks for future use in bile resistance studies.

2.2.3.3 Colony PCR

Colonies were identified through single colony 16S rDNA PCR. A single colony was isolated using a sterile toothpick and transferred to 10ul of ultra-pure H_2O. This was then boiled at 95°C for 5 minutes, and 1ul was used as a template for PCR.

The PCR reaction was set up as follows;

Reagent	Quantity (ul)
Template	1
5x buffer	10
dNTP	0.4
Primer Forward	1
Primer Reverse	1
goTaq	0.25
upH20	36.35
Total	50

Table 2-3 PCR amplification materials

The following universal primers were used as per *Baker et al* (2004)

AMP_F 5' GAG AGT TTG ATY CTG GCT CAG

AMP_R 5' AAG GAG GTG ATC CAR CCG CA

Stage Number	Number of Cycles	Temperature (°C)	Time
Stage 1	1	95	2 minutes
Stage 2	25	95	30 seconds
		55	30 seconds
		72	90 seconds
Stage 3	1	72	5 minutes

Table 2-4 PCR programme used for amplification of 16S rRNA genes

In order to confirm DNA amplification gel electrophoresis was performed. A 1% (w/v) agarose gel was prepared by dissolving 5g agarose powder in 500ml of 0.5mM Tris/borate/ethylenediaminetetraacetic acid (TBE) buffer. This was then microwaved (800 watts) for 2 minutes at full power to aid dissolution. The 1% (w/v) agarose solution was added to an electrophoresis gel tray in a horizontal gel electrophoresis system with a 6mm toothed comb fitted and allowed to cool and set. TBE (5 mM) was added to cover the gel. 2 ul Bioline Hyperladder I was added to the outer well to act as a molecular weight marker. 2 ul of DNA and 0.5 ul 10x loading dye were added to the remaining wells. The gel was run at 100 volts until the samples had migrated to the end of the gel, as indicated by the loading dye. The gels were submerged in ethidium bromide solution for 30 minutes and rinsed with water. DNA fragments were visualised and photographed using the AlphaImager HP system under ultra-violet transillumination.

Once DNA amplification was confirmed, the amplicons were purified, using QIAquick PCR purification Kit. 5 volumes of buffer PB with pH indicator were added to 1 volume of the PCR sample. If the sample was not yellow 10ul of 3M sodium acetate was added to ensure pH was <7.5. The sample was then transferred to a binding column and centrifuged at 17 900 xg for 60 seconds. The column was then washed twice with Buffer PE before DNA was eluted with 10mM Tris-HCl pH 8.5.

15ul of purified DNA and 2 ul of either forward or reverse primer (10pmol) was added to a barcode and sent to Eurofins for 16s rDNA Identification of isolate bacteria

The quality of the 16S rDNA sequencing data was initially assessed using FinchTV software (Geospiza, Inc.). The paired samples which had been sequenced with the AmpF and AmpR primers were assembled into a single contig using SeqMan

(DNASTAR, Inc). This was then checked for errors or mismatches through FinchTV.

These quality-checked sequences were then uploaded to The Ribosomal Database Project Sequence Matching tool (Cole et al., 2014). The search options enabled were both type and non-type strains, uncultured and isolate sources, near full length sequences and good quality sequences. The identification of the bacterial isolates was determined on the basis of the highest similarity (S_ab) score. An S_ab score of greater than 95% allows for classification in the same genus, below that in the same family. S_ab scores of less than 90% were discarded.

2.2.4   Metagenomics

2.2.4.1   Bacterial DNA extraction

Bacterial DNA was extracted using an optimized protocol for the Invitrogen Purelink Genomic DNA Mini Kit adapted from the manufacturers protocol for Gram Positive Bacterial Cell Lysate, with the addition of mutanolysin to promote bacterial wall breakdown.

Following the addition of 96% ethanol to Genomic Wash Buffer 1 and 2. Lysozyme digestion buffer was prepared using the following recipe:

Ingredient	Volume (in 10ml Ultra Pure H_2O)	Final Concentration
Tris-HCl 1M	250 ul	25 mM
EDTA 0.5M	50 ul	2.5 mM
Triton X-100	100 ul	1%

Table 2-5 Composition of Lysozyme Digestion Buffer

Fresh lysozyme was added to obtain a final lysozyme concentration of 20 mg/mL followed by mutanolysin at a final concentration of 10 U/mL.

Samples were thawed and 1 ml of bile was centrifuged for 15 minutes at 4°C at 14 500 xg. The supernatant was then removed and the pellet was re-suspended in 180ul of lysozyme digestion buffer. The solution was then mixed through vortexing before being left to incubate in a water bath set at 37°C for 30 minutes.

20 ul of Proteinase K (a protease widely used for digestion of proteins in nucleic acid preparations) was added to the solution. The solution was vortexed before adding 200ul PureLink Genomic Lysis/Binding Buffer. The solution was then left to incubate in a water bath set at 55°C for 30 minutes.

200 ul of 96% Ethanol was added to the lysate and mixed through vortexing for 5 seconds to yield a homogenous solution.

The lysate was added to a PureLink Spin Column in a collection tube and centrifuged at 10 000 xg for one minute at room temperature. The collection tube was discarded and the spin column placed in a clean PureLink Collection Tube.

The sample was "washed" with 500ul Wash Buffer 1 and centrifuged at 10 000 xg at room temperature for 1 minute. The spin column was placed in a fresh collection tube and 500 ul of Wash Buffer 2 was added before being centrifuged at 14 000 xg for 3 minutes at room temperature.

The spin column was placed in a sterile 1.5 mL Eppendorf tube, and 25 ul of PureLink Genomic Elution Buffer was added to the column to obtain a maximal yield of DNA. The sample was left to incubate at room temperature for 3 minutes before being centrifuged at 14 000 xg for 1 minute. The process was then repeated with another clean 1.5mL Eppendorf in order to recover more DNA. The sample was centrifuged at 14 000 xg for 90 seconds.

The concentration of DNA in the samples was assessed via Nanodrop and the purified DNA stored at -20C.

2.2.4.2   Amplification and sequencing of 16S rDNA gene regions

PCR amplification of the 16S rRNA gene V4 region, and subsequent DNA sequencing for the first 12 samples was performed at the Animal Health and Veterinary Laboratories Agency (AHVLA) using 454 pyrosequencing as per Ellis et al (2013)

Subsequently all samples were sent to the Earlham Institute (Norwich), and DNA sequencing was performed using the MiSeq Illumina platform as per standard protocols (Caporaso et al., 2012)

2.2.4.3   Bioinformatic analysis of 16S rDNA data

Samples were analysed using Quantitative Insights Into Microbial Ecology (QIIME), an open-source bioinformatics pipeline for performing microbiome analysis from raw DNA sequencing data. QIIME enables the analysis of raw data generated by sequencing platforms to generate graphics and statistics including taxonomic assignment, relative number of operational taxonomic units (OTUs) and diversity analyses.

2.2.5   Bile salt analysis

2.2.5.1   Sample preparation

1 ml of bile was centrifuged at 14 000 xg for 15 minutes at 4°C. The supernatant was then removed and transferred to fresh tubes that were frozen at -20°C prior to analysis.

2.2.5.2   Bile salt Mass-Spectrometry analysis

The composition of bile acids in the gallbladder and common bile duct samples were determined using HPLC-MS/MS by Mr Mark Philo at the Quadram Institute. In summary, a 20 ul aliquot was taken and diluted with 5% methanol – 1000x for bile duct samples and 10 000 xg for gallbladder samples. Reference standards of Lithocholic acid (LCA), cholic acid (CA), chenodeoxycholic acid (CDCA), deoxycholic acid (DCA), Glycochenodeoxycholic acid (GCDCA) were added at a final concentration of 0.5ug/ml. All samples were analysed according to the instrument and standard preparation conditions for the determination of bile acids in mouse liver and digestive tract (see Standard operating procedure in Appendix 2)

2.3 Results

2.3.1 Optimization of DNA extraction

Several attempts at DNA extraction were made before an optimal protocol was achieved. Initially bacterial DNA was extracted using a modified protocol for FastDNA Spin Kit for soil (MPBio). Wu et al (2013) had used lysis through mechanical disruption in order to extract DNA from gallbladder bile samples and this was incorporated into the method used for this study.

Initially 200ul of bile obtained from the gallbladder was defrosted and used as had been described in previous studies. (Wu et al., 2013). Unfortunately, this did not yield a measurable concentration of DNA, therefore 1ml was used and centrifuged for 15 min at 14 500 xg at 4°C. The supernatant was removed and the pellet re-suspended in 978ul of sodium phosphate buffer. 122ul of MT buffer was added and vortexed for 20 seconds. This solution was then left to stand for 1 hour at 4°C (being vortexed every 15 mins).

Approximately 1ml of sample was then transferred into a Lysing Matrix E Tube.

Samples were then lysed using the FastPrep Instrument for 1 minute at 6.5m/s. This was repeated 3 times allowing the samples to cool for 5 mins in between steps.

The lysing matrix tubes were then centrifuged at 14 900 xg for 1 minute. The supernatant was then transferred to a clean Eppendorf tube. 250ul of PPS reagent was added and the solution hand mixed 10 times.

The solution was then recentrifuged at 14 900 xg for 5 minutes to pellet precipitate. The supernatant was then transferred to a sterile 15ml tube. 1 ml of Binding Matrix Suspension was added to the supernatant and inverted by hand for 2 minutes. The tubes were then left to stand for 3 minutes to allow the settling of the silica matrix.

1 ml of the supernatant was then removed and discarded. The binding matrix was re-suspended in the remaining supernatant.

600ul of mixture was then transferred to into a "spin filter tube" and centrifuged for 1 minute. The matrix was washed 3 times with 500 ul SEWS-M and centrifuged at 14 500 xg for 1 minute with each wash. Following the final wash, the tubes were

centrifuged a final time at 14, 500 xg for 2 minutes to "dry" the matrix of residual SEWS-M wash solution.

The spin filters were then left to air dry for 2 mins before 50 ul of DNase/Pyrogen Free water was added. The tube was then centrifuged for 1 minute in order to elute DNA.

Good results were obtained from initial samples from the gallbladder, although DNA yields were low in comparison to faecal samples. However, when repeat DNA extraction was performed on samples isolated from undiseased bile ducts, the levels of DNA were often unrecordable by using Nanodrop (<0.1 mg/ul)

A review of the literature revealed that another group had extracted DNA from 400 ul noncentrifuged bile, obtained during ERCP for common bile duct stones, using the Invitrogen Purelink Genomic DNA Mini Kit, following manufactor's blood DNA extraction protocol. (Shen et al., 2015)

However, this technique resulted in similar yields of DNA as obtained from mechanical disruption. A hybrid method was performed whereby DNA extraction was attempted through enzymatic lysis followed by mechanical disruption, with repeated poor yields. Given that the majority of bacteria previously isolated were gram positive, a method was adapted based on lysozyme digestion which is described in section 2.2.4.1.

2.3.2   Demographics

In total 55 patients were screened for this study. One patient did not consent for the study. 12 patients were immediately excluded due to antibiotics or previous biliary intervention, a further 3 patients were excluded at the time of surgery due to a decision not to proceed with resection due to cancer progression and six patients were then excluded as the gallbladder was perforated at the time of removal, or biliary samples were unable to be aspirated from the common bile ducts.

Therefore 39 samples of bile were obtained from 33 patients (6 patients had samples taken from the gallbladder and common bile duct).

The bile samples were taken from 4 groups depending on pathology; "normal" Gallbladder (GB), "normal" Common bile duct (CBD), "diseased" Gallbladder, and "diseased" Common bile duct. The pathological reason for surgery are summarized in Table 3-6, whilst the demographics of patients are summarized in Table 3-7.

Due to the relatively small numbers in the "normal' GB and "diseased" CBD groups, there is a discrepancy in average age. However, the age range across all groups is comparable. The male: female ratio, smoking history, and alcohol consumption is similar across the groups.

Bile Sample Source	"Normal" GB n = 5	"Normal" CBD n = 15	"Diseased" GB n = 14	"Diseased" CBD n = 4
Surgical procedure	Liver Resection n = 5	Cholecystectomy n = 13	Cholecystectomy n = 15	ERCP
Reason for surgery	• **Colorectal Cancer metastases x 3** • **Adenoma** • **Carcinoid**	• Gallstones x 13	• Gallstones x 13 • Chronic Cholecystitis • Polyp	• Biliary stricture in PSC
Surgical procedure		Pancreatic resection n = 2		Cholecystectomy n = 3
Reason for surgery		• Duodenal adenoma • Pancreatic cancer		• Gallstones with positive cholangiogram x 3

Table 2-6 Bile sample source, surgical procedure and underlying pathology

Patient Group	"Normal" GB N = 5	"Normal" CBD N = 15	"Diseased" GB N = 15	"Diseased" CBD N = 4
Average Age (Years)	67 (37 – 82)	61 (33 – 76)	59 (37 – 82)	47 (25 – 78)
Male:Female ratio (%)	40:60	53:47	47:53	25:75
Smoking % (Current, Never, Ex)	20:60:20	7:80:13	13:80:7	0:100:0
Alcohol % (YES, NO)	20:80	40:60	40:60	0:100

Table 2-7 Demographics of patients

2.3.3 Metataxonomics

2.3.3.1 Bacterial growth

In total 149 discrete morphological colonies were identified from the 39 samples. On obtaining 16S PCR sequencing results some of these were identified as the

same species grown from the same sample but on different media. Therefore, a total of 115 bacterial taxa were grown from 39 samples and of these, 91 were identified with an S_ab score of greater than 95%. Of the remaining 24 samples, nine of these the microbial growth spread to form a lawn on the agar plates, and on microscopic examination had hyphae in keeping with them being fungi. The remaining 14 samples had S_ab scores of less than 80% despite repeat PCR and sequencing.

The bacteria isolated from the different samples are summarized in Table 3-8. The frequencies of bacteria by diseased group are summarized in Table 3-9.

The most abundant genera isolated were *Staphylococcus, Bacillus, Micrococcus* and *Enterococcus.* Samples taken from "diseased" gallbladders tended to contain more bacteria (in terms of colonies per ml) than was found in "diseased" common bile ducts. In total 79 different species were isolated across all patient groups; 24 different species (excluding unidentified bacteria) were isolated from "diseased" gallbladders, 16 from "normal" common bile ducts, 10 from "diseased" common bile ducts and 6 from "normal" gallbladder. Increased bacterial growth was observed on BHI and blood media agar. All anaerobic bacteria were subsequently found to grow in aerobic conditions making them facultative anaerobes. This also suggests that any obligate anaerobes present may have not survived the transfer from the surgical theatre to the anaerobic cabinet.

Interestingly, several samples, particularly those taken from the common bile duct, contained more bacterial colonies per ml when plated at 1/1000 dilution compared to 1/10, thereby suggesting an inhibitory effect on replication by a substance within the sample. This also means that it was not possible to accurately calculate the numbers of colonies per ml.

Samples 2 (Hepatic resection for carcinoid) and 10 (Primary Sclerosing Cholangitis and ERCP) grew motile bacteria; *Paenibacillus lactis* and *Pseudomonas aeroginosa.* Several other colonies were also present on these samples, but these were unable to be isolated and identified due to contamination from the motile bacteria. For sample 25 (gallbladder and common bile duct) a similar problem was encountered however repeat plating eventually yielded single morphological colonies.

Table 2-8 (Over 5 pages) Morphology of colonies, bacteria Identified by 16S rRNA gene sequencing, similarity scores (s_ab) of extracted sequences and origin of sample. Colonies marked "unidentifed" either had sequences reads that were too short or s_ab scores that were below 0.80. GB = gallstones, CBD = common bile duct, PSC = primary sclerosing cholangitis. ERCP = endoscopic retrograde cholangiopancreatogram

Sample ID	Morphology	Sequencing result	s_ab score	Origin
RW1	Small white circular	*Intrasporangiacae janibacter*	0.974	Gallstones and cholecystitis
RW2	Small white circular	*Janibacter sanguinis*	0.981	Gallstones and cholecystitis
RW3	Swarming	*Paenibacillus lactis*	0.963	Carcinoid
RW4	Large white circular	*Enterococcus faecium*	0.983	Gallstones and cholecystitis
RW6	Small translucent	*Unidentified*	N/A	Gallstones and cholecystitis
RW7	Small translucent	*Citrobacter freundii*	0.989	Gallstones and cholecystitis
RW8	Small cream circular	*Enterobacter asburiae*	0.94	Gallstones and cholecystitis
RW10	Small white circular	*Enterococcus faecalis*	0.986	Gallstones and cholecystitis
RW11	Swarming	*Proteus*	0.896	Gallstones and cholecystitis
RW15	Large yellow	*Unidentified*	N/A	Gallstones and cholecystitis
RW16	Large irregular	*Clostridium perfringens*	0.983	Gallstones and cholecystitis
RW17	Large irregular	*Cronobacter sakazakii*	0.98	Gallstones and cholecystitis
RW19	Small white circular	*Enterococcus faecalis*	1	Gallstones and cholecystitis
RW20	Large white circular	*Bacillus Circulans*	0.987	Benign lesion
RW23	Cream circular	*Staphylococcus epidermidis*	1	gallstones and cholecystitis

RW24	Large white circular	*Stapylococcus hominis*	0.979	gallstones and cholecystitis
RW25	Yellow circular	*Micrococcus luteus*	0.986	gallstones and cholecystitis
RW26	Swarming	*Paenibacillus lactis*	0.965	Gallstones and cholecystitis
RW27	Cream circular	*Staphylococcus epidermidis*	0.994	Colorectal cancer metastases
RW29	White circular	*Staphylococcus hominis*	1	PSC and stents
RW30	Small white circular	*Enterococcus faecium*	0.983	PSC and stents
RW31	Mucoid	*Pseudomonas aeroginosa*	0.998	PSC and stents
RW33	Yellow circular	*Micrococcus luteus*	0.973	Colorectal cancer metastases
RW34	white semi opaque	*Staphylococcus epidermidis*	1	Colorectal cancer metastases
RW35	White circular	*Staphyloccis hominis*	0.976	Chronic cholecystitis
RW36	White fan-like	*Corynebacterium aurimucosum*	0.967	Chronic cholecystitis
RW39	Large white circular	*Staphylococcus epidermidis*	0.994	Chronic cholecystitis
RW40	Yellow circular	*Staphylococcus pasteuri*	0.997	Colorectal cancer metastases
RW41	White circular	*Bacillus licheniformis*	1	Colorectal cancer metastases
RW43	Off white circular	*Staphylococcus pasteuri*	0.994	Duodenal adenoma
RW44	White circular	*Staphylococcus epidermidis*	0.994	Duodenal adenoma
RW45A	White circular	*Staphylococcus epidermidis*	0.996	Pancreatic Cancer
RW45B	Yellow circular	*Acinetobacter iwoffi*	0.987	Pancreatic Cancer

RW45C	Yellow circular	*Micrococcus luteus*	0.996	Pancreatic Cancer
RW46L	White small	*Staphylococcus epidermidis*	0.994	Pancreatic Cancer
RW46S	Yellow large	*Staphylococcus pasteuri*	0.997	Pancreatic Cancer
RW48	White circular	*Staphylococcus hominis*	1	Gallstones CBD sample
RW50	Yellow circular	*Staphylococcus capitis*	0.996	Gallstones CBD sample
RW51	Cream circular	*Bacillis firmis*	1	Gallstones CBD distal stone
RW52	Yellow circular	*Micrococcus luteus*	0.988	Gallstones CBD distal stone
RW53	Small white	*Lactobacillus rhamnosus*	1	Gallstones CBD sample
RW54	Large white	*Staphylococcus hominis*	0.999	Gallstones CBD sample
RW56	White circular	*Unidentified*	N/A	Gallstones CBD sample
RW57	Yellow circular	*Unidentified*	N/A	Gallstones CBD sample
RW58	Small white	*Unidentified*	N/A	Gallstones CBD sample
RW59	Large yellow	*Micrococcus luteus*	1	Gallstones CBD sample
RW60	Large white	*Staphylococcus epidermidis*	1	Pancreatitis CBD
RW62	White circular	*Staphylococcus lugdunesis*	1	Pancreatitis CBD
RW63	Yellow circular	*Micrococcus yunnanensis*	1	Pancreatitis CBD
RW65	White ragged	*Unidentified*	N/A	Pancreatitis CBD
RW66	White circular	*Staphylococcus hominis*	1	Pancreatitis CBD

RW68	White circular	*Staphylococcus hominis*	0.991	GS CBD distal stone
RW69	Yellow circular	*Micrococcus luteus*	0.984	GS CBD distal stone
RW70	White circular	*Staphylococcus epidermidis*	0.999	GS CBD distal stone
RW71	Yellow ragged	*Massila sp*	0.984	GS CBD distal stone
RW73	Large white	*Klebsiella varicola*	0.981	Gallstones - Prev ERCP
RW74	Small white	*Enterococcus faecalis*	0.991	Gallstones - Prev ERCP
RW83	Yellow circular	*Micrococcus luteus*	0.996	Gallstones GB sample
RW84	White circular	*Staphylococcus epidermidis*	0.989	Gallstones GB sample
RW85	Small yellow circular	*Staphylococcus aureus*	0.995	Gallstones GB sample
RW86	Yellow Circular	*Staphylococcus epidermidis*	1	Gallstones CBD sample
RW87	Yellow circular	*Staphylococcus aureus*	0.995	Gallstones CBD sample
RW88	Large white circular	*Bacillus cereus*	1	Gallstones
RW89	Grey ragged	*Unidentified*	N/A	Gallstones
RW92	Small off white	*Streptococcus sanguinis*	1	Gallstones
RW93	Large white circular	*Staphylococcus hominis*	1	Gallstones
RW94	White circular	*Unidentified*	N/A	Gallstones CBD sample
RW95	Grey circular	*Staphylococcus epidermidis*	0.999	Gallstones CBD sample
RW96	White circular	*Variovorax*	1	Gallstones CBD sample

RW99	White circular	*Staphylococcus capitis*	1	Gallstones CBD sample
RW100	Grey circular	*Staphylococcus hominis*	1	Gallstones GB sample
RW101	White fan-like	*Acintomyces viscosus*	1	Gallstones GB sample
RW102	Yellow circular	*Neisseria perflava*	0.996	Gallstones GB sample
RW103	White Spreading	*Unidentified*	N/A	Gallstones GB sample
RW104	White circular	*Bacillus subtilis*	1	Gallstones GB sample
RW105	Yellow circular	*Micrococcus luteus*	1	Gallstones GB sample
RW106	Bright White	*Staphylococcus haemolyticus*	1	Gallstones GB sample
RW107	Spreading	*Unidentified*	N/A	Gallstones CBD sample
RW108	Spreading	*Unidentified*	N/A	Gallstones CBD sample
RW109	Spreading	*Unidentified*	N/A	Gallstones GB sample
RW110	Off white circular	*Bacillus cereus*	1	Gallstones GB sample
RW111	Spreading	*Unidentified*	N/A	Gallstones GB sample
RW112	Spreading	*Unidentified*	N/A	Gallstones GB sample
RW113	Spreading	*Unidentified*	N/A	Gallstones GB sample
RW114	Spreading	*Unidentified*	N/A	Gallstones CBD sample
RW115	Yellow small circular	*Corynebacterium imitans*	0.926	Gallstones CBD sample
RW116	White circular	*Enterococcus faecalis*	0.995	Gallstones CBD sample

RW117	White coned	*Rothi dentocariosa*	0.995	Ductal Stones CBD
RW118	White circular	*Staphylococcus epidermidis*	0.992	Ductal Stones CBD
RW119	Small white circular	*Bacillus cereus*	1	Ductal Stones CBD
RW120	White circular	*Staphylococcus epidermidis*	1	Gallstones GB
RW121	Yellow small circular	*Corynebacterium imitans*	0.968	Gallstones GB
RW122	Yellow circular	*Bacillus subtilis*	0.97	Gallstones GB
RW123	Yellow circular	*Micrococcus luteus*	1	Ductal Stones GB
RW124	Dot like	*Staphylococcus salivarius*	1	Ductal Stones GB
RW125	Yellow circular	*Bacillus cereus*	0.998	Ductal Stones GB
RW126	White circular	*Staphylococcus pasteuri*	0.989	Ductal Stones GB
RW127	White circular	*Staphylococcus epidermidis*	1	Ductal Stones GB
RW128	White circular	*Staphylococcus salivarius*	0.947	Gallstones GB
RW129	Ragged White	*Staphylococcus hominis*	0.988	Gallstones GB
RW130	Haemolysis	*Bacillus cereus*	0.87	Ductal Stones CBD
RW131	Haemolysis	*Bacillus cereus*	1	Gallstones GB
RW132	White ragged	*Unidentified*	N/A	Gallstones CBD
RW133	White circular	*Unidentified*	N/A	Gallstones CBD sample
RW134	White ragged	*Unidentified*	N/A	Gallstones GB sample
RW135	White circular	*Unidentified*	N/A	Gallstones GB sample

RW136	Yellow circular	*Micrococcus luteus*	1	Gallbladder polyp
RW139	White circular	*Staphylococcus epidermidis*	1	Gallbladder polyp
RW140	Spreading	*Unidentified*	N/A	Gallbladder polyp
RW141	White circular	*Staphylococcus hominis*	0.986	Gallbladder polyp
RW142	White circular	*Unidentified*	N/A	Gallbladder polyp
RW144	White circular	*Staphylococcus caprae*	1	Gallstones CBD sample
RW146	Yellow circular	*Corynebacterium imitans*	0.832	Gallstones CBD sample
RW147	White with haemolysis	*Staphylococcus haemolyticus*	1	Gallstones CBD sample
RW149	White circular	*Unidentified*	N/A	Gallstones CBD sample

"Normal" GB n = 5	"Diseased" GB n = 15	"Normal" CBD n = 15	"Diseased" CBD n = 4
Bacillus circulans Micrococcus luteus Staph epidermidis Staph pasteuri Bacillus licheniformis Paenibacillus lactis	Staph epidermidis x 6 Micrococcus luteus x 4 Staph hominis x 4 Bacillus cereus x 3 Staph salivarius x 2 Bacillus subtilis x 2 Enterococcus faecium x 2 Citrobacter freundii Clostridium perfringens Corynebacterium aurimucosum Corynebacterium imitan Cronobacter sakazakii Enterobacter asburiae Enterococcus faecalis Intrasporangiacae janibacter Janibacter sanguinis Neisseia perflava Paenibacillus lactis Proteus Staph aureus Staph haemolyticus Staph pasturi Unidentified x 11	Staph epidermidis x 6 Staph hominis x 4 Micrococcus luteus x 4 Corynebacterium imitans x 2 Staph capitis x 2 Staph haemolyticus x 2 Staph pasteuri x 2 Bacillus cereus Entererococcus faecalis Lactobacillus rhamnosus Micrococcus yunnanensis Staph aureus Staph caprae Staph lugdenesis Strep sanguinis Variovorax Unidentified x 12	Staph epidermidis x 2 Staph hominis x 2 Enterococcus faecalis x 2 Bacillus firmis Bacillus cereus Entereococcus faecium Klebsiella varicola Massila sp Micrococcus luteus Pseudomonas aeroginosa Rothi dentocariosa

Table 2-9 Frequency of bacteria per disease cohort

2.3.4 Metagenomics

In total, all 39 samples underwent DNA extraction using the optimised protocol for DNA extraction from blood, and were sent for amplification of the V4 and V5 variable regions of the 16S rRNA gene before being sequenced commercially via the MiSeq illumina platform. Prior to this, 12 gallbladder samples had been sent for sequencing via the 454-pyrosequencing platform using the modified protocol for soil. Enough extracted DNA remained from 4 of these samples to send for sequencing via Illumina, thereby allowing direct comparison of the 2 techniques. Table 3-10 summarizes the total sequence reads per sample.

Sample	Number of reads per sample	
	Illumina	454 pyrosequencing
Gallstones + cholecystitis Gallbladder bile	92568	9288
Hepatic adenoma Gallbladder bile	93181	3236
Carcinoid Gallbladder Bile	72172	12140
Colorectal Cancer Metastases Gallbladder Bile	173776	1881

Table 2-10 Sequence reads per sample comparing Illumina versus 454 pyrosequencing methods. Numbers were generated using Qiime

Illumina generated sequence reads roughly 10 times the number of sequence reads per sample than that of 454 pyrosequencing.

The quantity of DNA was generally low when quantified using nanodrop (ranging between 1.3 and 150.1 ng/ul) when compared to faecal samples where the concentrations were > 250 ng/ul (See Appendix for full list of results). Each sample had the DNA extracted twice using the revised method. In total, 27/39 samples passed quality control and were sent for library reconstruction; 5 "normal" GB samples, 5 "diseased" GB samples, 14 "normal' CBD samples and 3 "diseased" CBD samples. The biggest fail rate was within the "diseased" GB cohort (10/15 samples), but this may reflect the fact these samples had been stored for the

longest period of time at -20°C, or had had been thawed the largest number of times which may have led to DNA degradation.

The sequences were analysed using the QIIME pipeline (section 3.4.2). This produced 3317945 high quality reads, with an average of 114412 ± 51247 reads per sample.

2.3.4.1   Biliary microbiota composition

The relative abundance of each bacterial genus within each sample is represented as a percentage proportion of each operational taxonomic unit within the microbiota as a whole. The samples have been separated into disease groups (Figure 2-2). In order to assess the differences in microbiota across groups the mean proportion of each phylum and genus was calculated and displayed as a pie chart (Figures 2-3 and 2-4).

In total six phyla were isolated across the disease groups. The most abundant phylum in all the disease groups was Proteobacteria, followed by Firmicutes. There was a marked shift in Proteobacteria: Firmicutes ratio in the "diseased" gallbladder group compared to the other 3 groups. Bacteroidetes only made up a small proportion of all bacterial taxa.

Figure 2-4 Pie charts showing relative abundance of each bacterial phylum by disease group. Each colour represents a bacterial phylum weighted by percentage contribution to overall bacterial population.

In total, 34 genera had percentage proportions greater than 0.5%. The most diverse microbiota was found in the "Normal" GB group, with diversity observed within both CBD groups.

Across the 4 groups the most abundant genus was *Pseudomonas,* representing 34% (SD 25%), 69% (SD 21%), 34% (SD 39%), 51% (SD 33%) for "normal" GB, "normal" CBD, "diseased GB" and "diseased" CBD, respectively. The standard deviations suggest that there is variability throughout the samples in regards to proportion of *Pseudomonas.* However, when looking at the samples individually *Pseudomonas* is the most prevalent genus in 13/14 "Normal" CBD samples, 4/5 "Normal" GB samples, 2/5 "Diseased" GB, and 3/3 "Diseased" CBD.

There is an emerging dysbiosis in the "Diseased" GB group with the relative abundance of *Enterococcus* being greater than the other groups (21% ± 33%) although this does not reach statistical significance (p = 0.3). This is reflected in 2/5 samples having *Enterococcus* as the predominant genus.

The variation in *Pseudomonas* is generally related to changes in one other genus within the group. In the "Diseased" GB group *Pseudomonas* is decreased due to an increased abundance of *Enterococcus.* Within the CBD groups there is an increase in relative abundance of *Pseudomonas* due to a lack of *Klebsiella* when compared to the GB groups.

When the standard deviations of the remaining genera are compared, there is a stability of the proportions of these bacteria across the samples, with 20 of the genera having SD < 1%. The most prevalent genera after *Pseudomonas, Enterococcus* and *Klebsiella* are *Janthinobacterium* (5.3% SD 2%), *Ruminococcus* from the *Ruminococcaceae* family (3% SD 1.5%) and *Acinetobacter* (2% SD 0.8%). The "Other" bacteria (those that were present at less than 0.5% of total operational taxonomic units) made up 5.1% ± 2.7%.
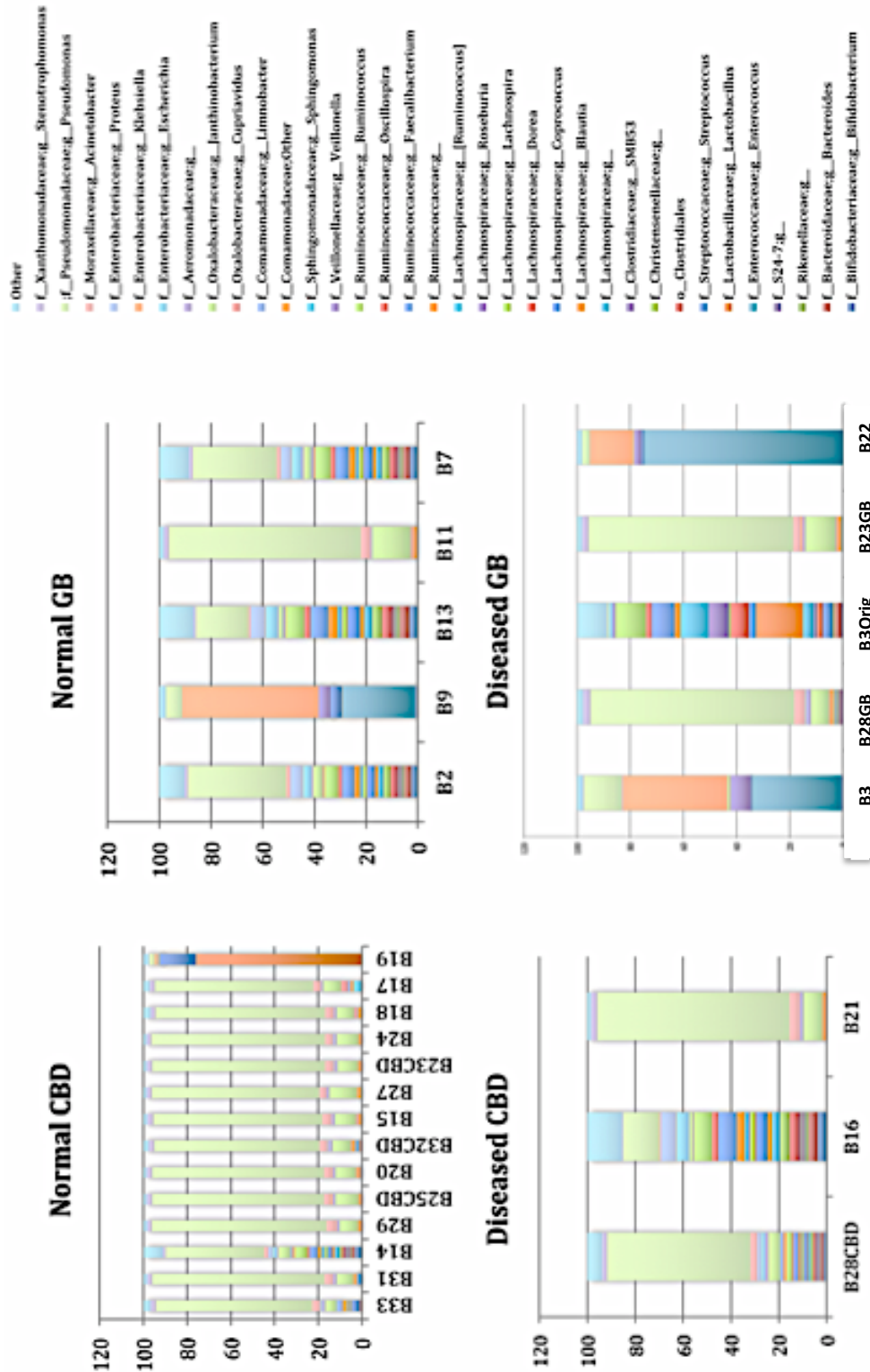
Figure 2-5 Column charts representing bacterial taxa identified in each sample through sequencing of 16S rDNA. Each colour represents a bacterial taxon weighted by % contribution to total bacterial population. Only bacterial taxa contributing >0.5% are shown. Those taxa representing <0.5% are shown as "other"

Legend (taxa):
- f__Bifidobacteriaceae;g__Bifidobacterium
- f__Bacteroidaceae;g__Bacteroides
- f__Rikenellaceae;g__
- f__S24-7;g__
- f__Enterococcaceae;g__Enterococcus
- f__Lactobacillaceae;g__Lactobacillus
- f__Streptococcaceae;g__Streptococcus
- o__Clostridiales
- f__Christensenellaceae;g__
- f__Clostridiaceae;g__SMB53
- f__Lachnospiraceae;g__
- f__Lachnospiraceae;g__Blautia
- f__Lachnospiraceae;g__Coprococcus
- f__Lachnospiraceae;g__Dorea
- f__Lachnospiraceae;g__Lachnospira
- f__Lachnospiraceae;g__Roseburia
- f__Lachnospiraceae;g__[Ruminococcus]
- f__Ruminococcaceae;g__
- f__Ruminococcaceae;g__Faecalibacterium
- f__Ruminococcaceae;g__Oscillospira
- f__Ruminococcaceae;g__Ruminococcus
- f__Veillonellaceae;g__Veillonella
- f__Sphingomonadaceae;g__Sphingomonas
- f__Comamonadaceae;Other
- f__Comamonadaceae;g__Limnobacter
- f__Oxalobacteraceae;g__Cupriavidus
- f__Oxalobacteraceae;g__Janthinobacterium
- f__Aeromonadaceae;g__
- f__Enterobacteriaceae;g__Escherichia
- f__Enterobacteriaceae;g__Klebsiella
- f__Enterobacteriaceae;g__Proteus
- f__Moraxellaceae;g__Acinetobacter
- f__Pseudomonadaceae;g__Pseudomonas
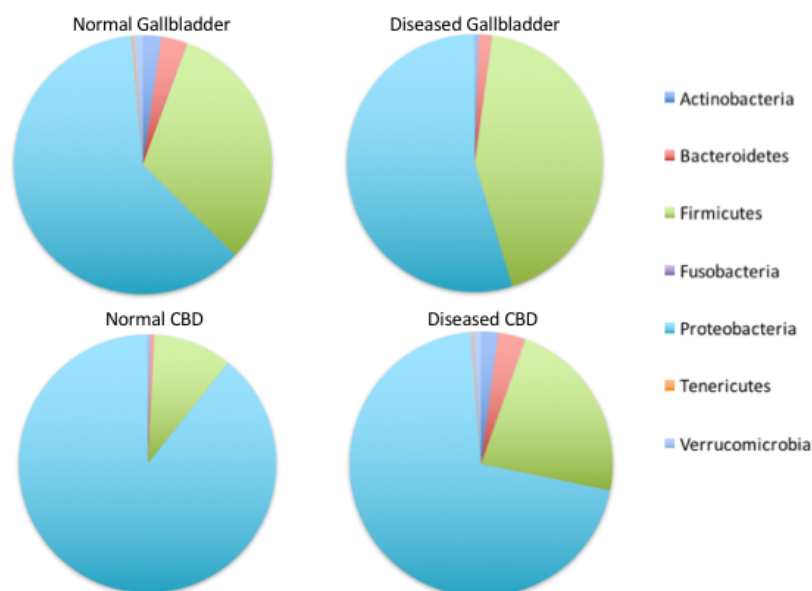- f__Xanthomonadaceae;g__Stenotrophomonas
- Other

Figure 2-6 Pie charts showing relative abundance of each bacterial taxa by disease group. Each colour represents a bacterial taxon weighted by percentage contribution to overall bacterial population. Only those taxa that were present at >0.5% of the total bacterial population are shown individually. Bacteria that contributed <0.5% are shown as "Other"

To see if there were any similarities in the microbiota composition between samples principal coordinates analysis (PCoA) was performed on the sequencing data. When CBD and GB samples were compared, the GB samples were disparate suggesting variation within microbiota. However, in regards to the CBD samples there was definite clustering with 13/17 samples being in close proximity, thereby suggesting a core microbiome.
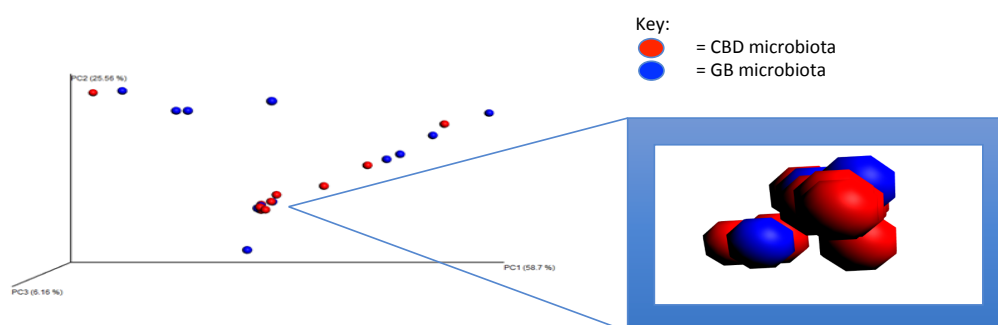
Figure 2-7: PCoA analysis comparing CBD samples (red dots) and GB samples (blue dots). The central cluster is expanded to reveal close clustering of CBD samples. Figure generated using QIIME 1.9.0 pipeline.

2.3.5   Bile Salt composition

The concentrations of bile salts in each of the 39 samples were analyzed. The average concentration of each of the bile salts by disease sample is summarized in table 2-11.

As expected, the concentrations of bile salts from the gallbladder were approximately 10 times that of the common bile duct. However, as the standard deviations for each demonstrate, there is great variation between samples. The concentration of the primary bile salt cholic acid is comparable between "normal" and "diseased" common bile ducts. There is an apparent difference between "normal" and "diseased" gallbladders (446 ug/ml vs 149 ul/ml). However, due to the standard deviations no statistical difference was observed between the groups (*P* – value of 0.75443 using unpaired 2 –tailed t test).

Deoxycholic acid, a secondary bile acid, had the most stable concentrations across the four groups. As secondary bile salts are solely produced through bacterial deconjugation and 7α dehydroxylation, this suggests that there may be a core

microbiome in human bile, and bile salt concentration does not affect the microbiota composition.

Bile Acid (ug/ml)	Normal Common Bile Duct (SD+/-)	Diseased Common Bile Duct (SD+/-)	Normal Gall Bladder (SD +/-)	Diseased Gallbladder (SD+/-)
Cholic Acid	12.96 (1.93)	17.53 (9.61)	446.72(587.65)	149.52(155.05)
Deoxycholic acid	22.15 (1.97)	22.69(2.85)	229.89(40.43)	213.32(106.53)
Glycocholic acid	2470.44	2565.47(2541.63)	33474.03(16654.38)	12200.38(22679.39)
Glycochenodeoxycholic acid	2193.30	1807.65(1751.91)	39061.25(20845.66)	10908.55(18332.28)
Glycodeoxycholic acid	2208.71(2653.35)	1483.24(2013.05)	37916.88(39887.74)	8826.06(12520.58)
Glycohyodeoxycholic acid	136.04(246.91)	66.94(81.43)	16673.00(28752.48)	1060.12(2350.53)
Glycolithocholic acid	123.71(175.09)	47.19(66.91)	873.25(1306.18)	385.38(635.75)
Glycoursodeoxycholic acid	138.62(254.90)	69.44(82.78)	16811.42(29201.51)	1102.41(2444.61)
Taurocholic acid	1147.39	927.61(839.52)	11217.64(9946.11)	6304.90(12901.01)
Taurochenodeoxycholic acid	866.55 (1096.84)	609.29(447.98)	14280.87(16236.25)	5282.68(10115.00)
Taurodeoxycholic acid	593.23(1007.82)	252.00(116.33)	1846.92(2088.08)	2414.74(4476.51)
Taurolithocholic acid	17.22(38.27)	0.00 (0.00)	25.27(56.50)	50.66(96.83)
Tauroursodeoxycholic acid	35.10(65.29)	19.04(23.30)	6906.60(13130.58)	366.42(795.42)

Table 2-11 Average concentrations of each of the bile acids (ug/ml) by disease group. Samples in orange reflect primary bile acid. The sample in blue reflect secondary bile acid. Samples in yellow reflect conjugated bile salts.

To explore this further the average proportion of each bile salt was calculated and expressed as a percentage of the total bile salt number for each disease group. This was then compared to the average sequence reads per sample produced through NGS (Figure 2.6). There is a significantly higher proportion of taurocholic acid and taurochenodeoxycholic acid in the "diseased" common bile duct (P –value *0.015)* and a non-significant lower proportion of deoxycholic acid and a corresponding rise in glycine conjugated derivatives (glycodeoxycholic acid, glycohyodeoxycholic acid, and glycoursodeoxycholic acid) in the "normal" gallbladder group (P – value *0.6).* However, this does not correlate with the average number of sequence reads per sample, which have no statistical difference between groups.

This is clearer when the samples are compared individually (Figure 2.7). There is no correlation between the proportion of primary, secondary and conjugated bile salts in these samples and the number of sequence reads per sample, meaning a larger number of secondary bile salts in a sample is not as a result of a higher number of bacteria.

Although there is a large proportion of *Pseudomonas* in "normal" common bile duct samples (section 3.5.3) there does not appear to be any difference in the bile acid composition of these samples when compared to the other groups.
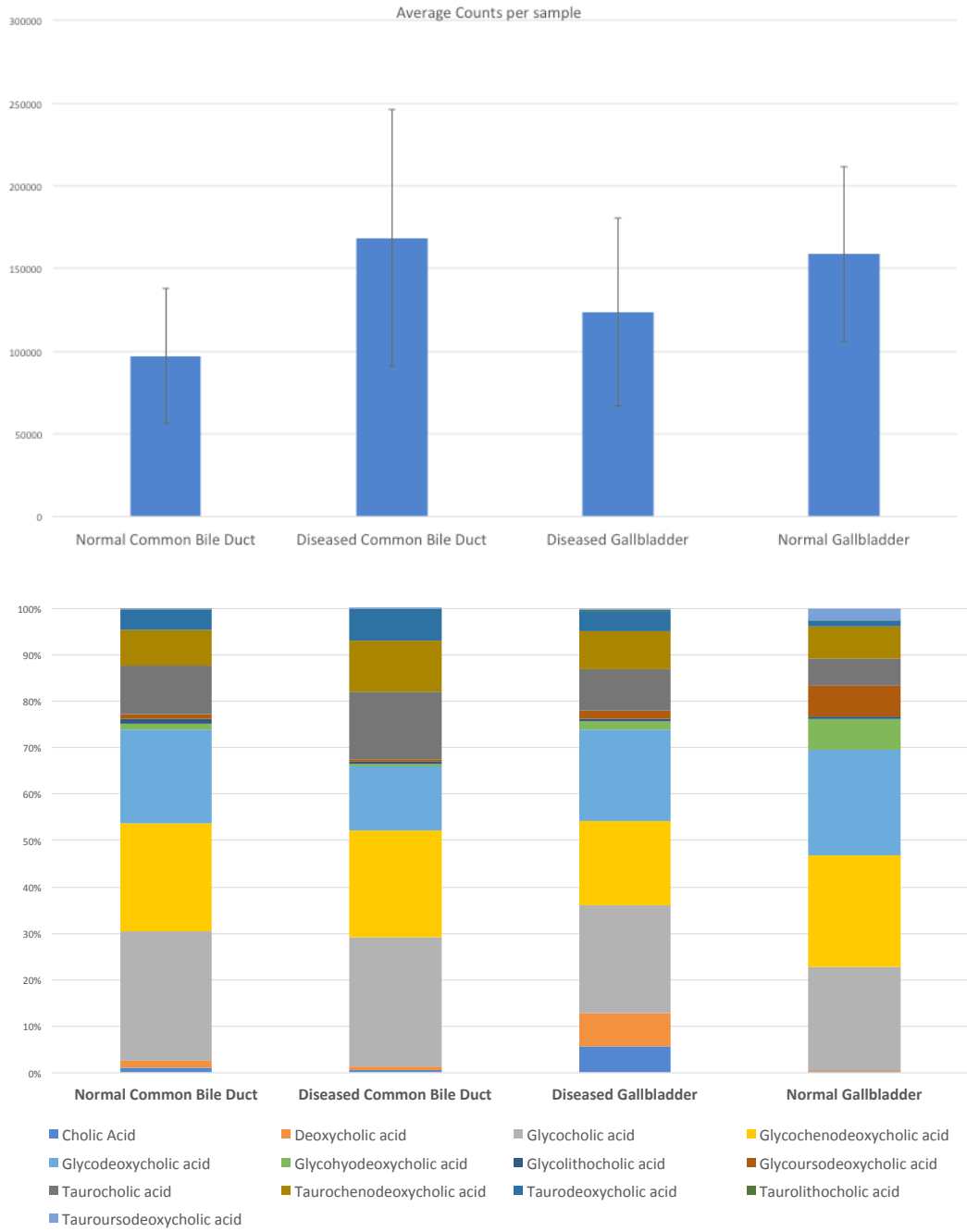
Figure 2-8 Average reads per sample by disease group and proportion of bile salts per disease group
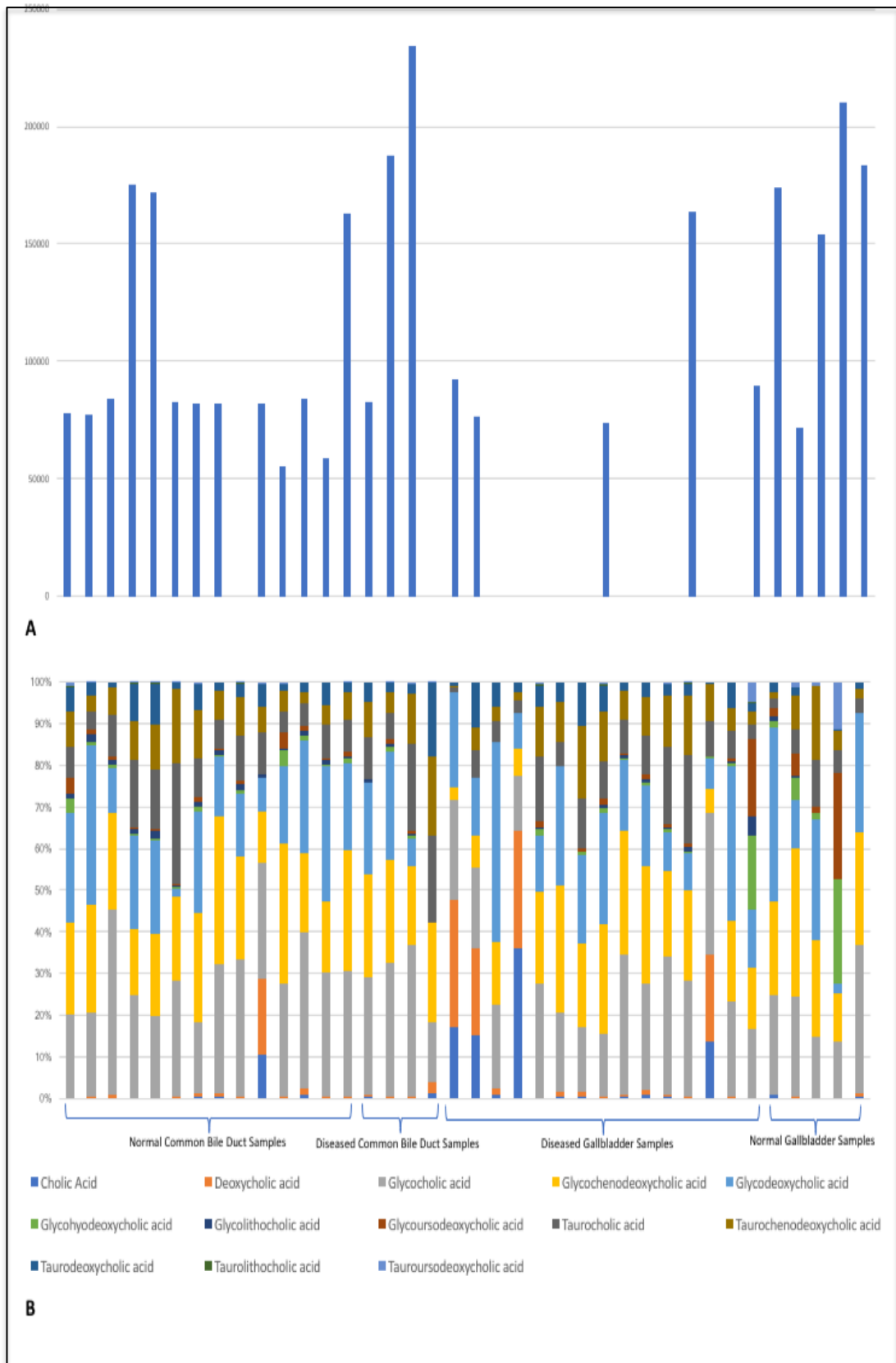
Figure 2-9 Sequence reads per sample (A) as sequenced by Illumina with corresponding proportions of bile salts per sample, with each colour corresponding to individual bile salts.

2.4 Discussion

2.4.1 Bile is not sterile

This study is the first to have shown that normal human bile is not sterile and contains a core microbiome. Culture-dependent techniques isolated a wide range of bacteria, and in patients with known gallstones or diseased biliary tract, the most frequently isolated bacteria tended to be pathogenic, due to an association with sepsis. *Klebsiella, Enterococcus, Enterobacter, Pseudomonas, Proteus*, and *Citrobacter* are all associated with biliary sepsis in humans (Csendes et al., 1996; Brook, 1989). One study reported that the bacterial family *Enterobacteriaceae* were responsible for 35% of all positive cultures taken at ERCP in symptomatic patients (Hakalehto et al., 2010). Therefore, the types of bacteria isolated in this study are in agreement with what would be expected to be found within a diseased and inflamed gallbladder.

However, there were also many bacteria from other families isolated both from the "diseased" and 'normal" systems, which are not typical of biliary sepsis. These included *Staphylococcus*, *Micrococcus*, and *Bacillus*. It could be argued that the Staphylococci*, especially *Staphylococcus epidermidis,* may represent contaminants. However, Staphylococci are frequently isolated from diseased bile ducts and gallbladders in humans (Hakalehto et al., 2010; Brook, 1989). In a recent study using pigs it was found that *Staphylococcus epidermidis* was one of the most common isolates from bile samples (Jimenez et al., 2014). The study team also swabbed the outer walls of the gallbladders to ensure this was not a contaminant, and these were found to be sterile.

When culture-dependent techniques and the results from the next generation sequencing were compared it became clear that 63% of the bacteria present in bile were not culturable. There are several explanations for this. Firstly, the study used general non-selective media and more bacteria may have been isolated if we had used additional selective media. Secondly, no obligate anaerobes were cultured, which may be due to the time taken to transfer samples from the surgical theatre to the anaerobic cabinet. Thirdly, it may be possible that some of the bacteria isolated could inhibit the growth of other bacteria within the same sample. It has been shown that bile resistant *Staphylococcus aureus* has an inhibitory effect on

coliform growth when in mixed cultures, whilst also encouraging its own growth (Hotterbeekx, A et al. 2017).

The metataxonomic data from NGS suggests that there is a diverse microbiota within normal human bile. Although the amount of DNA extracted was low when compared with faecal studies, the actual range and diversity of the bacteria isolated suggests that there is a complex microbiome within the biliary system. This is particularly evident in the "normal" gallbladder group, whereas in the "diseased" gallbladder group there is an emerging dysbiosis for *Enterococcus* and *Klebsiella.* This suggests that in the diseased gallbladder, pathogenic bacteria colonize the bile at the expense of the core microbiota. This may be due to colonization of gallstones, or impaired flow of bile and cholestasis. The loss of 10/15 "diseased" samples due to failure of quality controls may have had an impact on these results. However only 1/5 "diseased" samples showed the same high diversity as the "normal" samples and 2/5 samples had a large predominance of *Enterococcus* suggesting that bigger numbers may have made this finding more significant. Equally the small numbers of samples in the "normal" gallbladder group were still representative given that 4/5 samples showed consistent microbial diversity.

No data has been published from next generation sequencing regarding bacteria at the genus level for the biliary microbiota. However, there is some information on phyla level break down. In a study looking at bile in gallstone disease in an Eastern population, *Wu et al* (2013) measured 2493 counts per sample, whereas *Jimenez et al* (2014) measured roughly 48 000 counts per sample when examining pig bile from normal gallbladders. In comparison, this study measured an average of 136 973 counts per sample. These other studies used 454 pyrosequencing whereas this study used the Illumina platform, which is known to generate considerably more reads than 454. When comparing phyla level data, the most predominant phyla were Proteobacteria, Firmicutes, Bacteroidetes, and Actinobacteria, which corresponds with the findings in these other two studies. However, both these studies found Firmicutes to be the predominant phyla, followed by Proteobacteria in pig bile, and Bacteroidetes in the human study. When the "diseased" gallbladder group is taken in isolation there is a change in the Firmicutes: Proteobacteria ratio, which more closely resembles the findings of *Wu et al.*

Pseudomonas was the predominant genus throughout all 4 groups as well as in 21/27 samples. *Pseudomonas* is a gram-negative bacillus, and is a common inhabitant of soil and surfaces in aqueous environments (Gellatly and Hancock,

2013). It is an opportunistic pathogen that can cause serious health issues in immunocompromised patients (Hassett et al., 2010; Bicking et al., 2017), and is a leading cause of morbidity and mortality in patients with serious underlying medical conditions. It is also known to colonise the human host, for example it is the leading cause of chronic lung infection in patients with cystic fibrosis (Langton Hewer and Smyth, 2017). One of the reasons for this is that it has multiple adaptive mechanisms that make it resistant to antibiotics, drugs, and host defense mechanisms (Gellatly and Hancock, 2013). As such, it is reasonable to assume that it would be able to survive in the harsh environment of the human biliary tree. *Pseudomonas* is known to cause biliary sepsis, and in a recent study looking at gallbladder bile in Colombian patients with gallstones, *Pseudomonas* was the most prevalent isolate *(Arteta et al., 2017)*.

The bile salt concentration between samples showed great variability. The concentrations of primary bile salts were low, with greater numbers of conjugated bile salts. Given that most conjugation occurs within the liver, this is to be expected. The concentrations of secondary bile salts were relatively stable across the samples and disease groups. The fact that secondary bile salts were identified within the bile suggests that bacteria are producing these prior to excretion into the bowel. The stability across the samples also suggests that there may be a core microbiota which can utilize the primary bile salts. There did not seem to be a correlation between bile salt composition and relative proportions of bacterial taxa. Therefore, the core microbiota is able to survive within the bile ducts regardless of the composition of bile salts.

The difficulty with analysis of the microbiota of human bile is that it is not possible to obtain normal bile samples. The same accusation could be leveled at this study as even the normal groups were undergoing surgery for gallstones, pancreatic/ampullary disease or hepatic tumours. This has been minimized by selecting biliary colic patients who had not had previous biliary intervention, had never had abnormal liver function tests, had not required treatment for biliary sepsis, and had normal bile duct imaging both pre- and peri-operatively. However, it is possible that in the gallstone patients, small stones may have passed through the common bile duct causing disruption to the Sphincter of Oddi, and therefore allowing for reflux of small bowel bacteria. Equally, in the duodenal adenoma and pancreatic cancer patients there was the possibility of impaired biliary flow and subclinical cholestasis. Therefore, perhaps the most important group with regards

to sterility is the "normal" gallbladder group. Although these patients were having surgery for liver tumours, this should not affect the microbial composition of the gallbladder and biliary tract as there was no evidence of biliary disease or inflammation, and the background liver in these patients was normal. These samples therefore give a good approximation of the core biliary microbiome.

Regarding the bowel, there is a certain level of disagreement over whether the faeces, mucus layer, or mucosal biopsies are best for sampling and assessing the colonic microbiota, and the same question could be asked with regards to the biliary tract. However, in studies to date, fluorescent in situ hybridization of the diseased bile ducts has failed to identify any viable bacteria (Swidsinski et al., 2005). Equally, examination of the mucus layer of gallbladders in pigs revealed bacterial counts much lower than those found in bile (Jimenez et al., 2014).

The amount of bacterial DNA from biliary samples was much lower than other sites, such as faeces, as reflected by a third of samples failing quality control prior to NGS due to insufficient DNA. In order to try and optimize this, multiple methods were used and adapted. The method that yielded the highest results relied upon enzymatic lysis and lysozyme digestion. It is not clear if this is the best method for extracting small concentrations of DNA from samples. Optimisation of this method meant that biliary samples needed to be repeatedly thawed, which could have caused denaturation of DNA and therefore a reduction in yields.

3 Functional assessment of the biliary microbiota

3.1 Introduction

Bacteria resident in the human intestine have a dualistic relationship with bile acids. Whereas bile acids are toxic to a large proportion of pathogenic and some commensal bacteria, many have evolved to survive in the harsh environment of the gastrointestinal tract and even convey a beneficial effect through the production of secondary bile acids, and the removal of toxic bile acid compounds (see section 1.3.6)

In the previous chapter 115 different bacterial species were isolated. These bacteria would have had to adapt to be able to survive in a bile salt rich environment. This poses the question as to their mechanism of survival. Are these bacteria able to upregulate pathways that protect them from oxidative stress and DNA damage? Are they able to upregulate efflux pumps to expel bile acids from the cell? Or do they transform bile salts and use then as energy sources through the bile salt hydrolase and 7σ dehydroxylase.

The aim of this chapter is to assess if the bacteria isolated from the human biliary tract are able to survive and replicate in the presence of bile salts at physiologically relevant concentrations, and therefore establish if they have the potential to be commensal or simply transient residents. Bile salt hydrolase (BSH) activity of the most bile salt resistant bacteria will also be assessed.

3.2 Materials and Methods

3.2.1 Bile Salt Resilience/Resistance

3.2.1.1 Growth curve analysis by optical density

To assess the isolated bacteria's ability to survive in the presence of bile salts, stored bacterial glycerol stocks were thawed and 10 ul inoculated into 10ml BHI and allowed to grow overnight at 37°C.

Initial testing resulted in poor growth curves at even low concentrations of bile salts from many of the bacteria tested. It was hypothesized that during storage the bacteria may have downregulated non-essential pathways. As such, samples were

inoculated into media containing Bile Salts (Oxoid No 3) at a concentration of 0.15% (w/v).

Bile salts were added to BHI media at the following concentrations; 0.2%, 0.5%, 1%, 3%, 5% and 10% (w/v)

Bile salts were dissolved in 100ml of BHI placed in a 37°C water bath before the media solution was steri-filtered into a new sterile bottle. These concentrations were used to mimic concentrations of bile salts seen in the gallbladder (up to 10%), common bile duct (up to 1%), and small bowel (0.5%).

Two 100 well Bioscreen plates were set up with triplicate samples of each concentration plus a 0% control (250ul in each well), and 10ul of the fresh inoculate was added to the appropriate wells. Growth curves were generated over a 48-hour period using a Bioscreen C microbiological growth monitoring system, which incubates, agitates and measures turbidity of up to 200 wells simultaneously.

Following the initial bile salt studies, it became clear that a large proportion of bacteria were unable to replicate in the presence of bile salts. As many of these bacteria were isolated from the common bile duct where concentrations of bile salts are only up to 1% it was possible that the bacteria isolated may be able to survive transiently without proliferating, or were growing at a rate not detectable by the Bioscreen C machine. Thus, an experiment was designed to measure colony forming units to determine growth at 24 and 48 hours and therefore assess whether bacteria were able to survive when exposed to low dose bile salts at a concentration similar to that of the common bile duct.

3.2.1.2   Determination of growth by colony forming units

Glycerol stocks were defrosted and 10 ul of each were added to separate 10ml aliquots of BHI broth. The cultures were left to incubate aerobically at 37°C overnight.

BHI agar plates were prepared and air-dried in the sterile laminar flow hood. Liquid BHI media containing 0.15% Bile salts was prepared as described previously.

The BHI cultures were removed from the incubator and serial dilutions were performed by taking 100 ul of culture and adding to 900 ul of BHI broth. This was

repeated to obtain dilutions to 10^{-8}, and 20ul of the appropriate dilutions were spotted in triplicate on the BHI agar plate. After drying, plates were left to incubate aerobically at 37°C overnight.

10 ul of each inoculated media was then added to 990 ul of BHI media containing 0.15% bile salts and placed in the incubator for 24 hours to assess growth following exposure to bile salts. After 24 hours samples underwent the same process for dilutions as above, before being incubated for a further 24 hours at 37°C.

The plates were examined for bacterial growth. The dilution upon which separate colonies could be identified and counted (between 10 and 30) per spot was used. The number of colonies were averaged and a total colony count per ml in the initial sample was calculated. This was repeated for baseline, 24 hours post exposure to bile salts, and 48 hours post exposure to bile salts.

3.2.1.3 Bile Salt Hydrolase activity

A protocol adapted from Sedlackova et al. (Sedlackova, 2015) was used to assess if any of the most resilient bacteria survived following exposure to bile salts was due to bile salt hydrolase activity.

Glycerol stocks of the 10 most resilient bacteria were thawed, 10ul were added to 10ml PBS and 20ul was spread on BHI plates and incubated aerobically overnight at 37 ° C.

Soft agar plates (0.75%) containing calcium chloride 0.375 g/L and 0.5% Bile salts no. 3 were prepared.

Colonies were stabbed into the agar and left to incubate at 37°C for 72 hours. If BSH was active then a halo would develop around the puncture site, and results were assessed by measuring the diameter of the halos. These measurements were repeated 3 times.

To see if exposure to bile salts had an effect on inducing BSH activity following glycerol storage, the same bacteria were also incubated in BHI liquid media containing 0.15% bile salts (made as previously described in 3.3.2.1) overnight before being plated on BHI plates. The same protocol was then followed.

3.3 Results

3.3.1 Bile salt resistance studies

To observe if bacteria cultured from bile could survive physiological concentrations of bile salts, resistance studies were performed. 132 colonies from 39 samples were tested in triplicate. In total 20/132 colonies grew in media containing >5% bile salts. Of these several colonies were identified as the same genus grown from the same bile sample but on different media. Therefore, 12 distinct bacterial species were found to be resistant to high concentrations of bile salts. Growth curves for these bacteria are shown in figure 2-1.

Nine of the bacteria were isolated from four diseased gallbladders. The other three were isolated from three different common bile ducts, two normal and one at time of ERCP.

11/12 of the bacteria grew best in media containing no bile or 0.2% bile salts and then growth was progressively inhibited as bile salts concentrations increased. *Citrobacter freundi* grew best in low concentrations of bile salts but negative effects were observed at higher doses. The minimal inhibitory concentration for most of the bacteria was between 5 and 10%.

As bile salt concentration increased, growth was delayed by up to 24 hours. This suggests that the bacteria were able to induce pathways which had been downregulated during storage, to enable survival and proliferation in media containing bile salts.

Citrobacter, Klebsiella, Enterobacter, Proteus, Cronobacter and one of the unidentified bacteria had an initial rapid growth phase in low dose bile salts (0.2 to 0.5%) before growth slowed and then plateaued suggesting that they were using bile salts as a source of energy until the reserves became depleted within the media.
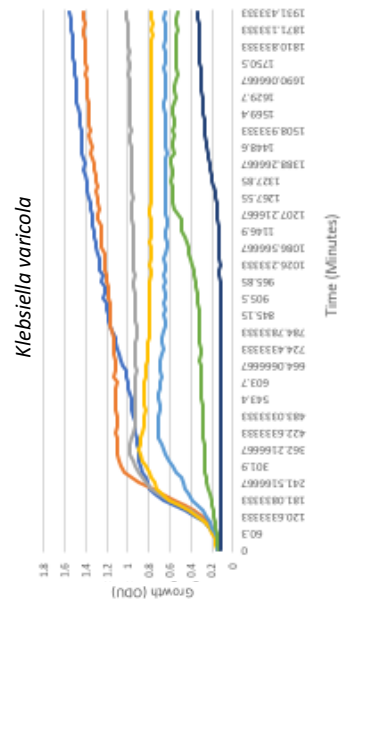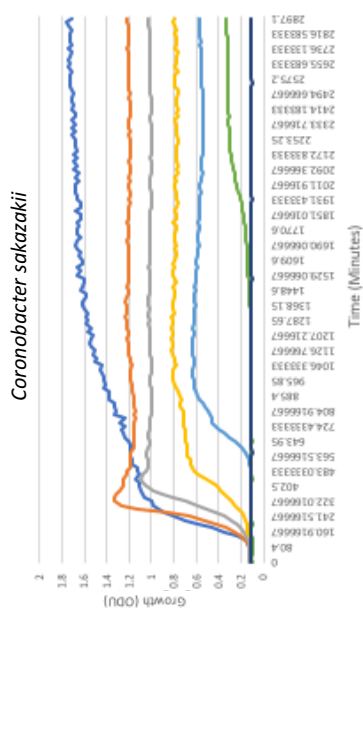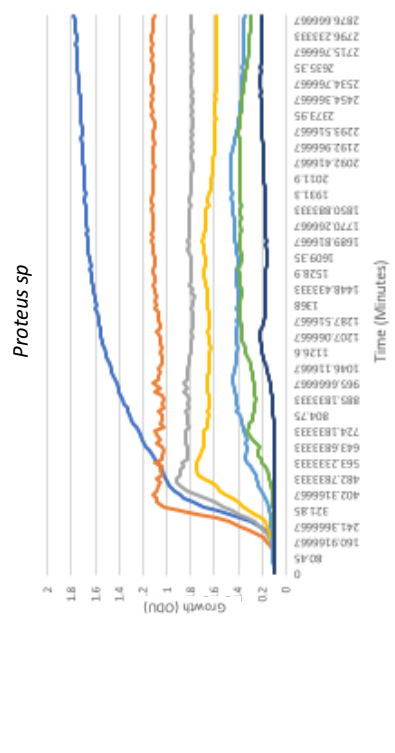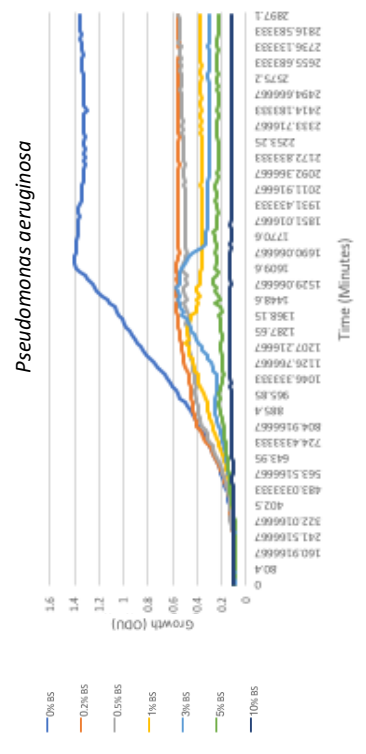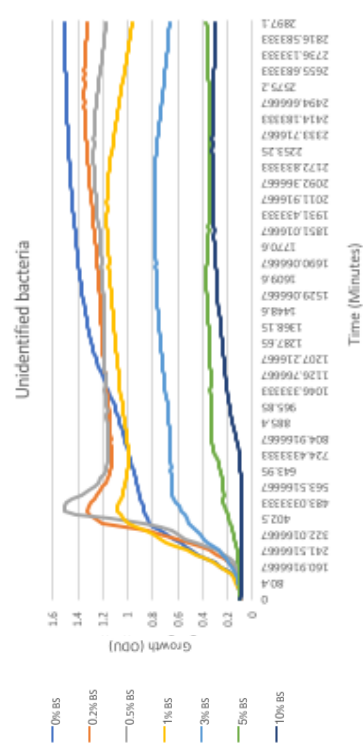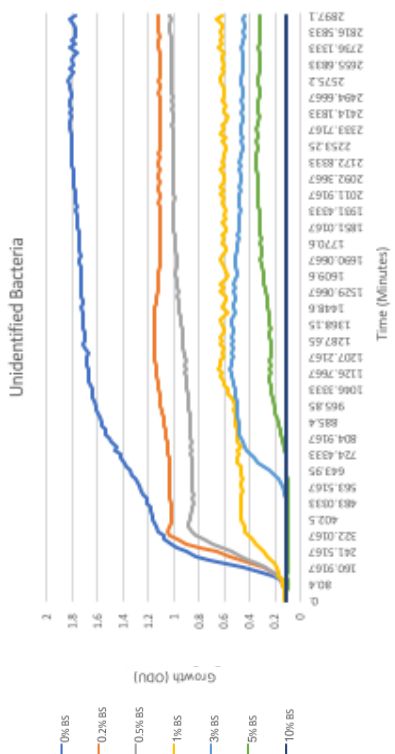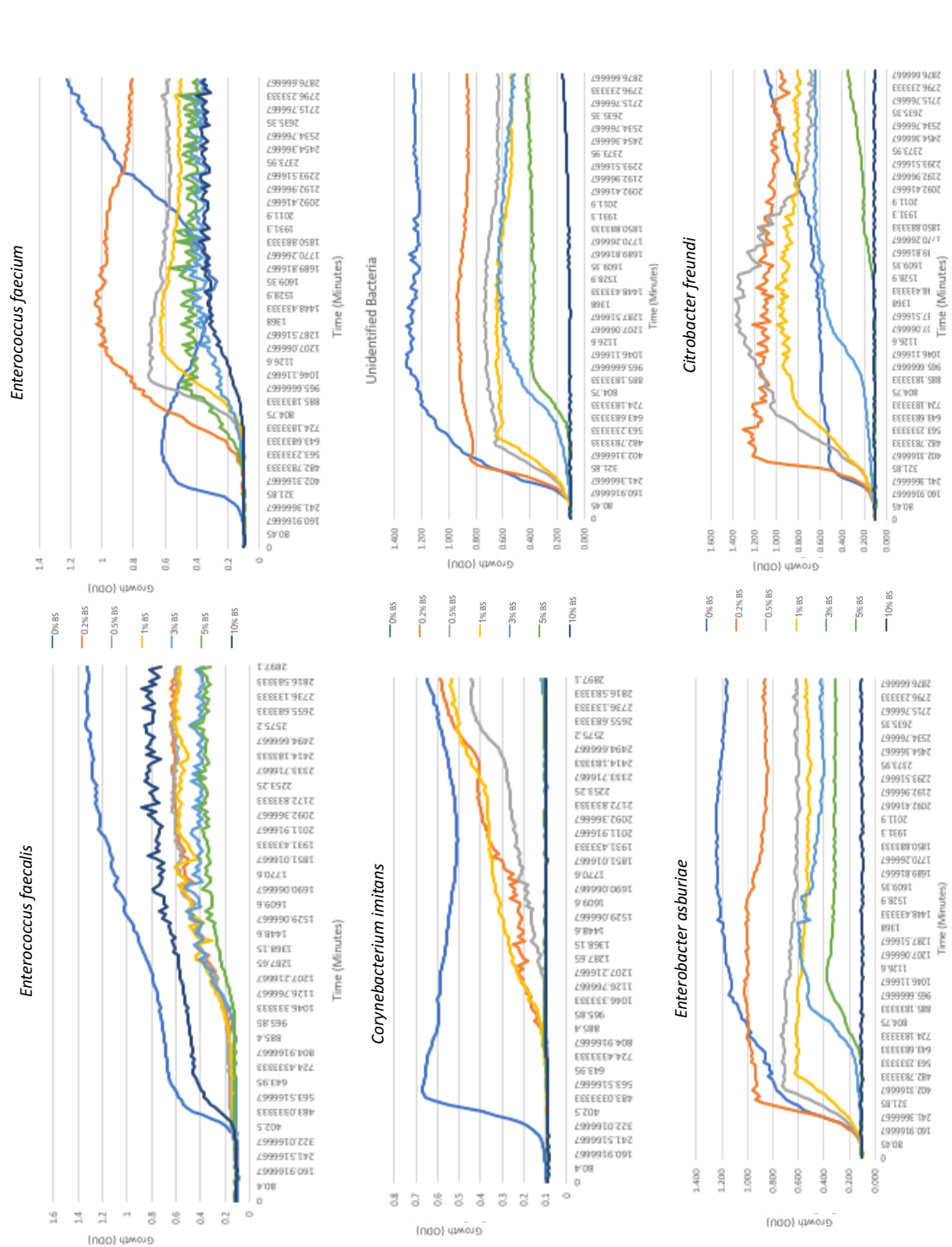
Figure 3-1 Growth curves for 12 bacterial species in increasing concentrations of bile salts. Growth curves were generated through optical density 260xnm using the Bioscreen C microbiological growth monitoring system. Each bacterial species was grown in triplicate and the growth curves generated represent an average of the 3 experiments

3.3.2　Bile salt tolerance studies

As many of the bacteria were unable to proliferate in media containing bile salts, it was decided to examine whether bacteria were able to survive in media containing 0.2% modified bile salts, which is physiologically relevant for the common bile duct (Coleman et al., 1979).

116 bacterial colonies were analysed, with the number of colony forming unit (CFU) per ml calculated at baseline, 24 hours and 48 hours.

Three patterns (A, B and C) were observed and are summarised in figures 3-2, 3-3 and 3-4.

Pattern A demonstrated an initial decrease in the number of colonies within the first 24 hours, and colony numbers remained stable between 24 and 48 hours. Bacteria that followed this pattern were generally isolated from the gallbladder and are highlighted in table 3-2.

Pattern B showed a progressive decline in the number of colonies from baseline to 48 hours. Although this suggests that bile salts may restrict the growth of these bacteria the bacteria were still viable at 48 hours. These bacteria were predominantly isolated from the common bile duct, which suggests that they may only be present transiently. These bacteria are highlighted in table 3-3.

Pattern C showed an initial fall in numbers at 24 hours with subsequent recovery and proliferation between 24 and 48 hours thereby suggesting the induction of pathways to allow survival following exposure to bile salts. These bacteria were isolated from the common bile duct and gallbladder. The bacteria that followed this pattern are highlighted in table 3-4.

S*taphylococcus epidermidis, Staphylococcus pasteuri* and *Micrococcus luteus* displayed more than one of the patterns observed. This suggests that there is variability within species with regards to bile salt tolerance, depending on the source of the bacteria and pathways, which were up or down regulated in storage.
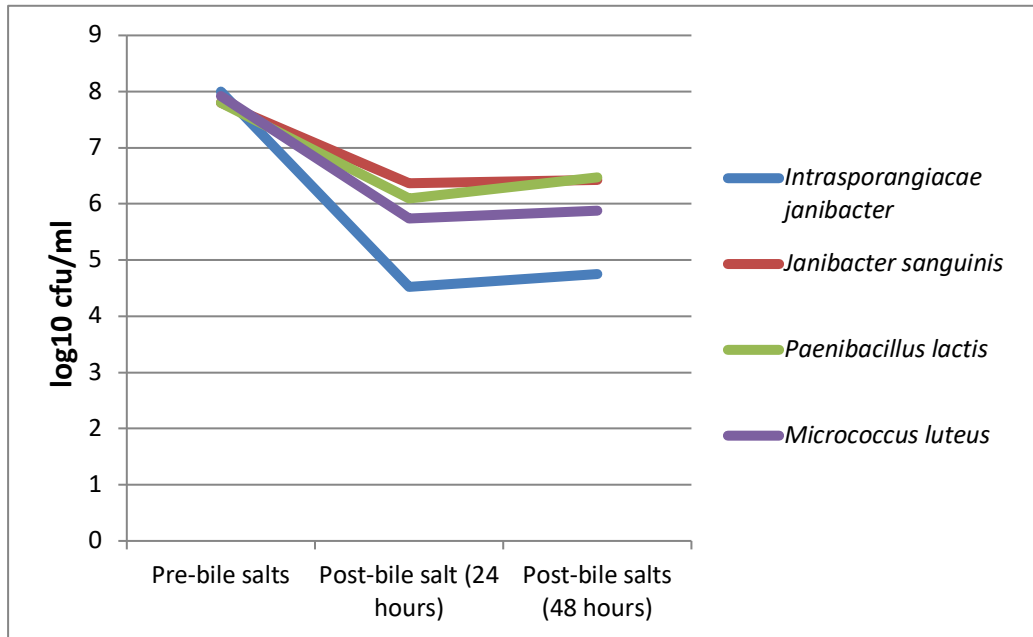
Figure 3-2: Pattern A; CFU per ml of four bacterial species following exposure to 0.2% (W/v) bile salts. Numbers of colonies are presented as log10. Pre-bile salts = Bacteria inoculated in BHI media for 24 hours and then plated on BHI agar and counted. Post-bile salts = Bacteria subsequently inoculated into BHI media containing 0.2% bile salts before being plated on normal BHI and counted. Each experiment was performed in triplicate and the average shown.

Bacteria	Patient Group
Intrasporangiacae Janibacter	Diseased GB
Janibacter sanguinis	Diseased GB
Enterococcus faecalis	Diseased GB
Micrococcus luteus	Diseased GB
Enterococcus faecium	Diseased CBD
Neisseria Perflava	Diseased GB
Staphylococcus pasteuri	Normal GB
Bacillus licheniformis	Normal GB
Staphylococcus epidermidis	Normal GB
Paenibacillus lactis	Normal GB
Staphylococcus epidermidis	Normal CBD

Table 3-1; Identity of bacteria that show initial decline in growth following exposure to bile salts with subsequent stability of numbers between 24-48 hours. GB = gallbladder, CBD = common bile duct.
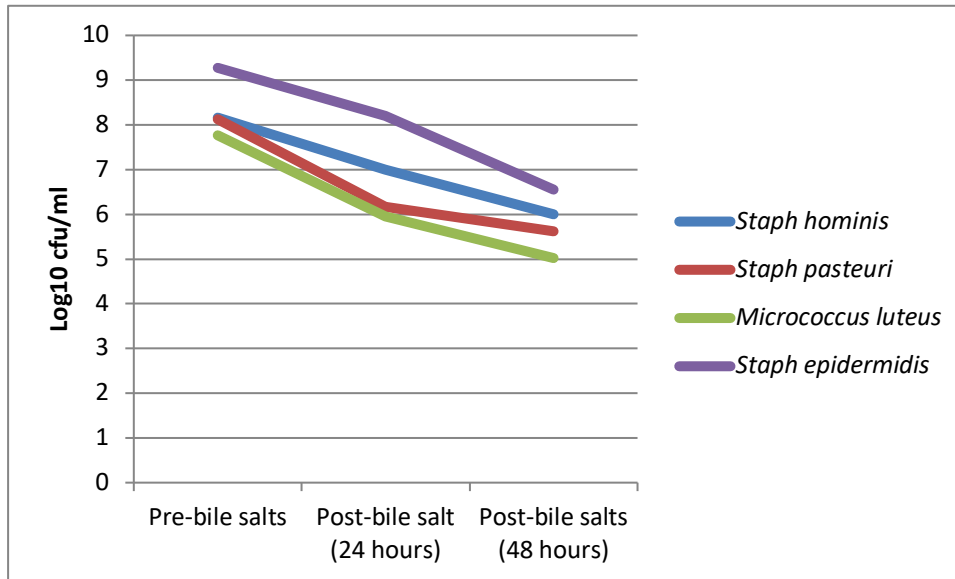
Figure 3-3 Pattern B; CFU per ml of four bacterial species following exposure to 0.2% (W/v) bile salts. Numbers of colonies are presented as log10. Pre-bile salts = Bacteria inoculated in BHI media for 24 hours and then plated on BHI agar and counted. Post-bile salts = Bacteria subsequently inoculated into BHI media containing 0.2% bile salts before being plated on normal BHI and counted. Each experiment was performed in triplicate and the average shown.

Bacteria	Patient Group
Clostridium perfringens	Diseased GB
Staphylococcus epidermidis	Diseased GB
Staphylococcus sanguinis	Diseased GB
Acintomyces viscosus	Diseased GB
Bacillus subtilis	Diseased GB
Massila sp	Diseased CBD
Bacillus firmis	Diseased CBD
Staphylococcus hominis	Diseased CBD
Micrococcus yunnanensis	Normal CBD
Staphylococcus lugdunesis	Normal CBD
Variovorax	Normal CBD
Micrococcus luteus	Normal CBD
Micrococcus luteus	Normal GB

Table 3-2 Identity of bacteria that show progressive decline in growth over 48 hours following exposure to bile salts. GB = gallbladder, CBD = common bile duct
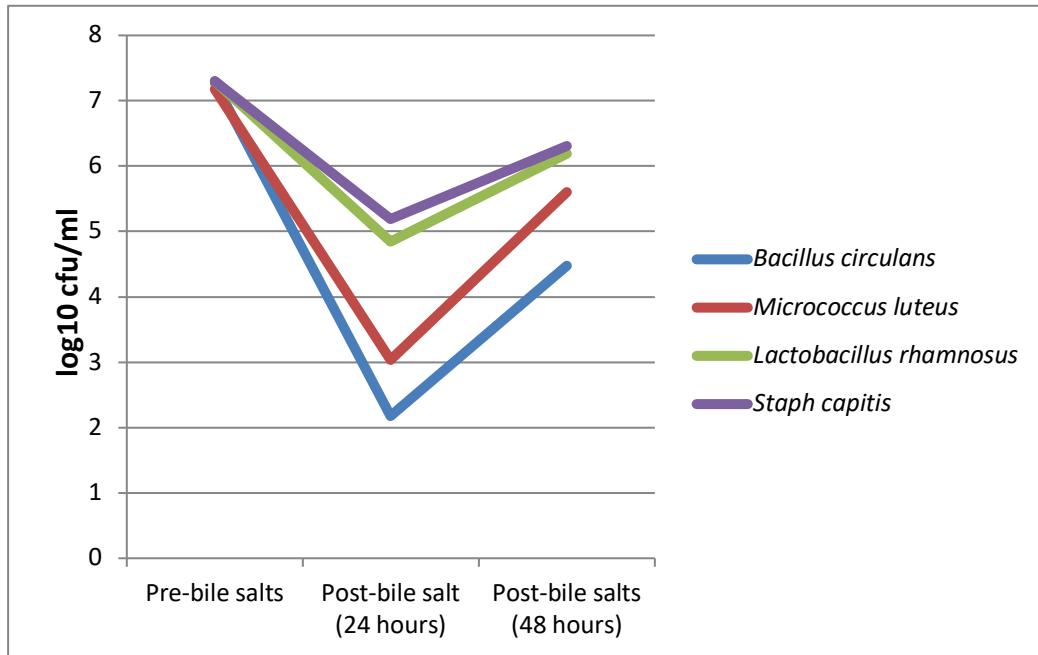
Figure 3-4 Pattern C; CFU per ml of four bacterial species following exposure to 0.2% (W/v) bile salts. Numbers of colonies are presented as log10. Pre-bile salts = Bacteria inoculated in BHI media for 24 hours and then plated on BHI agar and counted. Post-bile salts = Bacteria subsequently inoculated into BHI media containing 0.2% bile salts before being plated on normal BHI and counted. Each experiment was performed in triplicate and the average shown.

Bacteria	Patient Group
Bacillus circulans	Normal GB
Micrococcus luteus	Normal CBD
Staphylococcus hominis	Normal CBD
Staphylococcus capitis	Normal CBD
Staphylococcus aureus	Normal CBD + Diseased GB (paired sample)
Lactobacillus rhamnosus	Normal CBD
Corynebacterium imitans	Normal CBD
Corynebacterium aurimucosum	Diseased GB
Klebsiella varicola	Diseased GB
Staphylococcus salivarius	Diseased GB
Micrococcus luteus	Diseased CBD

Table 3-3 Identity of bacteria that show initial decline following exposure to bile salts with subsequent proliferation between 24 and 48 hours. GB = gallbladder, CBD = common bile duct

3.3.3 Bile Salt Hydrolase (BSH) activity

The bacteria that exhibited growth in higher concentrations of bile salts to identify whether they demonstrated bile salt hydrolase activity were stabbed into soft agar plates containing bile salts and calcium chloride. A white precipitate will form around the inoculation point of the bacteria that have bile salt hydrolase activity

The bacteria were recovered from glycerols and inoculated in bacterial growth media with and without bile salts to see if exposure to bile salts prior to inoculation resulted in an increase in BSH activity.
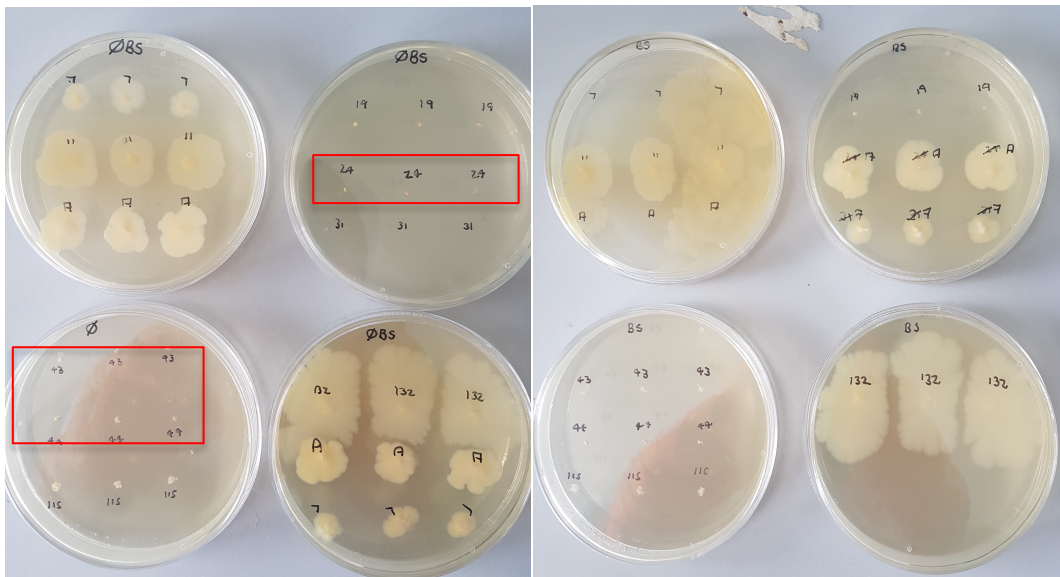
Figure 3-5 Growth plates assessing Bile Salt Hydrolase activity. Colonies were stabbed into soft agar containing bile salts and calcium chloride. If bacteria have bile salt hydrolase activity then a white precipitate is seen after 72 hours. Red boxed samples represent negative controls.

Seven bacteria that had shown strong resistance to bile salts were chosen for testing, along with three controls that had not been able to proliferate during the bile resistance experiments. The results are shown in figure 3-5 with the measurements and bacteria tested shown in table 3-4.

Unidentified bacteria (132), *Proteus sp* (11) and *Cronobacter sakazakii* (17) had the strongest reaction to the bile salts producing the largest halos. As expected, the negative controls had no bile salt hydrolase activity. *Pseudomonas aeroginosa (31)* and *Enterococcus faecalis (19)* also showed no bile salt hydrolase activity,

thereby suggesting that these bacteria have other mechanisms by which they are resistant to bile. There appeared to be no difference in the size of halo following pre-exposure to bile salts (table 3-4).

Bacteria	Sample Number	Average halo diameter (no bile salts) (mm)	Average halo diameter (bile salts) (mm)
Citrobacter freundii	7	8	9
Proteus sp	11	18	18
Cronobacter sakazakii	17	16	15
Enterococcus faecalis	19	1	1
Staphylococcus hominis	24	0	NA
Pseudomonas aeroginosa	31	0	NA
Staphylococcus pasteuri	43	<1	1
Staphylococcus epidermidis	44	<1	1
Corynebacterium imitans	115	2	2
Unidentified bacteria	132	25	25

Table 3-4 Average maximum diameter (mm) of halos measured after 72 hours' incubation of bacteria grown in either normal media or media containing 0.2% bile salts. Bacteria highlighted in blue did not proliferate in bile salts on previous testing and therefore acted as controls.

3.4 Discussion

Bile salts provide an important barrier to bacterial infection through their bactericidal properties. However, bacteria have adapted and evolved so that many are bile salt resistant and able to survive in even the high concentrations of bile salts in the gallbladder. In this chapter, the bacteria that had been isolated from the human biliary tree were grown in similar concentrations of bile salts as seen in the common bile duct (0.2-1%) and gallbladder (6-9%) (Coleman et al., 1979). Only 12 of the bacteria that were tested were able to proliferate in concentrations of >5%.

Unsurprisingly, the majority of these bacteria were isolated from gallbladders. *Enterobacter, Enterococcus, Citrobacter, Klebsiella, Proteus, Pseudomonas, Cronobacter sakazakii*, and *Corynebacterium* are all known to be resistant to bile salts *(Nami et al., 2015; Hakalehto et al. 2010; Fakruddin et al., 2014).* These bacteria are also known to be pathogenic, with most identified as causes of biliary sepsis *(Hakalehto et al., 2010). Cronobacter sakazakii* is a food-borne pathogen that has been linked with outbreaks of food poisoning, especially in infants due to contaminated infant formula milk, (Ueda, 2017; Endersen et al., 2017) however asymptomatic carriage has been reported.

Staphylococcus species are known to have bile resistant properties. However, in this study, none of the isolated Staphylococci were able to grow in concentrations of >5% bile salts. However, many of the isolated species were able to survive and even proliferate in low concentrations of bile salts (0-0.2%). This suggests that the isolated species were able to induce pathways to allow survival in the presence of bile salts. It is also possible that whilst in storage as glycerols these bacteria downregulated non-essential pathways in order to conserve energy. Therefore, if exposed to higher concentrations of bile salts for a longer period of time, there may have been increased growth beyond the end of the bile salt resistance studies. All of the samples were exposed to bile salts when removed from storage so an experimental control measuring growth over 48 hours without bile salts to see if bacteria had adapted to bile salts for survival would be beneficial.

Although only a few bacteria could actively proliferate in >5% bile salts, the majority of them were able to survive for at least 48 hours in concentrations of bile salts similar to those expected in the common bile duct. All of the bacteria that were isolated from the biliary tract are also known to inhabit the normal human intestine. It is therefore possible that those bacteria which were not proliferating in the presence of bile salts were there transiently following reflux from the small intestine. It has previously been shown that patients with confirmed sphincter of Oddi laxity and cholangiolithiasis have a more varied biliary microbiota than those that do not when bile was sampled at ERCP (Liang et al., 2016). Also, previous biliary intervention has been shown to be a risk factor for positive biliary cultures at the time of endoscopic sampling (Rupp et al., 2016). Therefore, it is possible that bacteria refluxing from the small intestine may have an influence on the core biliary microbiome, which equally may have a role to play in the development of biliary disease. Little is known about the duodenal microbiota but it is unlikely that variations in small bowel microbiota between patients influenced the results given

that patients with risk factors for small bowel bacterial overgrowth and coeliac disease were excluded.

Seven of the most resilient bacteria were assessed for BSH activity, and four of these formed clear halos, suggesting that they exhibited strong BSH activity. *Citrobacter* had a smaller halo when compared to *Proteus* and *Cronobacter*. However, when the growth curves were examined, *Citrobacter* had a stronger response in low concentrations of bile salts than the other bacteria, suggesting that they were able to utilize bile salts as a source of energy as well as the media nutrients. Conversely, three of the bacteria did not show any BSH activity, and these tended to grow slower, and with a lower maximum load, than those bacteria who were able to utilize BSH. Therefore, they likely have other pathways that provided protection against the negative effects of bile salts, which they were able to upregulate in order to replicate. Thus, bacteria isolated from the human biliary tract have different methods by which they are able to survive, and in some cases, proliferate. These adaptations may also have an effect on the host, and therefore be a possible mechanism through which biliary disease occurs.

4 Assessment of faecal microbiota in cholestatic liver disease

4.1 Introduction

Chronic cholestasis refers to impairment of bile formation and flow. If untreated it can lead to hepatocyte damage and bile duct destruction through retention of bile salts constituents. This in turn can lead to hepatic fibrosis and ultimately cirrhosis (de Vries and Beuers, 2017). The two most common chronic cholestatic liver diseases are Primary Biliary Cholangitis (PBC) and Primary Sclerosing Cholangitis (PSC).

PBC is a chronic, immune mediated, progressive liver disease. It is characterized by inflammation of the small and medium sized intrahepatic ducts which then leads to fibrosis and eventually cirrhosis. It generally effects middle aged women and has an indolent course. Symptoms are non-specific and include pruitis, fatigue and in the later stages jaundice and chronic liver failure. As such it is often not diagnosed until quite advanced, or coincidentally on blood testing for other causes.

PSC is characterized by irregularities, stricturing and upstream dilatation effectiveness of the intra and extra-hepatic ducts, which like PBC can progress to fibrosis and cirrhosis. It effects men more than women and is generally diagnosed at an earlier age. Symptoms generally consist of pruitis, abdominal pain, fatigue, weight loss and symptoms of biliary sepsis. There is an association between PSC and inflammatory bowel disease with a co-existence of around 70%.

Treatment options for these conditions are limited. Ursodeoxycholic acid (UDCA), a secondary bile acid, is the first line treatment for PBC. 40% of patients do not respond to UDCA. It is thought to work through stimulating biliary secretion of bile acids thereby reducing bile acid cytotoxicity, protecting hepatocytes from bile acid-induced apoptosis through inhibition of mitochondrial membrane permeability transition and protection of cholangiocytes from cytotoxicity of hydrophobic bile acids by modulating the composition of mixed phospholipid-rich micelles (Purohit and Cappell, 2015). These patients have a worse prognosis in terms of transplant free survival. The effectiveness of UDCA in PSC is less clear. Although liver biochemistry improves there is no proven benefit in terms of survival (EASL, 2009). Recently obeticholic acid, a farcenoid X receptor agonist, has been licensed for use in UDCA non-responders for patients with PBC (Nevens et al., 2016). Fibrates, for example bezafibrate, have also been shown to improve liver biochemistry and

can be used off license (Cheung et al., 2016; Suraweera et al., 2017). No new treatments have been shown to be effective in PSC.

The aetiology of these conditions is unclear. Genetic, auto-immune and environmental factors have been implicated and likely predispose an individual to development of the conditions, but do not fully explain pathogenesis. There is emerging evidence that bacteria may have a role to play in the development of these conditions. Molecular mimicry, whereby antibodies in the sera of PBC patients that bind to self – peptides also cross-react to conserved bacterial proteins, which include *Escherichia coli* and *Lactobacillus delbrueckii.* This may explain the breakdown of self-tolerance seen in PBC. The bile of PBC patients undergoing liver transplant has been found to contain predominantly Gram-positive cocci on 16S RNA analysis, in comparison to Gram-negative bacteria in patients with gallstones. However, this study did not identify bacteria in the bile of patients without hepatobiliary disease in contrast to this and other studies (see also chapters 2 and 3). More indirect evidence includes the association of recurrent urinary tract infections with PBC.

Given that PSC has a close association with IBD, recent research has focussed on the gut microbiota and cholestatic liver disease. In PSC, a distinct dysbiosis in patients with PSC and ulcerative colitis (UC) has been observed which is separate from the dysbiosis seen in UC alone (Sabino et al., 2016; Quraishi et al., 2017). Within the last few weeks work has been published suggesting that there is a dysbiosis associated with PBC which may be partially reversed with UDCA therapy (Tang et al., 2017).

The aims of this chapter were to confirm whether there is a dysbiosis associated with PBC and PSC, to see if any change is related purely to stage of liver disease, to compare PSC and PBC microbiota and to see if UDCA therapy has an impact on microbiota in these conditions.

4.2   Sample collection

Performing a search of the Norfolk and Norwich University Hospital patient letter database identified patients with primary sclerosing cholangitis and primary biliary

cholangitis. The letters were then reviewed and patients were excluded if they were on long-term antibiotics including rifaximin, had current decompensated liver disease or previous liver transplant.

Patients were then contacted in writing with a subsequent follow up phone call unless they expressed a wish to not be included in the study.

Exclusion criteria at the time of interview were use of antibiotics within the preceding 6 weeks, current or recent use of probiotics, use of laxatives, recent diarhoeal illness, current decompensation, and recent variceal bleed.

Control patients were identified through the bowel cancer screening programme. These patients are asymptomatic but have tested positive for faecal occult blood on stool testing and therefore are invited for colonoscopy. Patients were consented at the pre-assessment clinic.

Exclusion criteria for controls were recent use of antibiotics or probiotics, recent use of laxatives, bowel resection, history of liver disease and recent diarrhoea. Samples were collected prior to colonoscopy and bowel cleansing. If subsequently positive for bowel cancer on endoscopy then these samples were ommited from the study.

Patients were given a home collection kit for faeces and urine and samples were then collected from their homes within 2 hours. Given that patients were recruited from the whole of Norfolk this proved to be unsustainable in terms of distance traveled and availability to collect samples. Therefore postal kits were subsequently provided.

On arrival at the laboratory faecal samples were then aliquoted into 6 faecal collection tubes and stored at -20°C. Urine samples were frozen directly.

4.3   Materials and Methods

4.3.1   DNA extraction

DNA was extracted using the modified protocol for the FastDNA Spin Kit for Soil (MPBio) (Maukonen et al., 2006)

0.2 g of frozen faecal sample was weighed and placed into a 10ml sterile test tube. 978 ul of sodium phosphate buffer and 122 ul of MT buffer was added and vortexed for 20 seconds. This solution was then left to stands for 1 hour at 4°C (being vortexed every 15 mins).

Approximately 1ml of sample was then transferred into a Lysing Matrix E Tube.

Samples were then lysed using the FastPrep Instrument for 1 minute at 6.5m/s. This was repeated 3 times allowing the samples to cool for 5 mins in between steps.

The lysing matrix tubes were then centrifuged at 14 900 xg for 1 minute. The supernatant was then transferred to a clean Eppendorf tube. 250 ul of PPS reagent was added and the solution hand mixed 10 times.

The solution was then centrifuged at 14 900 xg for 5 mins to pellet precipitate. The supernatant was then transferred to a sterile 15ml tube. 1 ml of Binding Matrix Suspension was added to the supernatant and inverted by hand for 2 minutes. The tubes were then left to stand for 3 minutes to allow the settling of the silica matrix.

1 ml of the supernatant was then removed and discarded. The binding matrix was re-suspended in the remaining supernatant.

600 ul of mixture was then transferred into a "spin filter tube" and centrifuged for 1 minute. The matrix was washed 3 times with 500ul SEWS-M wash solution and centrifuged at 14, 500 xg for 1 minute with each wash. Following the final wash the tubes were centrifuged a final time at 14, 500g for 2 minutes to "dry" the matrix of residual SEWS-M wash solution.

The spin filters were then left to air dry for 2 mins before 50ul of DNase/Pyrogen Free Water was added. The tube was then centrifuged for 1 minute in order to elute the DNA.

The quantity of DNA isolated was measured using a Nanodrop spectrophotometer (Thermofisher).

4.3.2 Amplification and sequencing of 16S rDNA gene regions

All samples were sent to the Earlham Institute and DNA sequencing performed using the Illumina platform as per standard protocols as described in section 2.1 (Caporaso et al., 2012)

4.3.3 Bioinformatic analysis of 16S rDNA

Samples were analysed using Quantitative Insights Into Microbial Ecology (QIIME), an open-source bioinformatics pipeline for performing microbiome analysis from raw DNA sequencing data. QIIME enables the analysis of raw data generated by sequencing platforms to generate graphics and statistics including taxonomic assignment, relative number of operational taxonomic units (OTUs) and diversity analyses.

4.3.4 Preparation of faecal and urinary samples for metabolomics analysis

Approximately 50 mg of frozen faecal material was transferred into a 2ml Eppendorf tube and kept on ice.

Phosphate buffer solution (PBS) was made by the following specifications; 100 ml heavy water (D_2O), Monosodium Phosphate 0.51 g, Dipotassium Phosphate 2.82 g, Trisodium Phosphate 0.0345 g, Sodium Azide 0.1 g.

PBS was added to the faecal material at a ratio of 12 ul/1mg. The faecal matter was then homogenized with a mechanical pestle for 1 minute. This suspension was centrifuged for 15 minutes at 15 000 xg at 4°C

The supernatant was transferred to a new sterile 1.5ml Eppendorf tube and then centrifuged for a further 15 minutes at 15 000 xg at 4°C

The supernatant was then transferred into a new 1.5ml Eppendorf and stored at -20° C until ready for NMR analysis.

Urine samples were defrosted and 2mls transferred to a sterile Eppendorf. This was then centrifuged at 14, 500 xg for 15 minutes at 4° C. The supernatant was then removed and transferred to a clean Eppendorf before analysis.

NMR was then performed by Dr Gwen LeGall as per standard protocol (Bouatra et al., 2013) and the results returned for analysis.

4.4   Results

4.4.1   Patient Demographics

In total 58 volunteers were screened and consented; 19 in the control cohort, 18 in the Primary Biliary Cholangitis (PBC) cohort and 22 in the Primary Sclerosing Cholangitis (PSC) cohort. 3 were immediately excluded due to recent antibiotic or probiotic use. 4 control patients were excluded post colonoscopy due to pathology discovered at the time of endoscopy (3 for colorectal cancer, 1 for previously undiagnosed active inflammatory bowel disease). A further 3 did not provide faecal samples. Therefore 48 patients were included in the final study. The demographics of these volunteers is summarised in table 4.1.

Patient Group	Control (n=13)	PBC (n=14)	PSC (n=21)
Mean Age (Years)	64.5 (55 to 74)	60.4 (45 to 78)	62.1 (33 to 77)
Male:Female	7:6	1:13	8:13
Cirrhosis (Yes:No)	0:13	5:9	4:17
On UDCA (Yes:No)	0:13	12:2	15:6

Table 4-1 Demographics of patients included in the study. PBC = Primary Biliary Cholangitis, PSC = Primary Sclerosing Cholangitis, UDCA = Ursodeoxycholic acid.

There are more volunteers in the PSC cohort. This is due primarily to volunteers in the control cohort being excluded for cancer and volunteers in the PBC cohort failing to provide samples. The average and maximum ages are comparable although there were 2 patients in the PSC cohort under the age of 50 and 1 in the PBC cohort. The predominance of females in the PBC cohort reflects the worldwide Male:Female ratio of 1:9 for patients diagnosed with PBC. There were also more females in the PSC cohort, which contradicts the national prevalence of 2:1 although the total number of volunteers in this study was limited.

40 of the samples were collected directly from the patient and transported on ice back to the laboratory before being separated into 1 g aliquots and stored in the -80°C freezer. 8 samples were posted directly to the laboratory using biohazard containers. These were then frozen directly in the collection tubes on receiving. The maximum length of time from providing the sample to freezing was 34 hours. Urine samples were frozen in the collection containers. In total 49 faecal and 38 urine samples were collected from 48 patients.

4.4.2 Metataxonomics

DNA was extracted from 48 samples. The concentration of DNA measured by nanodrop ranged from 9.0 to 885.1 ng/ul, although more than 75% of samples ranged between 200 and 400 ng/ul. Samples were analysed using the QIMME pipeline. In total there were 5364455 high quality reads with an average of 111759 ± 29561 sequence reads per sample.

In total 8 phyla were identified from the samples. The most abundant phylum in all the cohorts was Firmicutes (Figure 5.1). There is an emerging dysbiosis for Proteobacteria within the PSC cohort but this does not reach statistical significance when compared to the control (P-value = 0.17) and PBC cohorts (P-value = 0.1). In the PBC cohort there is a small increase in the relative abundance of Bacteroidetes and Actinobacteria compared to the control and PSC cohorts but this was not statistically significant (P-value = 0.64 and 0.19 respectively).
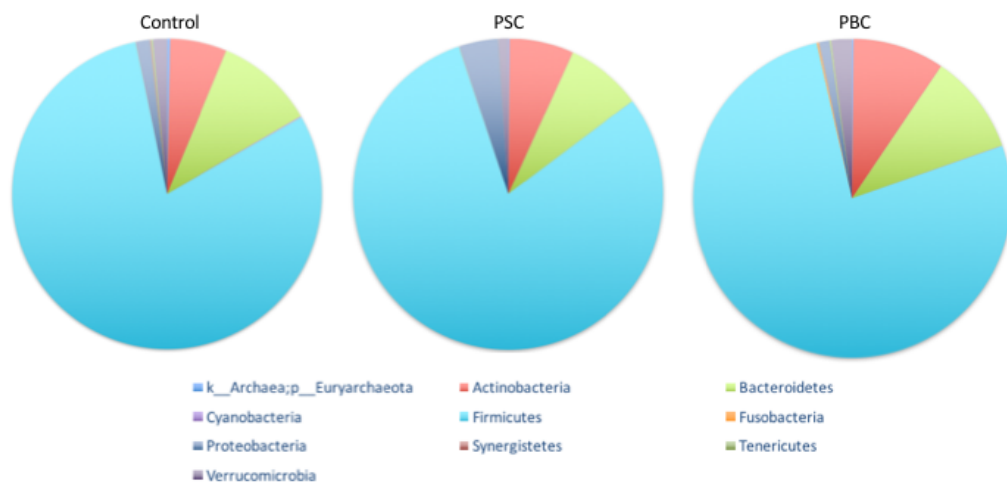
Figure 4-1 Pie charts showing relative abundance of each bacterial phylum by disease group. Each colour represents a bacterial phylum weighted by percentage contribution to overall bacterial population.

In total 62 bacterial genera were identified with relative abundances of > 0.5%. There was a wide range of microbial diversity across all disease groups (Figure 5.2).

The most abundant genus in all 3 cohorts was *Blautia* representing 11.45% (SD 3.46%), 13.4% (SD 8.93%) and 14.8% (SD 6.51%) for control, PSC and PBC respectively.

In both the PSC and PBC group there was an increase in the genus *Faecalibacterium* when compared to the control group. *Bifidobacterium* and *Bacteroides* are increased in the PBC cohort which explains why there is an increase at phyla level in Bacteroidetes and Actinobacteria. *Escherichia* is increased in the PSC cohort thereby increasing the abundance of Proteobacteria in this cohort.

Figure 4-2 Stacked column charts showing relative abundance of each bacterial species by disease group. Each colour represents a bacterial species weighted by percentage contribution to overall bacterial population. Only those taxa that were present at >0.5% of the total bacterial population are shown individually. Bacteria that contributed <0.5% are shown as "Other"

To see if these differences between controls and disease could be explained through development of cirrhosis and the changes in bowel integrity that develops as a result, the relative proportion of bacteria was compared at phyla level between PSC and PBC patients with cirrhosis (n = 9) and those without (n = 24) (Figure 5-3).
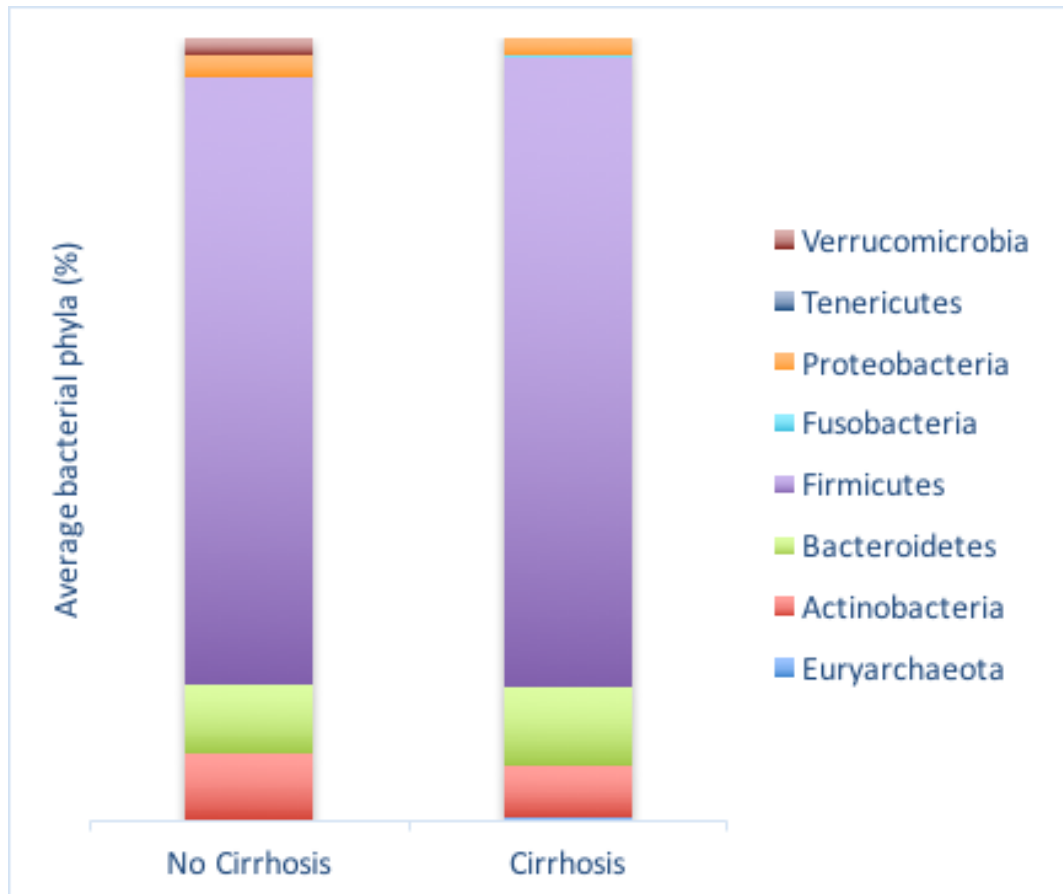
Figure 4-3 Column charts representing bacterial phyla identified in PSC and PBC patients with and without cirrhosis. Each colour represents a bacterial phylum weighted by % contribution to total bacterial population.

The principal difference between the groups is a significant loss of Verrucomicrobia in patients with cirrhosis (2% vs 0.04% p=0.04). There is a slight increase in Bacteroidetes of 1.3% and a fall in Actinobacteria of 2% in the cirrhosis group.

The effect of ursodeoxycholic acid (UCDA) on the gut microbiota composition was also examined. The relative proportion of bacteria were compared at phyla level between those PBC and PSC patients who were taking UCDA (n = 25) and those who were not (n = 8) (Figure 4.4).
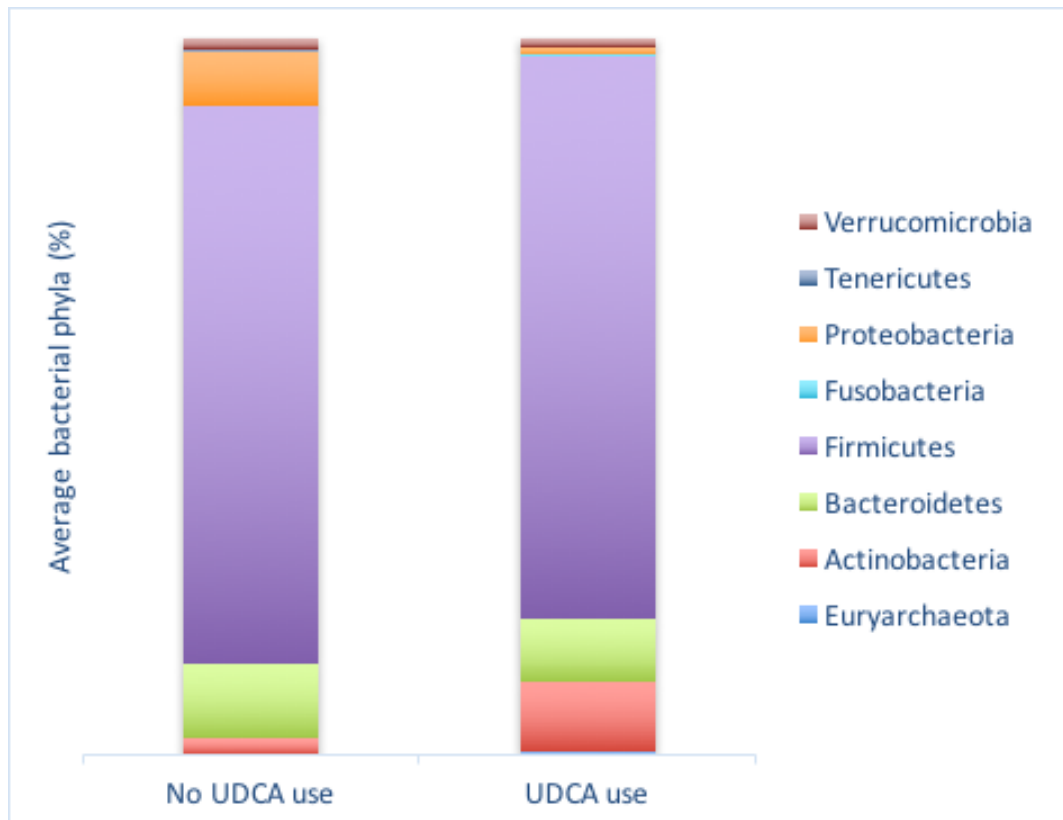
Figure 4-4 Column charts representing bacterial phyla identified in PSC and PBC patients who are taking UDCA acid and those who are not. Each colour represents a bacterial phyla weighted by % contribution to total bacterial population. UDCA = Ursodeoxycholic acid.

There is significantly larger proportion of Proteobacteria within the no UDCA cohort (7.5% vs 0.4%; *p* = 0.004). This is associated with a statistically significant larger proportion of Actinobacteria within the UDCA cohort (2.2% vs 9.84%; *p* = 0.02).

To see if there was any similarity within the disease cohort's, principal component analysis (PCoA) was performed on the 16S sequencing data (figure 4-5). This showed that the PSC patients had very varied microbiotas. There was also a separation of these patients from the PBC and control groups. The PBC and control cohorts showed some clustering but there is no definite separation between the two.
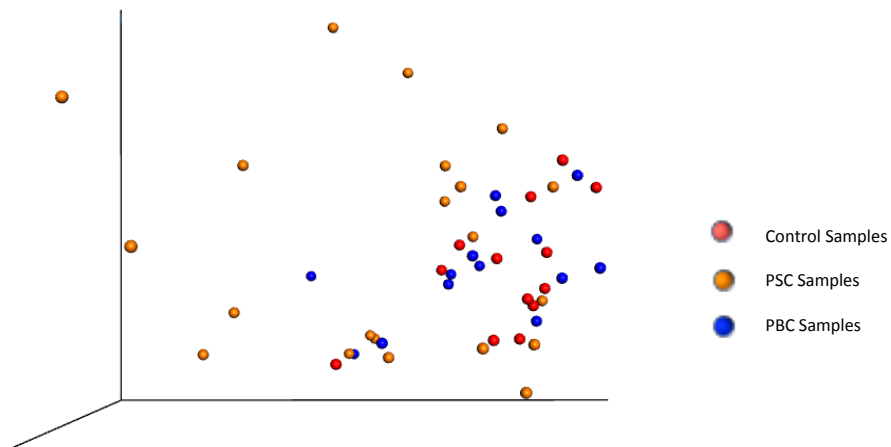
Figure 4-5 : PCoA analysis comparing control samples (red dots), PBC samples (blue dots) and PSC samples (orange dots). Figure generated using QIIME 1.9.0 pipeline.

To see if the patterns observed were due to other influences PCoA plots were also generated for presence of cirrhosis, inflammatory bowel disease, and UDCA use (Figures 4-6, 4-7, 4-8). None of these factors generated patterns of separation thereby suggesting the separation seen for the PSC cohort is not due to these variables.
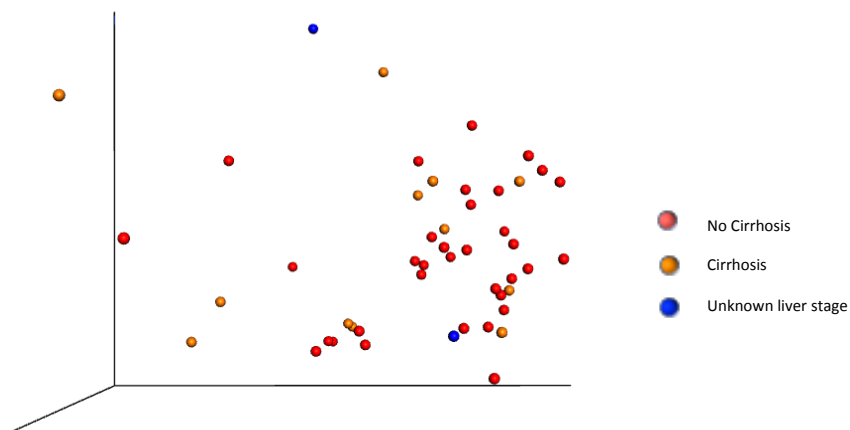
Figure 4-6 PCoA analysis comparing patients without cirrhosis (red dots), patient with cirrhosis (orange dots) and Unknown stage of liver disease (blue dots). Figure generated using QIIME 1.9.0 pipeline.
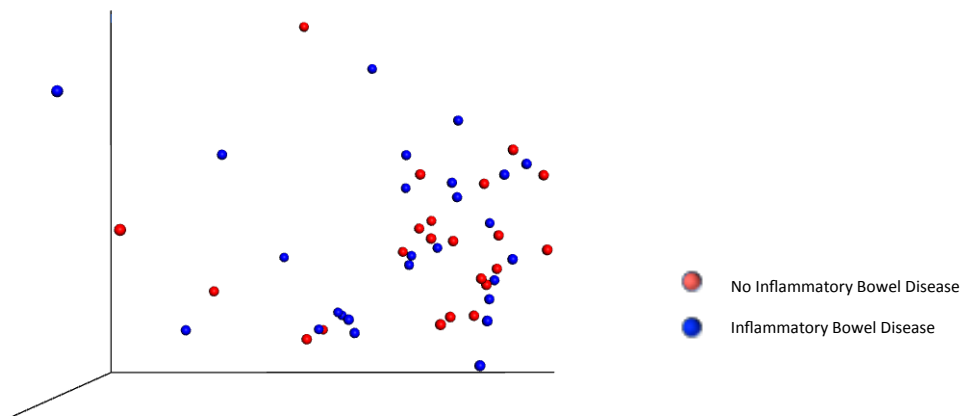
Figure 4-7 PCoA analysis comparing patients with inflammatory bowel disease (blue dots) and patients without inflammatory bowel disease (red dots). Figure generated using QIIME 1.9.0 pipeline.
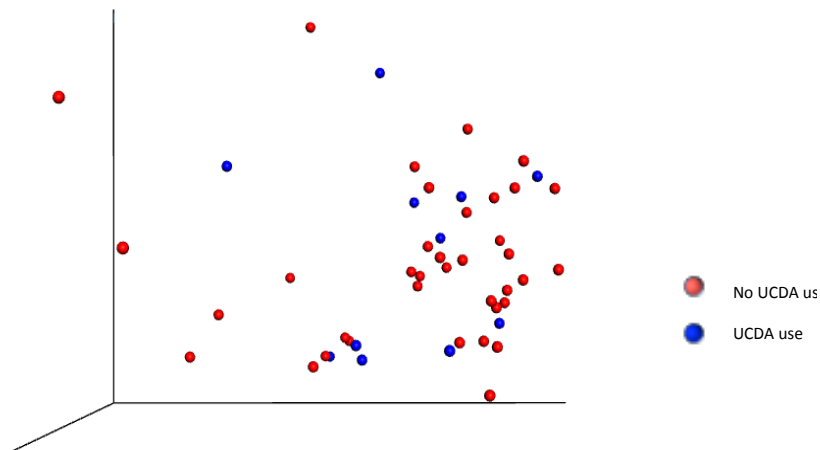
Figure 4-8 PCoA analysis comparing patient use of UDCA (blue dots) to those not taking UDCA (red dots). Figure generated using QIIME 1.9.0 pipeline.

4.4.3   Metabolomics

To assess if metabolites varied between sample groups and thereby could have a role to play in pathogenesis disease, metabolomic analysis was performed on faecal water and urinary samples. In total 49 faecal samples (13 control samples, 15 PBC samples and 21 PSC samples) and 38 urine samples (21 PSC samples, 11 PBC samples and 6 control samples) were analysed. The full results for the 89 metabolites are given in Appendix 3. In faeces the most abundant short chain fatty

acids were Acetate (average concentrations 59.25 mmol/kg) and butyrate (average concenetration 17.11 mmol/kg) and the most abundant amino acid was Glutamate (average concentration 3.58 mmol/kg). In urine the concentrations of metabolites were much lower. Creatinine was the most abundant compound (average concentration 7.822 mM/kg). Glucose was also relatively abundant (average concentration 1.96 mM/kg) but patients with diabetes may skew this. The concentrations of short chain fatty acids and amino acids were in keeping with the published data (Bouatra et al., 2013; Fernandes et al., 2013; Francavilla et al., 2012; Rahat-Rozenbloom et al., 2014).

PCoA plots were generated using the metabolomic data (Figures 4-9, 4-10, 4-11). These do not show any separation between the disease groups or the controls. This mirrors the metataxonomic data whereby only subtle shifts are seen between the three groups.
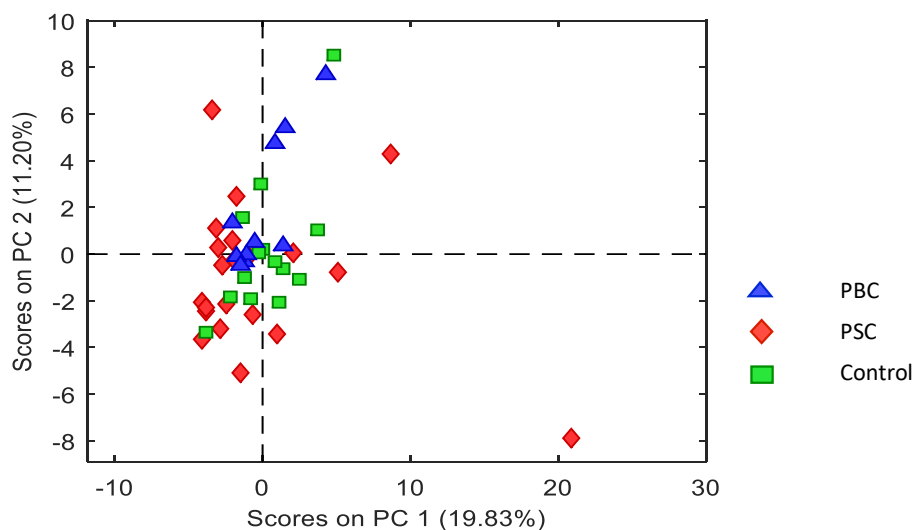
Figure 4-9 PCoA of faecal metabolomic data obtained through nuclear magnetic resonance spectroscopy (NMR). Figure provided by Dr Gwen Le Gall. PBC =Primary Biliary Cholangitis, PSC = Primary Sclerosing Cholangitis
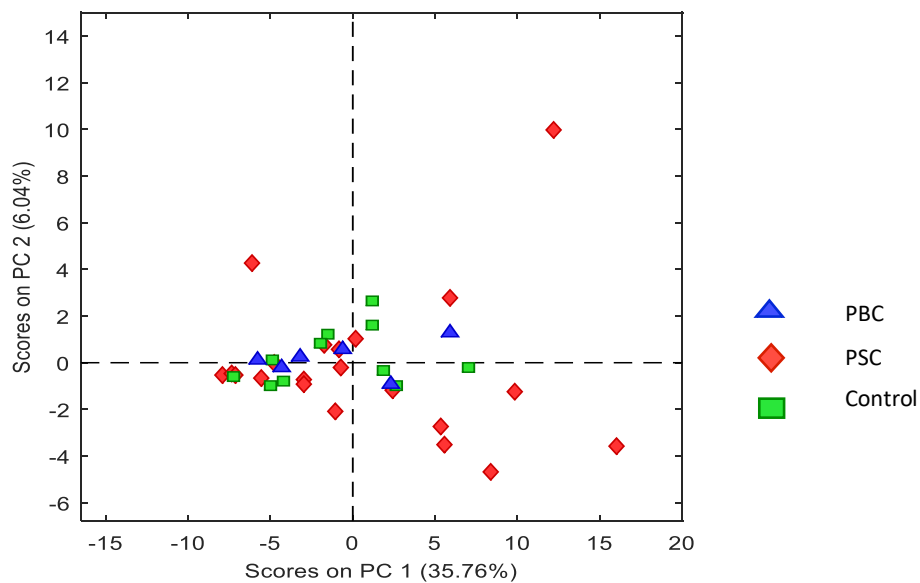
Figure 4-10 PCoA of urinary metabolomic data obtained through nuclear magnetic resonance spectroscopy (NMR). Figure provided by Dr Gwen Le Gall. PBC =Primary Biliary Cholangitis, PSC = Primary Sclerosing Cholangitis

4.5 Discussion

Previous studies have suggested that alterations in the microbiota have a crucial role to play in the development of chronic liver disease (Sabino et al., 2016; Tuomisto et al., 2014; Zhu et al., 2013). In this chapter the gut microbiota of patients with PBC and PSC was assessed through a 16S rDNA sequencing approach. At phyla level the relative abundance Bacteroidetes and Acintobacteria were slightly increased in the PBC cohort, although this does not reach statistical significance. A recent study looking at treatment of naïve PBC patients reported that Bacteroidetes was decreased when compared to healthy controls, with a corresponding over-representation of Proteobacteria and Fusobacterium (Tang et al., 2017). At genus level, an increase in *Bacteroides* and *Faecalibacterium* was observed, whereby in the study by *Tang et al (*2017) there was an increase in *Streptococcus*, *Klebsiella, Pseudomonas* and *Haemophilus.*

In comparison, previous studies have reported an increase in Bacteroidetes and Fusobacterium, with a decrease in Firmicutes, in patients with PSC (Quraishi et al., 2017; Sabino et al., 2016), whereby this study reports an increase in Proteobacteria, largely due to an increase in *Escherichia.* This compares to

another study that showed *Escherichia* was increased in patients with Non-alchololic steatohepatitis (Zhu et al., 2013). Interestingly the study by Sabino *et al* (2016) also reported a less significant increase in Bacteroidetes and Faecalibacterium in patients with UC alone when compared to healthy controls, whereas other studies have reported a decrease in Bacteroidetes and Firmicutes (Rossen et al., 2015).

The data presented in this chapter reflects the problems associated with microbiota research in that sampling can only ever provide a snap shot of the bacteria within the gut at any given time. There are multiple factors that can affect the microbiota from day to day including medications, infections, antibiotics and diet. Therefore there is great variability between subjects and samples, which leads to conflicting results between publications being produced. Larger numbers of patients with repeated sample collections over time would give a better reflection on the stability of the gut microbiota in these conditions.

The studies in PSC and PBC so far have not accounted for the stage of liver disease. There is now good evidence that end stage liver disease and portal hypertension may cause alterations in gut permeability and alterations in gut microbiota through prolonged gut transit and small bowel bacterial overgrowth (Reiberger et al., 2013; Kalaitzakis, 2014). Therefore this study examined the differences between patients with PBC/PSC and cirrhosis and those without. The principle findings were that Verrucomicrobia was not detected and Fusobacteria became slightly more apparent in the cirrhosis group. Although the Verrucomicrobia result was skewed by a single patient sample it is interesting to note that 6/13 control patients and 7/23 non- cirrhotic patients had abundances of Verrucomicrobia of greater that 1.5% all due to the presence of *Akkermansia sp,* whereas the cirrhosis patients had a maximum abundance of 0.1%. This bacterium is the sole representative of Verrucomicrobia in human stools and has been shown to be depleted in patients with diabetes and obesity, both of which are risk factors for liver disease (de Vos, 2017). It promotes beneficial interactions with the host through signalling immune and metabolic pathways (de Vos, 2017). Therefore, it is possible that this is a significant result that requires further investigation.

Until recently UDCA has been the only approved treatment for PBC (Rudic et al., 2012). It is thought that its effect is through displacement of endogenous hepatotoxic bile through expansion of the hydrophilic bile acid pool (Stiehl et al., 1999), although its exact mechanism of action is unclear. It is a secondary bile acid

produced by intestinal bacteria and unabsorbed UDCA undergoes further transformation to lithocholic acid by colonic bacteria (Kullak-Ublick et al., 2000). Due to this it is possible that the UDCA may have an effect on dysbiosis associated with chronic liver disease. A recent study has looked at this particular hypothesis by examining treatment naïve PBC patients and examining faecal microbiota before and after UDCA. They showed that treatment with UDCA resulted in partial amelioration of gut dysbiosis, in particular *Haemophilus, Streptococcus* and *Pseudomonas sp* were decreased (Tang et al., 2017). In this chapter it has been shown that the most marked differences in microbiota are seen when patients taking UDCA are compared to those that are not. There is a significant decrease in the relative abundance of Proteobacteria in the UDCA cohort. Although these are not comparing the same patients this result reflects the findings published by Tang *et al. (2017).* This suggests that UDCA has a positive effect on dysbiosis, although whether this is due to a direct impact on harmful bacteria or because beneficial bacteria are able to utilize UDCA to aid proliferation is not clear. The effect of UDCA may also be the reason that greater changes were not seen between the control group and patients with disease. Future work could include using the *in vitro* colonic model to see if UDCA has a direct affect on the faecal microbiota, or whether this is an effect generated by interactions with the host.

The interaction between cholestatic liver disease, bile salts and the gut microbiome is likely to be a complex one involving some of the pathways described in figure 1.5. It is possible that it involves a 2- way process with changes in the microbiota effecting metabolic pathways through alterations in bile salts on the one hand and changes in expression of pathways such as FXR due to genetic predisposition causing changes in bile salt synthesis/reabsorption which alter microbiota on the other.

One of the reasons that these results do not fully mirror previous studies may be due to means by which samples were transferred and stored. Several of the samples were delivered via post and therefore not kept on ice. Equally all the samples were frozen until required and DNA not extracted directly from fresh samples. There is therefore the possibility that DNA within the samples is progressively denatured during transport and on thawing from frozen. Recent studies however have shown that technical reproducibility and stability within samples is generally high when samples are frozen immediately or incubated at room temperatuere for 96 hours and then stored (Vogtmann et al., 2017). There

are also comparable cure rates for *Clostidium difficile* infection when frozen and fresh faecal microbial transplants are used (Jiang et al., 2017).

5 General Discussion

5.1 Summary of results and completion of aims

The gastrointestinal tract in humans is home to 100 trillion bacteria. This complex bacterial organ forms an important symbiotic relationship with the host and has been shown to be vital in health. Alterations in the microbiota structure and function can occur due to environment, diet, infections, disease and drugs. Equally alterations, or dysbiosis, within the microbiota are linked with a number of gastrointestinal and non- gastrointestinal diseases, with emerging evidence that this dysbiosis can induce or interfere with host pathways and therefore cause pathogenesis, and not simply occur as a result of disease.

The liver receives 70% of blood flow directly from the gut. The biliary system also drains directly into the small intestine. Therefore the liver is continuously exposed to bacterial components from the gut. As such the gut microbiota has been linked to the pathogenesis of several liver conditions including alcoholic liver disease, non-alcoholic steatohepatitis and primary sclerosing cholangitis. Biliary diseases such as cholesterol gallstones are thought to have a bacterial trigger. However there is little data on the normal biliary system and to date no studies have accurately assessed if there is a normal biliary tract microbiota.

The overall aim of this study was to determine whether a biliary microbiota exists, its complexity and whether bacteria isolated from the biliary tree have the properties required to survive in this environment. The faecal microbiota from patients with Primary Biliary Cholangits and Primary Sclerosing Cholangitis, two chronic auto-immune biliary conditions, was also examined to see if there was any dysbiosis that could be linked to biliary damage

5.1.1 Aim 1: "To see whether bile isolated from the normal biliary tract is truly sterile"

Bacteria were repeatedly and successfully isolated from both the diseased and normal human biliary tract. The predominant species were *Bacillus, Micrococcus, Enterococcus* and *Staphylococcus.* Bacterial DNA was also successfully extracted

from biliary samples from healthy and diseased status for 16S-based metataxonomic analysis that revealed a diverse microbial population. This also highlighted that many of the bacteria within the human biliary tract that could not be cultured using standard microbiological growth media. . The predominant species identified in both normal and diseased biliary tract was *Pseudomonas.* The findings confirm for the first time that there may be a core biliary microbiota.

5.1.2   Aim 2: "To see if there is a difference in microbial biodiversity between bile isolated form diseased and normal biliary tracts"

Bile isolated from the normal gallbladder showed the greatest biodiversity. There was an emerging dysbiosis for *Enterococcus* within the diseased gallbladder group. *Pseudomonas* was the predominant species in all four diseased groups, especially within the normal common bile duct group.

5.1.3   Aim 3: "To assess whether bacteria isolated from the biliary tract have bile resistant properties"

A small proportion of bacteria, predominantly isolated from the diseased gallbladder, were able to proliferate in the presence of high dose bile salts. The other bacteria were all able to survive in physiologically relevant concentrations of bile salts. A subset of bacteria were able to proliferate following an initial decline suggesting that these bacteria were able to induce pathways to utilise bile salts. Some of the bacteria were shown to express bile salt hydrolase (BSH), an enzyme that deconjugates bile salts which may aid in the bacterial resistance to bile salts . Others were able to survive without BSH activity, thereby suggesting alternative mechanisms for bile salt resistance.

5.1.4   Aim 4: "To investigate changes in microbial biodiversity between Primary Biliary Cholangits (PBC) and Primary Sclerosing Cholangitis (PSC) when compared to healthy controls"

There was a higher proportion of Proteobacteria in the gut microbiota within the PSC cohort when compared to healthy controls. There was also a higher proportion of Bacteroidetes and Actnobacter within the PBC cohort. At genus level there was a relative abundance of *Escherichia* in the PSC cohort and *Bacteroides*

and *Bifidobacterium* in the PBC cohort. None of these findings reached statistical significance. There was a great deal of individual variablility within the 3 cohorts.

5.1.5 Aim 5: "To see if treatment and stage of liver disease has an impact on faecal microbiota"

Patients with cirrhosis had no detectable levels of *Akkermansia species* within samples when compared to controls and patients with only fibrosis. This bacterium is known to play a beneficial role has a role to play in gut health and may be a significant finding in terms of understanding increased gut permeability and translocation seen in cirrhosis and portal hypertension.

There was a statistically significant change between patients taking UDCA and those that did not. Patients on UCDA had significantly more Actinobacter and less Proteobacteria when compared to patients not taking this medication. UDCA may also be the reason that a greater difference was not seen between patients with cholestatic liver disease and controls.

5.1.6 Aim 6: "To assess the metabolic activity of the gut microbiota in PBC and PSC"

There were no significant differences between the metabolomic profile of patients with disease and healthy controls. This corresponds with the microbiota findings that produced no statistically significant differences in the metataxonomics between the groups.

5.2 Future Work

This study was aimed at proving if there was a core biliary microbiota. Having shown that such microbiota exists, that it is now important that this work is carried on to see how this extension of the bacterial organ has a role in the pathogenesis of disease. The study has raised many questions that require future research to be able to answer. Having identified that *Pseudomonas* is a prevalent species within

the normal biliary tract research can now focus on what interactions it has with the host and whether it has a role in immune mediated pathways. Several other bacteria have been isolated which previously had not been considered important in the pathogenesis of liver disease and may have a role to play. The most obvious of these is *Staphylococcus,* which is often a considered a contaminant. However, it is frequently isolated from GI and biliary studies and may be an important bacterium to study. A core microbiota has been identified but it is not clear whether alterations to this can cause or prevent disease. Manipulation of this microbiota through probiotics and antibiotics could provide further insight into the pathogenesis of biliary and liver disease.

The way in which these bacteria utilise and survive in bile salts may provide a therapeutic option for cholestatic disease. The bacteria isolated could be inoculated into media containing bile salts and the total concentrations of bile salts and metabolites measured following growth, thereby providing a mechanism through which interactions with the host take place. UDCA is a secondary bile acid and obeticholic acid (OCA) is a powerful FXR agonist. Both of these drugs have clear potential to interact with the gut microbiota and should be studied further in this context. It would be possible to examine these compounds using the *in vitro* colonic model to see if they cause direct changes to the microbiota or whether the effect seen is because of interactions with the host.

The finding that *Akkermansia* is reduced in cirrhotic patients is an interesting discovery from this study. It already has links to diabetes and obesity and clearly looks like it may have a role to play in the pathogenesis of liver disease. It would be interesting to see if the metabolic pathways these bacteria are associated with may play a role in the prevention of cirrhosis.

Finally, this study has isolated several fungi from the biliary tract. To date there is limited data on the role fungi have in the development of liver, biliary or gastrointestinal disease. It would be possible to identify these fungi through sequencing of their 18S rDNA and this 'mycobiomics' needs further research.

5.3   Conclusion

The work presented in this study provides evidence that there is a normal diverse biliary microbiota that alters with disease. Some of the bacteria isolated from the biliary tracts are able to utilise bile salts that may aid their survival and thereby provide a potential therapeutic target that requires further investigation. Treatment for chronic liver disease has an impact on the gut microbiota further highlighting the importance of the gut-liver axis in our understanding of liver disease.

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A MICROBIOLOGICAL ASSESSMENT OF PATIENTS WITH CHRONIC LIVER DISEASE

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Introduction

The gastrointestinal tract in humans is home to 100 trillion bacteria with at least 1000 different bacterial species (Shanahan, 2012). In the last decade research has begun to focus on this bacterial "organ" to see if it has an impact on the pathogenesis of disease. Many auto-immune conditions are thought to have an inflammatory or infective trigger so it is conceivable that changes in the gut microbiome could play a role in the development of some auto-immune disease. In Crohns disease it has been shown that there is abnormal microbial composition, increased mucosal-associated bacteria (Sartor, 2008) and that exposure to intestinal contents causes recurrent disease after loop ileostomy (D'Haens et al., 1998). It has been shown that there is a loss of biodiversity of the bacterial species (Noor et al., 2010) and an altered metabolic activity of the gut microbiota in patients with ulcerative colitis (Le Gall et al., 2011). A similar finding has been found in early rheumatoid arthritis with proportionally less bacteria, especially *Bifidobacterium* species, when compared to fibromyalgia patients (Vaahtovuo, Munukka, Korkeamaki, Luukkainen, & Toivanen, 2008). Increased intestinal permeability and increased intestinal lymphocytes have also been cited as possible causes in the pathogenesis of Type 1 diabetes and Hasimoto's thyroiditis (Mori, Nakagawa, & Ozaki, 2012; Bosi et al., 2006).

The liver receives 70% of its blood supply directly from the gastrointestinal tract via the portal vein. This results in continual exposure to gut bacteria and bacterial cell components and metabolites (Son, Kremer, & Hines, 2010). Indeed small bowel bacterial overgrowth has been associated with bacteraemia in spontaneous bacterial peritonitis (SBP) and hepatic encephalopathy and antibiotic therapy is a mainstay of treatment in the management and prevention of a number of complications of chronic liver disease (Quigley, Stanton, & Murphy, 2013). It is therefore conceivable that this complex gut microbiome has an important role in the development of chronic liver disease via this "gut-liver axis" and recent research has begun to explore this hypothesis. Recently it has been shown that there is a significant difference in the composition of the gut microbiome between cirrhotics and healthy controls with a significant increase in *Enterocoocus* and *Enterobacteriaceae* species (Liu et al., 2012). Changes in the microbiota have also been identified in non-alcohol related fatty liver disease, alcohol related liver disease and intestinal failure associated liver disease (Quigley et al., 2013).

The aim of this thesis is to expand our knowledge of the gut microbiome and its association with liver and biliary disease, in particular the pathogenesis of primary biliary cirrhosis (PBC), the impact of antibiotics in chronic liver disease and the investigation of bile especially the possibility that it is not sterile and may have a role in the composition of the gastrointestinal microbiome. The thesis will therefore be undertaken in 3 parts which are discussed below.

1. Is Bile Sterile?

Summary

Aim

Conventional wisdom dictates that bile is sterile. However, recent studies have identified bacterial populations within the gallbladder, gallstones and bile of symptomatic patients undergoing cholecystectomy. It is therefore feasible that bacteria are present within healthy individuals and this may a role in the development of liver and biliary disease. The aim of this study is to analyse bile from patients undergoing liver resection, cholecystectomy or pancreatectomy to assess whether bile is truly sterile.

Approach

A prospective study of the bacterial composition of bile sampled at the time of elective biliary surgery. Metaxonomic analysis will be performed on any positive growth. Bile resistance studies will also be performed on positive colonies to assess whether they have pathogenic potential. Metabolomic analysis will also be performed using NMR and LC-MC

Study population

Patients undergoing elective liver resection, pancreaticoduodenectomy (Whipple's procedure) and laparascopic cholecystectomy for gallstone disease.

Scientific background

It has now been established that cholesterol gallstones contain bacteria (Monstein, Jonsson, Zdolsek, & Svanvik, 2002) and bacteria including *H. pylori* has been detected within the mucosa of diseased gallbladders (Griniatsos et al., 2009). More recently studies have identified a diverse bacterial community within the bile of gallstone patients (Wu et al., 2013) and within diseased pancreatic ducts and biliary stents (Swidsinski et al., 2005). To date studies have been unable to culture bacteria directly from bile and no studies have assessed bile from patients not known to have biliary infection. The pathogenesis of hepatobiliary diseases such as PBC and primary sclerosing cholangitis are thought to include an infectious trigger (Pollheimer, Halilbasic, Fickert, & Trauner, 2011) although studies have failed to show any significant bacteriaemia in mesenteric and peripheral blood samples (Weismuller, Wedemeyer, Kubicka, Strassburg, & Manns, 2008). The immune response in PBC is restricted to the epithelial cells of the intrahepatic ducts (Selmi, Bowlus, Gershwin, & Coppel, 2011). It is therefore possible that bile is not sterile and may contain bacteria which could trigger disease in genetically susceptible patients.

As gallbladders are routinely removed during liver resections and the bile duct swabbed during pancreatic cancer resection we have the opportunity to sample and examine sterile bile using new sequencing technology to assess whether the human micro biome also has a role in liver and biliary disease.

Objectives

Overall objective

To assess whether "normal" bile is sterile.

Specific objectives

1) To culture "normal" bile.
2) To identify bacteria within bile through DNA extraction and 454 sequencing
3) To assess whether patients with cholelithiasis have bacterial colonization of bile
1. If bacteria are cultured, to assess their resistance to bile salts
2. To obtain information regarding the metabolic activity of bacteria within bile.

Study design

Recruitment policy

Patients under the care of Mr Weymss-Holden (Consultant hepato-biliary surgeon at the Norfolk and Norwich University Hospital) and Mr Harper

(Consultant hepato-biliary surgeon at Addenbrookes Hospital, Cambridge) undergoing liver resection and cholecystectomy, laparoscopic cholecystectomy or a pancreatectomywill be approached and consented pre-operatively.

Screening criteria

All patients undergoing elective liver resection and cholecystectomy, laparoscopic cholecystectomy or pancreatectomy procedure at the Norfolk and Norwich University Hospital or Addenbrookes hospital.

Basic Exclusion Criteria

Emergency procedures.
Current septicaemia.
Previous biliary sepsis

Screening Exclusion Criteria

Biliary intervention which would may increase the risk of colonization of the biliary tree such as biliary stents and sphincterotomy.
Inability to perform liver resection/ surgery such as disease progression.
Current antibiotic use or antibiotics within previous 30 days.
Current probiotic use or probiotic use within previous 30 days.

Procedures and timelines

Recruitment and samples will be collected between April 2013 and April 2015
Processing of the samples will be completed by January 2016.

Methods

Sampling

Bile samples will be collected at the time of surgery from resected gallbladders. This will be done under sterile conditions with the bile being placed in the sterile containers (which have previously been kept in anaerobic conditions for a minimum of 24 hours) for transport to the laboratory for processing.

Processing

Bile will be immediately inoculated onto different media both aerobically and anaerobically. Growth will be assessed at 24 and 48 hours. Colonies will be

separated and regrown before undergoing single colony 16S PCR. RNA will then be purified before being sent for 454 sequencing.

The remaining bile will then be frozen at -80C to preserve DNA before undergoing DNA extraction using the modified Qiagen protocol.

Glycerols will be taken of all colonies and stored at -80C for future investigation.

Analysis

Metaxonomic analysis on sequenced samples will be analysed using the QIIME computer programme to provide details of composition and quantitive measurement of the microbiota.

Bile resistance studies will be performed on colonies using the bioscreen and recording growth curves over 48 hours in increasing concentration of bile salts up to 10% to mimic the concentrations experienced in the gall bladder.

Metabolomic analysis will be by NMR and LC-MC

1. **Primary Biliary Cirrhosis**

Summary

Aim

The aetiology of primary biliary cirrhosis (PBC) is unclear but there is increasing evidence that the faecal microbiota plays an important role in the development of auto-immune disease and chronic liver disease. The aim of this project is to assess the faecal microbiota in PBC patients with and without cirrhosis as compared to controls. I also aim to look at the impact ursodeoxycholic acid has on the faecal microbiome, which may aid understanding of the mechanism of action for this drug.

Approach

This will be a prospective study with faecal and urine samples being collected from patients with PBC with and without liver cirrhosis. Bacterial DNA will be extracted from these samples and undergo metaxonomic and metabolomic analysis. These results will then be compared to age-matched controls.

Population

Patients with primary biliary cirrhosis will be selected from the liver database at the Norfolk and Norwich Hospital. Age-matched controls will then be selected from the tissue bank database.

Scientific background

The prevalence of primary biliary cirrhosis (PBC) has been increasing over the past 30 years and is now a significant cause of liver morbidity and mortality. The aetiology remains unclear although there is a clear auto-immune and genetic component as suggested by a weak association with HLA-B8, the discovery of 12 new susceptibility loci on genome-wide association studies (Mells et al., 2011), its association with extra hepatic autoimmune disease and a high concordance rate in monozygotic twins (Selmi et al., 2011). Several infectious and environmental factors are thought to contribute to the onset of PBC, as evidenced by clustering of cases near toxic waste sites in New York, the significantly higher rate of urinary tract infections in patients with PBC (Varyani, West, & Card, 2011) and the demonstration of molecular mimicry between mitochondrial and nuclear auto antigens in PBC (Shimoda et al., 2003). Given that PBC is a chronic inflammatory disorder it is possible that exposure to bacteria in a genetically susceptible individual may precipitate the development of the condition. Both pathogenic and non-pathogenic Gram-negative bacteria have been proposed but as yet not substantiated (Selmi et al., 2011). Through this study it may be possible to detect changes in the human microbiome in PBC patients which may provide further evidence to an infectious cause. At present treatment options for PBC are limited. Ursodeoxycholic acid (UDCA) isthe only treatment that has approval, but studies have shown that although it improves liver biochemistry and histological progression, it may have no effect on mortality, progression to liver transplantation or symptoms (Rudic, Poropat, Krstic, Bjelakovic, & Gluud, 2012). If a link between the gut microbiota and primary biliary cirrhosis can be established then it may open doorways to novel treatment strategies which could prevent and possibly treat established disease.

Objectives

Overall objective

To assess the role of the colonic microbiota in the pathogenesis of primary biliary cirrhosis.

Specific objectives

1) To perform quantitive assessment of the composition and phylogenic data of the gut microbiota in patients with PBC
2) To establish if the metabolite profiles derived from blood and urine samples can be correlated with differences in bacterial metabolism in PBC patients
3) To assess if the use of UCDA has an impact on the composition of the gut microbiota of patients with PBC.

Study design

Recruitment policy

Patients will be selected from the hepatology database at the Norfolk and Norwich University. They will then be invited to participate via letter with subsequent phone call to confirm participation. Patients may decline via e-mail or phone call at the time of receipt of the letter or at the time of follow up phone call. A meeting will be organised with those patients who wish to participate where a detailed explanation of the study and process for sample collection will take place.
Controls will age matched from samples collected in the tissue bank.

Screening Criteria

All patients identified as having PBC according to hepatology hospital database who live within 45 mins of the hospital. These patients will then be subdivided into those with cirrhosis and those without.

Basic Exclusion Criteria

Age > 80 or <18
Current antibiotic therapy
Concurrent liver disease of another aetiology

Screening Exclusion Criteria

Current antibiotic use including rifaximin and rifampicin
Antibiotic use within the last 30 days.
Current inclusion in banding programme.
Current diarrhoeal illness or diarrhoea within 2 weeks.
Current or recent use of laxatives within 30 days.
Acute decompensation and hospital admission within 30 days.

Procedures and Timelines

Recruitment and samples will be collected between April 2013 and April 2015. Processing of samples will be completed by January 2016.

Methods

Sampling

Arrangements will be made with patients at the time of consent for sample collection. This will either involve collecting the sample or delivery to the institute of food research by the participant. All samples will be delivered within 2 hours for processing.

Processing

Stool samples will be collected in sterile tubes and immediately stored at -80C until analysis. Total bacterial DNA will be extracted using the modified Qiagen soil protocol. The integrity of the nucleic acids will be determined visually using electrophoresis on 1% agarose gel containing ethidium bromide. Samples will then be sent for 454 sequencing.

Analysis

Metaxonomic analysis on sequenced samples will be analysed using the QIIME computer programme to provide details of composition and quantitive measurement of the microbiota. This programme will also provide information on the differences between PBC patients and controls.
Metabolomic analysis will be by NMR and LC-MC.

3. What is the evidence for prophylactic antibiotic use in increasing survival in cirrhosis?

Antibiotics are routinely used in prophylactic treatment of complications of cirrhosis such as variceal bleeding (Jalan & Hayes, 2000), spontaneous bacterial peritonitis (Moore & Aithal, 2006) and hepatic encephalopathy (Mullen et al.,

2013). It is also common practice to use antibiotics in acute liver failure (Lai, Lee, Han, & Kim, 2004). This is because patients with acute liver failure and acute decompensation of cirrhosis are prone to infection, especially *Staph. aureus* and *E. coli*, due to host defence mechanisms being impaired (Borzio et al., 2001). Despite this antibiotics are not routinely recommended in guidelines as there is uncertain survival benefit (Polson & Lee, 2005). The aim of this chapter is to perform a meta-analysis looking specifically at the role of prophylactic antibiotics in terms of survival in cirrhosis.

Ethical Approval

All aspects of this study have received ethical approval from the Faculty of Medicine and Health Research Ethics Committee, University of East Anglia

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Appendix 2: SOP for bile salt analysis

BILE ACID (BA) ANALYSIS FROM LIVER AND INTESTINAL SCRAPES

BA standards

d4 Internal standards

Each d4 internal standard is prepared at 1 mg/ml in MeOH

Prepare 5 solutions of these all at 40 µg/ml by taking 400µl of each stock standard to 10 ml in 70% MeOH

INT Std #	Int Std(s)
1	d4-GCA + d4 LCA
2	d4-CA
3	d4-CDCA
4	d4-DCA
5	d4-DCA/CDCA/CA/GCA/LCA

Calibration standards

Each BA is prepared at 1 mg/ml in MeOH and stored refrigerated.

Note: most are supplied as salt hydrates which must be taken into account when weighing out the standard.

A 10 µg mix of the BAs is prepared in 70% MeOH by taking 100 µl of each individual BA (at 1 mg/ml) into a pooled vial and making to 10 ml total volume.

Make all calibration standards to total volume of 500µl with methanol.

Note – where subsequent dilution made, you need to prepare 2 of these standards.

Std (ng/ml)	Vol (µl)	Of what std	Make up vol (µl) (MeOH)	Notes
4000	200	10 µg/ml mix	300	
2000	100	10 µg/ml mix	400	
1000	50	10 µg/ml mix	450	Prep x 2
500	25	10 µg/ml mix	475	
200	10	10 µg/ml mix	490	
100	50	1000 ng/ml mix	450	Prep x 2
25	12.5	1000 ng/ml mix	487.5	
15	75	100 ng/ml mix	425	
10	50	100 ng/ml mix	450	
5	25	100 ng/ml mix	475	
0	0	-	500	

Then to each of these, add 25μl of d4-Int Std mix #5 (40 μg/ml) to give 2000 ng/ml each.

BA standard identities

BIOCHEMICAL	Abbrev	Cat#	Supplier	IFR#
Chenodeoxycholic acid	CDCA	C9377	Aldrich	85
Deoxycholic acid	DCA	D2510	Aldrich	90
Dehydrocholic acid	DHCA	30830	Aldrich	348
Glycocholic acid	GCA	G2878	Aldrich	144
Glycochenodeoxycholic acid	GCDCA	G0759	Aldrich	146
Glycodeoxycholic acid	GDCA	G9910	Aldrich	143
Lithocholic acid	LCA	L6250	Aldrich	179
Taurocholic acid	TCA	T4009	Aldrich	262
Taurochenodeoxycholic acid	TCDCA	T6260	Aldrich	264
Taurodeoxycholic acid	TDCA	T0895	Aldrich	263
Ursodeoxycholic acid	UDCA	U5127	Aldrich	267
Taurolithocholic acid	TLCA	T7515	Aldrich	265
α-Muricholic acid	a-MCA	C1890-000	Steraloids	340
β -Muricholic acid	b-MCA	C1895-000	Steraloids	338
Cholic acid	CA	C1900-000	Steraloids	86
Glycolithocholic acid	GLCA	C1437-000	Steraloids	330
Hyodeoxycholic acid	HDCA	C0885-000	Steraloids	156
Muricholic acid	MCA	C1850-000	Steraloids	335
Tauro-α-Muricholic acid	T-a-MCA	C1893-000	Steraloids	333
Tauro- β -Muricholic acid	T-b-MCA	C1899-000	Steraloids	332
Glycohyocholic acid	GHCA	C1860-000	Steraloids	345
Glycoursodeoxycholic acid	GUDCA	C1025-000	Steraloids	341
Taurohyocholic acid	THCA	C1887-000	Steraloids	342

Taurohyodeocycholic acid	THDCA	C0892-000	Steraloids	343
Tauroursodeoxycholic acid	TUDCA	C1052-000	Steraloids	344
Glycohyodeoxycholic acid	GHDCA	C0867-000	Steraloids	346
Taurodehydrocholic acid	TDHCA	C2047-000	Steraloids	347
DEOXYCHOLIC ACID -D4	d4-DCA	C1070-015	Steraloids	
LITHOCHOLIC ACID -D4	d4-LCA	C1420-015	Steraloids	
CHOLIC ACID-D4	d4-CA	C1900-015	Steraloids	
GLYCOCHOLIC ACID -D4	d4-GCA	C1925-015	Steraloids	
CHENODEOXYCHOLIC ACID -D4	d4-CDCA	C0940-015	Steraloids	

Solutions required

70% Methanol

5% Methanol

Methanol

Sample preparation Liver extracts

Take 50mg (record weight) liver tissue in 2 ml screw cap tube + 4-6 1.4mm ceramic beads

Add 1 ml ice cold 70% MeOH

Add 25 µl of Int Std #4 (so is present at 1000 ng/ml)

Homogenise 30s 6000 (prog 4 in Precellys)

Centrifuge 5 min 3000 rcf 4°C

Take supernatant to new 1.5ml Eppendorf tube

Add 25 µl of Int Std #3

Rotary evaporate at 50°C for 70 minutes (removes the MeOH content)

This will not be to dryness, but make up by eye in eppendorf tube scaling to 1 mL with 5% MeOH

Add 25 µl Int Std #2

Solid Phase Extraction (SPE) Clean-up

Clean-up is via **Waters OASIS PRIME HLB 1 30mg** SPE cartridges.

Install cartridges into SPE vacuum system.

Load sample onto tube.

Engage vacuum as low as possible. Some tubes will empty quicker than others, but minimum of 3 minutes to empty tube and collect to waste.

As each tube becomes empty, close it off and increase vacuum as necessary with care to allow all remaining tubes to empty.

Once all empty, decrease vacuum and open all tubes. Re-apply vacuum to dry cartridges applying moderate vacuum for 2 minutes.

Vacuum off and close cartridges.

Wash with 1 ml of 5% MeOH

Apply same elution and dry procedure as above ensuring cartridges are dry.

Discard washings and replace collectors with labelled 1.5 ml Eppendorfs

Elute with 500 µl 100% MeOH using same procedure.

Add 25 µl of Int Std # 1 to eluate.

Summary of SPE

- Load sample (1 ml)
- Wash with 1 ml 5% MeOH
- Elute with 1 ml MeOH

The issue here is not to allow solutions to pass through too quickly. Different tubes elute at different rates so the vacuum is adjusted accordingly. Note there is no cartridge conditioning step necessary with this product.

Transfer samples to low volume autosampler tubes for LC-MS analysis.

LC-MS

Conditions

Column : Supelco Ascentis Express C18 150 x 4.6, 2.7µm

Flow: 600 µl/min

Mobile phase A : Water + 5mM Amm. Ac + 0.012% Formic acid

Mobile Phase B: Methanol + 5mM Amm. Ac + 0.012% Formic acid

Inj: 5µl

Column: 40°C

Mobile phase preparation

In one litre of MeOH or Water:

5 mM Ammonium Acetate = 0.385g

0.012% formic acid = 120 µl

LC Gradient

Time	%B
0	50
2	50
20	95
24	95
25	50
29	50

Source Conditions (negative mode)

SOURCE	
CUR:	25
TEM:	550
GS1:	40
GS2:	50
ihe:	ON
CAD:	-2
IS:	-4500
EP	-10
CXP	-9

MRM settings

ID	Q1	Q3	Dwell	DP	CE	RT
LCA	375.3	375.3	20	-90	-10	22.7
CDCA	391.3	391.3	20	-120	-10	20.85
DCA	391.3	391.31	20	-120	-10	21.17
HDCA	391.3	391.32	20	-120	-10	18.29
MCA	391.3	391.33	20	-120	-10	16.57
UDCA	391.3	391.34	20	-120	-10	17.52
DHCA	401.2	401.2	20	-90	-10	
a-MCA	407.3	407.3	20	-120	-10	15.93
b-MCA	407.3	407.31	20	-120	-10	16.29
CA	407.3	407.32	20	-120	-10	18.89
w-MCA	407.3	407.33	20	-120	-10	15.93
GLCA	432.3	432.3	20	-80	-10	19.77
GUDCA	448.2	448.3	20	-130	-40	14.15
GCDCA	448.3	448.31	20	-80	-10	17.72
GDCA	448.3	448.32	20	-80	-10	18.28
GCA	464.3	464.3	20	-80	-10	15.7
TLCA	482.2	482.2	20	-130	-10	19.19
TUDCA	498.2	498.3	20	-130	-60	13.66
TCDCA	498.3	498.31	20	-130	-10	17.1
TDCA	498.3	498.32	20	-130	-10	17.64
THDCA	498.3	498.33	20	-130	-10	14.26
T-a-MCA	514.3	514.3	20	-130	-10	10.81
T-b-MCA	514.3	514.31	20	-130	-10	11.06
TCA	514.3	514.32	20	-130	-10	15.08
THCA	514.3	514.33	20	-130	-10	13.62
d4-LCA	379.3	379.3	20	-90	-10	22.7
d4-CDCA	395.3	395.31	20	-120	-10	20.83
d4-DCA	395.3	395.3	20	-120	-10	21.13

d4-CA	411.3	411.3	20	-120	-10	18.86
d4-GCDCA	452.4	74	20	-80	-40	17.73
d4-GCA	468.4	74	20	-80	-40	15.71
tlc query	482.2	80	20	-130	-60	
tdc query	498.2	498.2	20	-130	-60	
P lipid query	153	153	20	-130	-10	

Qualifiers

ID	Q1	Q3	Dwell	DP	CE	RT
GLCA	432.3	74	20	-80	-40	20.59
GCDCA	448.3	74.11	20	-80	-40	18.31
GDCA	448.3	74.12	20	-80	-40	18.93
GCA	464.3	74	20	-80	-40	16.03
TCDCA	498.3	80.1	20	-130	-60	17.33
TDCA	498.3	80.11	20	-130	-60	17.87
T-a-MCA	514.3	80.1	20	-130	-60	10.68
T-b-MCA	514.3	80.11	20	-130	-60	11.02
TCA	514.3	80.12	20	-130	-60	15.12
TLCA	482.2	80	20	-130	-60	19.57

QTrap method file : **Bile salts (MP).dam**

Example Solvent Standard Chromatogram.
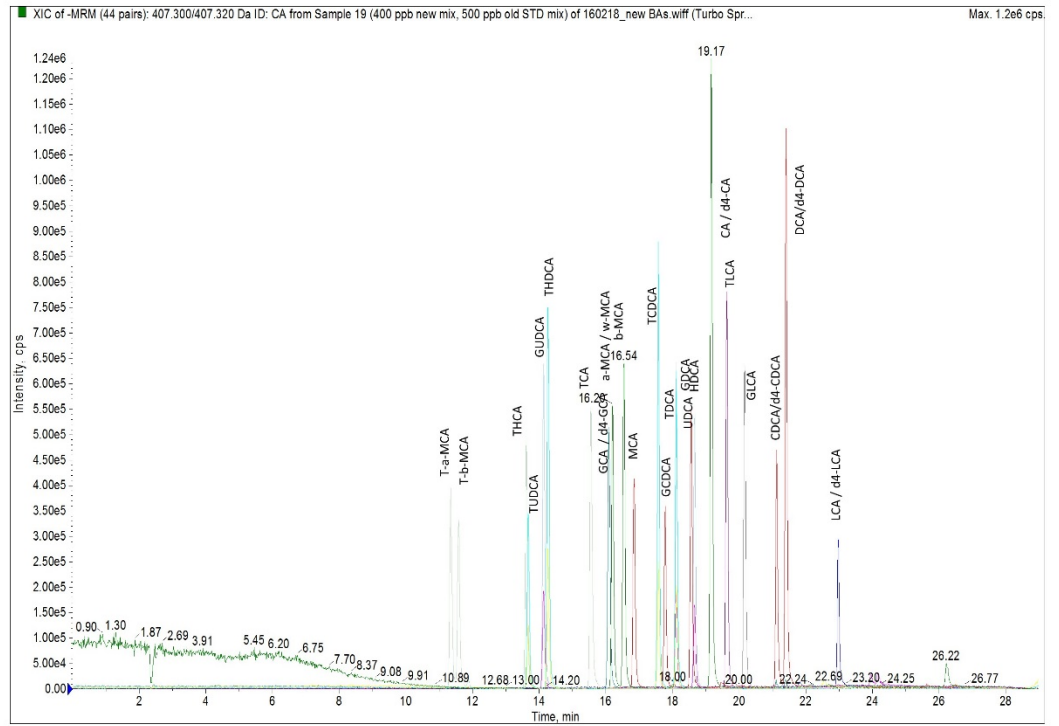
For publication:

Cleaned-up extracts were analysed using HPLC – mass spectrometry operated in multiple reaction monitoring (MRM) mode.

Each sample (5 µl) was analysed using an Agilent 1260 binary HPLC coupled to an AB Sciex 4000 QTrap triple quadrupole mass spectrometer. HPLC was achieved using a binary gradient of solvent A (Water + 5mM Ammonium Ac + 0.012% Formic acid) and solvent B (Methanol + 5mM Ammonium Ac + 0.012% Formic acid) at a constant flow rate of 600 µl/min. Separation was made using a Supelco Ascentis Express C18 150 x 4.6, 2.7µm column maintained at 40°C. Injection was made at 50% B and held for 2 min, ramped to 95%B at 20 min and held until 24 minutes. The column equilibrated to initial conditions for 5 minutes.

The mass spectrometer was operated in electrospray negative mode with capillary voltage of -4500V at 550°C. Instrument specific gas flow rates were 25ml/min curtain gas, GS1: 40 ml/min and GS2: 50 ml/min

Mass fragmentation was monitored as in the table below.

Quantification was applied using Analyst 1.6.2 software to integrate detected peak areas relative to the deuterated internal standards.

A portion of sample (50 mg accurately weighed) was taken into a tube along with 6 ceramic beads, 1 ml of 70% v/v methanol and 25µl of 40 µg/ml d4-DCA and then homogenised for 30 seconds at 6000 rpm. The slurry was centrifuged at 3000 rpm at 4°C and the supernatant transferred to a new tube with the addition of 25 µl of 40 µg/ml d4-CDCA. This was evaporated by centrifugal evaporation at 50° for 70 minutes to almost dryness and then made to 1 ml volume with 5% v/v methanol and addition of 25 µl of 40 µg/ml d4-CA.

The reconstituted sample was passed through a hydrophilic-lipophilic balance clean-up cartridge (Waters Oasis Prime HLB, 1cc, 30mg), washed with 1 ml of 5% methanol and eluted in 500 µl methanol and addition of 25 µl of 40 µg/ml d4-GCA and d4-LCA. Of the internal standards added, d4-GCA was the primary reference internal standard with the others monitored as checks in the extraction procedure.

The final sample was submitted for analysis my LC-MS/MS.

Appendix 3: Metabolomics results for urine

concentrations_urine_final.xlsx

Concentrations (mM)		amino acids					
reaction end-products		energy related (incl. citric cycle)	code	(mM)	PSC vs C	PBC vs C	PSC vs PBC
sugars		purines and pyrimidines	Urine ID	average	t-test	t-test	t-test
index	Compound	chenomx list	index (ppm)		12 markers	4 markers	7 markers
1	Acetate	Acetate	1.91 s	0.3539	0.66	0.41	0.63
2	Propionate	Propionate	1.05 t	0.0021	#DIV/0!	0.34	0.34
3	Isobutyrate	Isobutyrate	1.06 d	0.0532	0.98	0.27	0.24
4	Lactate	Lactate	1.32 d	0.2527	0.38	0.65	0.32
5	Formate	Formate	8.46 s	0.4973	0.39	0.37	0.41
6	Ethanol	Ethanol	1.17 t	0.0656	0.17	0.51	0.31
7	Methanol	Methanol	3.34 s	0.0844	0.01	0.28	0.14
8	Dimethylamine	Dimethylamine	2.72 s	0.3198	0.38	0.88	0.44
9	Trimethylamine	Trimethylamine	2.87 s	0.0121	0.12	0.22	0.31
10	Trimethylamine N-oxide	Trimethylamine N-oxide	3.25 s	0.4684	0.77	0.61	0.82
11	Dimethylglycine	Dimethylglycine	2.92 s	0.0359	0.63	0.72	0.97
12	Hippurate	Hippurate	7.54 t	1.5284	0.09	0.04	0.56
13	4-Hydroxyphenylacetate	4-Hydroxyphenylacetate	6.85 d	0.0998	0.84	0.85	0.99
14	N-Phenylacetylglutamine	N-Phenylacetylglycine	7.41 d	0.8364	0.94	0.08	0.09
15	3-Indoxylsulfate	3-Indoxylsulfate	7.73 d	0.2098	0.44	0.63	0.29
16	3-Phenylpropionate	3-Phenylpropionate	7.30 d	0.0756	0.01	0.30	0.06
17	Benzoate	Benzoate	7.46 t	0.1035	0.21	0.16	0.19
18	Quinolinate	Quinolinate	8.00 dd	0.0401	0.02	0.05	0.94
19	phenolic 6.93 ppm d	4-Hydroxy-3-methylbenzoic acid	6.93 d	0.5748	0.02	0.17	0.08
20	4-Aminohippurate	4-Aminohippurate	7.68 d	0.0021	0.33	#DIV/0!	0.33
21	4-Hydroxyhippurate	4-Hydroxybenzoate	6.97 d	0.0795	0.25	0.52	0.77
22	2-Hydroxyisovalerate	2-Hydroxyisovalerate	0.82 d	0.0048	0.63	0.13	0.18
23	2-Aminobutyrate	2-Aminobutyrate	0.97 t	0.0192	0.76	0.71	0.24
24	2-Hydroxybutyrate	2-Hydroxybutyrate	0.89 t	0.0080	0.32	0.98	0.14
25	2-Hydroxyisobutyrate	2-Hydroxyisobutyrate	1.35 s	0.0476	0.51	0.78	0.64
26	2-Hydroxyvalerate	2-Hydroxyvalerate	0.90 t	0.0065	0.61	0.75	0.21
27	2-Oxobutyrate	2-Oxobutyrate	1.04 td	0.0107	0.40	0.30	0.41
28	2-Oxoisocaproate	2-Oxoisocaproate	0.92 d	0.0088	0.11	0.33	0.38
29	3-Aminoisobutyrate	3-Aminoisobutyrate	1.18 d	0.1144	0.79	0.79	0.49
30	3-Hydroxyisobutyrate	3-Hydroxyisobutyrate	1.12 d	0.0106	0.29	0.20	0.61
31	3-Hydroxyisovalerate	3-Hydroxyisovalerate	1.26 s	0.0382	0.86	0.54	0.31
32	4-deoxyerythreonate	Propylene glycol	1.13 d	0.0474	0.30	0.20	0.34
33	4-deoxythreonate	L-Allothreonine	1.23 d	0.2994	0.52	0.34	0.37
34	Methylmalonate	Methylmalonate	1.22 d	0.1165	0.32	0.08	0.09
35	Methylsuccinate	Methylsuccinate	1.09 d	0.1075	0.26	0.91	0.13
36	Arabinose	Arabinose	4.51 d	0.1566	0.32	0.66	0.62
37	Fucose	Fucose	1.24 d	0.0926	0.71	0.74	0.98
38	Glucose	Glucose	4.64 d	1.9688	0.30	0.97	0.47
39	Ribose	Ribose	4.92 d	0.0925	0.70	0.76	0.92
40	Xylose	Xylose	4.57 d	0.1277	0.53	0.28	0.04
41	1,6-Anhydro-β-D-glucose	1,6-Anhydro-β-D-glucose	5.44 bs	0.0960	0.81	0.94	0.84
42	Ascorbate	Ascorbate	4.51 d	0.0608	0.09	0.70	0.11
43	Mannitol	Mannitol	3.86 dd	0.6415	0.53	0.35	0.13
44	myo-Inositol	myo-Inositol	3.53 dd	0.1367	0.27	0.29	1.00
45	Gluconate	Gluconate	4.29 d	0.3279	0.99	0.51	0.46
46	Glucuronate	Glucuronate	5.24 d	0.2679	0.08	0.15	0.67
47	Alanine	Alanine	1.47 d	0.2667	0.91	0.45	0.30
48	Asparagine	Asparagine	2.85 dd	0.0467	0.00	0.77	0.01
49	Aspartate	Aspartate	2.65 dd	0.0166	0.02	0.34	0.03
50	N-Acetylaspartate	N-Acetylaspartate	2.00 s	0.0249	0.30	0.25	0.92
51	Glutamine	Glutamine	2.46 m	0.2969	0.36	0.59	0.67
52	Pyroglutamate	Pyroglutamate	2.38 t	0.1509	0.83	0.88	0.92
53	Glycine	Glycine	3.55 s	1.0595	0.07	0.07	0.69
54	Histidine	Histidine	7.10 s	0.1462	0.88	0.56	0.61
55	Isoleucine	Isoleucine	1.00 d	0.0145	0.61	0.91	0.64
56	Valine	Valine	0.98 d	0.0330	0.51	0.94	0.50
57	Leucine	Leucine	0.96 t	0.0249	0.34	0.75	0.14
58	Lysine	Lysine	1.72 m	0.0820	0.82	0.54	0.05
59	Threonine	Threonine	1.32 d	0.1239	0.03	0.08	0.24
60	Serine	Serine	3.98 dd	0.2676	0.03	0.24	0.14
61	Tryptophan	Tryptophan	7.71 d	0.0558	0.87	0.30	0.37
62	Tyrosine	Tyrosine	6.88 d	0.1036	0.20	0.34	0.94
63	Taurine	Taurine	3.26 t	0.2931	0.08	0.62	0.03
64	Choline	Choline	3.19 s	0.0348	0.07	0.26	0.49
65	sn-Glycero-3-phosphocholine	sn-Glycero-3-phosphocholine	3.21 s	0.0110	0.00	0.01	0.86
66	Ethanolamine	Ethanolamine	3.14 t	0.2335	0.05	0.47	0.18
67	Betaine	Betaine	3.25 s	0.1070	0.55	0.39	0.55
68	Carnitine	Carnitine	3.21 s	0.0934	0.45	0.65	0.73
69	O-Acetylcarnitine	O-Acetylcarnitine	3.18 s	0.0362	0.90	0.80	0.64
70	Citrate	Citrate	2.54 d	2.8733	0.92	0.66	0.65
71	Fumarate	Fumarate	6.51 s	0.0067	0.86	0.96	0.80
72	Pyruvate	Pyruvate	2.37 s	0.0364	0.55	0.40	0.37
73	Malate	malic_ifr	2.63 dd	0.0162	0.33	#DIV/0!	0.33
74	Succinate	Succinate	2.40 s	0.0458	0.82	0.25	0.22
75	2-Oxoglutarate	2-Oxoglutarate	3.00 t	0.0530	0.93	0.86	0.79
76	Tartaric acid	Tartaric acid	4.33 s	0.4104	0.35	0.15	0.12
77	Glycolate	Glycolate	3.94 s	0.3774	0.63	0.75	0.40
78	Pantothenate	Pantothenate	0.87 s	0.0234	0.82	0.27	0.30
79	3-Hydroxybutyrate	3-Hydroxybutyrate	1.19 d	0.0197	0.82	0.80	0.33
80	Creatine	Creatine	3.02 s	0.2166	0.65	0.85	0.86
81	Creatinine	Creatinine	3.04 s	7.8322	0.18	0.96	0.18
82	Adenosine phosphate	Adenosine triphosphate	6.14 d	0.0099	0.55	0.30	0.54
83	Allantoin	Allantoin	5.38 bs	0.0990	0.67	0.78	0.53
84	Malonate	Malonate	3.11 s	0.0956	0.26	0.46	0.52
85	Hypoxanthine	Hypoxanthine	8.18 s	0.0421	0.01	0.41	0.03
86	Xanthine	Xanthine	7.95 s	0.1714	0.10	0.89	0.10
87	Pseudouridine	Pseudouridine	4.28 t	0.1489	0.27	0.82	0.31
88	Dihydrothymine	Dihydrothymine	1.07 t	0.0144	0.60	0.54	0.91
89	Inosine	Inosine	6.07 d	0.0025	0.32	0.36	0.01
90	2-py (N-methyl-2-pyridone-5-carboxamide)	Gentisate	6.65 d	0.0861	0.30	0.42	0.71
91	1-Methylhistidine	1-Methylhistidine	7.00 s	0.0733	0.31	0.93	0.23
92	3-Methylhistidine	3-Methylhistidine	7.05 s	0.0670	0.71	0.13	0.15
93	1-Methylnicotinamide	1-Methylnicotinamide	9.26 s	0.0401	0.03	0.02	0.82
94	Trigonelline	Trigonelline	9.11 s	0.1368	0.35	0.26	0.76

Appendix 4: Metabolomic results for faeces

concentrations_faecal_final.xlsx
Concentrations (mmol/kg)   final conc =values (mM) *0.0006(L)/0.00005 (Kg)=mmol/kg

index	metabolite	metabolite in chenomx coding coding	average amount (mmol/kg)
1	Butyrate	Butyrate	17.11
2	Acetate	Acetate	59.25
3	Propionate	Propionate	16.50
4	Valerate	Valerate	2.68
5	Isobutyrate	Isobutyrate	1.42
6	Isovalerate	Isovalerate	1.69
7	2-methylbutyrate	2-methylbutyric_ifr	0.76
8	Lactate	Lactate	0.32
9	Formate	Formate	0.37
10	Propylene glycol	Propylene glycol	0.01
11	Ethanol	Ethanol	6.95
12	Methanol	Methanol	0.90
13	Glycerol	Glycerol	1.49
14	Isopropanol	Isopropanol	0.11
15	Indole-3-lactate	Indole-3-lactate	0.01
16	Indoleacetate	Indoleacetic acid	0.03
17	Dimethylamine	Dimethylamine	0.06
18	Methylamine	Methylamine	0.20
19	Trimethylamine	Trimethylamine	0.13
20	Dimethylglycine	Dimethylglycine	0.04
21	Putrescine	Putrescine	0.13
22	Cadaverine	Cadaverine	0.18
23	Tyramine	Tyramine	0.02
24	4-Hydroxyphenylacetate	4-Hydroxyphenylacetate	0.07
25	3-(3-Hydroxyphenyl)-3-propanoic acid	3-(3-Hydroxyphenyl)-3-hydroxypropanoic acid	0.03
26	3-Hydroxyphenylacetate	3-Hydroxyphenylacetate	0.06
27	3-Phenylpropionate	3-Phenylpropionate	0.27
28	Phenylacetate	Phenylacetate	0.62
29	4-Aminohippurate	4-Aminohippurate	0.00
30	p-Cresol	p-Cresol	0.25
31	Phenol	Phenol	0.01
32	Benzoate	Benzoate	0.02
33	2-Hydroxyisovalerate	2-Hydroxyisovalerate	0.00
34	3-Methyl-2-oxovalerate	3-Methyl-2-oxovaleric acid	1.10
35	Malonate	Malonate	2.48
36	β-Alanine	β-Alanine	0.08
37	2-Aminoadipate	2-Aminoadipate	0.39
38	Glutarate	Glutarate	0.36
39	2-Oxoisocaproate	2-Oxoisocaproate	0.00
40	Methylsuccinate	Methylsuccinate	0.12
41	2-Piperidinone	2-Piperidinone	0.03
42	5-Aminovalerate	5-Aminopentanoic acid	0.40
43	Acetaminophen	Acetaminophen	0.02
44	analgesic compound 1 at 6.80 ppm	Gentisate	2.10
45	analgesic compound 2 at 6.85 ppm	Gentisic acid	0.24
46	analgesic compound 3 at 6.93 ppm	Protocatechuate	1.11
47	Arabinose	Arabinose	0.13
48	Fucose	Fucose	0.11
49	Galactose	Galactose	0.27
50	Glucose	Glucose	3.46
51	Ribose	Ribose	0.74
52	Alanine	Alanine	1.46
53	Asparagine	Asparagine	0.14
54	Aspartate	Aspartate	1.05
55	Glutamate	Glutamate	3.58
56	Glutamine	Glutamine	0.96
57	Glycine	Glycine	1.28
58	Histidine	Histidine	0.26
59	Isoleucine	Isoleucine	0.85
60	Methionine	Methionine	0.52
61	Valine	Valine	1.35
62	Leucine	Leucine	1.53
63	Lysine	Lysine	1.02
64	Threonine	Threonine	0.89
65	Serine	Serine	0.70
66	Phenylalanine	Phenylalanine	0.63
67	Proline	Proline	0.42
68	Tryptophan	Tryptophan	0.13
69	Tyrosine	Tyrosine	0.71
70	4-Aminobutyrate	4-Aminobutyrate	0.11
71	Taurine	Taurine	0.72
72	Choline	Choline	0.11
73	O-Phosphocholine	O-Phosphocholine	0.03
74	Citrate	citrate_ifr	0.01
75	Fumarate	Fumarate	0.06
76	Succinate	Succinate	2.08
77	Pyruvate	Pyruvic acid at 1.48 ppm s	0.79
78	Creatine	Creatine	0.07
79	Deoxycholate	Deoxycholic acid	0.16
80	Lithocholate	Chenodeoxycholic acid	3.31
81	Cholate	Cholate	0.09
82	Adenine	Adenine	0.01
83	adenosine phosphate	AMP	0.01
84	Hypoxanthine	Hypoxanthine	0.19
85	Inosine	Inosine	0.01
86	Uracil	Uracil	0.24
87	Uridine	Uridine	0.00
88	Xanthine	Xanthine	0.25
89	Nicotinate	Nicotinate	0.08

Appendix 5: Nanodrop results from biliary samples

Sample Number	Nanodrop result (ng/ul)
1GB	13.3
2GB	20.6
3GB	16.8
4GB	86.3
5GB	25.4
6GB	26
7GB	4.1
8GB	18.2
9GB	1.3
10CBD	583.2
11GB	11
12GB	151.5
13GB	9
14CBD	21
15CBD	2.7
16CBD	1.2
17CBD	3.4
18CBD	14.3
19CBD	1.6
20CBD	51.4
21CBD	58.1
22GB	5.6
23CBD	13.2
23GB	26.8
24CBD	16.3
25CBD	20.3
25GB	38.2
26CBD	8.4
26GB	22.8
27CBD	2.1
27GB	7.3
28CBD	19.4
28GB	57.2
29CBD	4.7
30GB	44.4
31CBD	4.3
32CBD	34.7
32GB	24.5
33CBD	27.9

Table Appendix 1: Nanodrop results following DNA extraction from samples. GB = gallbladder sample, CBD = common bile duct