Manipulating the Gut Microbiome using Faecal Microbiota Transplantation as an Alternative Treatment for Bile Acid Diarrhoea

School of Life Sciences

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1. Abbreviations

Abbreviation	Definition	Abbreviation	Definition
7-KDCA	7-ketodeoxycholic acid	LCA	Lithocholic Acid
7keto-LCA	7-keto-lithocholic acid	MS	Mass Spectrometry (HPLC-MS)
ASBT	Apical Sodium Bile Acid Transporter	OH	Hydroxyl
BA	Bile Acid	SeHCAT	75-selenium homocholic acid taurine
BAD	Bile Acid Diarrhoea	UDCA	Ursodeoxycholic Acid
BAI	bile acid-inducible		
BAM	Bile Acid Malabsorption		
BSH	Bile Salt Hydrolases		
BSM	Bile Salt Malabsorption		
C4	7α-Hydroxy-4-cholesten-3-one		
CA	Cholic Acid		
CDCA	Chenodeoxycholic acid		
CYP27A1	Sterol 27-hydroxylase		
CYP7A1	Cholesterol 7 alpha-hydroxylase		
DCA	deoxycholic acid		
EHC	Enterohepatic Circulation		
FGF15	Fibroblast Growth Factor 15		
FGF19	Fibroblast Growth Factor 19		
FMT	Faecal Microbiota Transplant		
FXR; NR1H4	Farnesoid X Receptor		
GCA	Glycocholic Acid		
GCDCA	Glycochenodeoxycholic Acid		
GI	Gastrointestinal		
GUDCA	glycoursodeoxycholic acid		
HPLC	High Performance Liquid Chromatography		
HSDH	Hydroxysteroid Dehydrogenase		
IBD	Inflammatory bowel disease		
IBS	Irritable Bowel Syndrome		
IBS-D	diarrhoea-predominant IBS		

2. Literature

2.1 Introduction to Bile Acid Diarrhoea

Bile acid diarrhoea (BAD) which was previously referred to as bile acid malabsorption (BAM) or bile salt malabsorption (BSM) is a condition that predominantly presents as chronic watery diarrhoea as well as bloating and abdominal pain [1]. BAD is associated with excessive bile acid (BA) synthesis secondary to a gastrointestinal (GI) disease or an idiopathic disorder (also known as primary BAD) [2]–[4]. Bannaga *et al* observed that over 90% of survey participant suffering from BAD also felt a negative impact on mental health [1]. They found depression, isolation, helplessness and low self-esteem are very common among patients living with BAD [1]. Smith *et al*. estimated that over one-third of patients diagnosed with irritable bowel syndrome (IBS) actually suffer with BAD [5]. Although figures of disease prevalence are estimations of estimates, it is thought that up to 1% of the population have primary BAD [6]. If non-primary BAD patients are also included, this potentially equates in excess of 700,000 individuals in the United Kingdom [7]. Consequently, BAD has wide-reaching economic impacts due to absence of work through sickness and cost of continued care and medications through The National Health Service.

BAD is a condition that results from excess BAs synthesis that includes insufficient absorption of BAs in the ileum causing, increased transit of BAs into the colon. High levels of BA in the colon triggers an increase of water secretion in the colon to combat irritation, this prevents stool from properly forming, leading to diarrhoea. BAD has several different causes that fall under one of three types. Type I, when the ileum is damaged due to inflammation or removal. Type II is idiopathic also known as 'primary BAD'. Type III, results from other diseases or conditions within the abdomen. Walters *et al* first proposed that type II BA malabsorption was not a result of reduced or impaired absorption but in fact unregulated BA synthesis [4]. Studies demonstrated that patients with BAD had significantly higher levels of 7α-Hydroxy-4-cholesten-3-one (C4, a precursor for BA) and significantly lower levels of fibroblast growth factor 19 (FGF19, a hormone that down regulates BA synthesis) compared with healthy individuals [4]. They also demonstrated that patients with secondary BAD (type I and III BAD) similarly have low levels of FGF19 in blood serum compared with healthy subjects, as expected [2]–[4]. Despite this, the cause of low levels of FGF19 remains widely unknown.

2.1.1 Diagnosing and Treating Bile Acid Diarrhoea

The 75-selenium homocholic acid taurine (SeHCAT) scan, a nuclear medicine test, is considered the gold standard for diagnosing BAD as it has a high sensitivity and specificity [8]. An artificial

BA (SeHCAT) is orally ingested and a preliminary scan is performed on the same day to establish the initial base amount of radioactive BAs in the body. One week later a second scan will show how much SeHCAT has been retained and thus whether sufficient absorption is taking place. C4, a BA precursor present in blood sera, can also be used as a marker in diagnosing BAD [9], [10]. In the absence of a SeHCAT scan doctors often prescribe treatment as the response to medication can provide a diagnosis, this is known as a trial of therapy.

Treatment for BAD includes diet changes and medication; however, these just relieve symptoms and there is currently no cure for BAD. Doctors advise a diet with reduced fat intake of 40g of fat per day [11]. BA sequestrant medicines are often prescribed and work by binding BA with high affinity and preventing them from irritating the large intestine. Current treatment for BAD has not changed since the 1960s and have adverse effects if the patients is taking other medications [12]. Frequently treatment for BAD is not administrated because it is often misdiagnosed as IBS as both diseases have similar clinical presentations. The pathophysiology of BAD is incompletely understood and to date, a clinically effective cure remains elusive.

1.1. Structure and Function of Bile Acids

BAs are the main component of bile and are amphiphilic molecules with 24 carbon atoms that possess a hydrophobic steroid nucleus to which are attached hydrophilic hydroxyl group(s) and an acidic aliphatic side chain (**Figure 2.1**). There are two types of BAs; primary BA and secondary BA. Primary BAs in humans can be further divided between 3α, 7α, 12α-trihydroxy-5β-cholan-24-oic acid (cholic acid, CA) and 3α, 7α-dihydroxy-5β-cholan-24-oic acid (Chenodeoxycholic acid, CDCA). These BAs differ at position C-12 where CA possess a hydroxyl (OH) group whereas CDCA does not. In mice the primary BAs are α and β-muricholic acid. Primary BAs are often conjugated (N-acyl amidation) with an amino acid, nearly always (95%) glycine or taurine conjugated at position C-24 (**Figure 2.1**) [13]. Of this 95%, approximately 75% are conjugated to glycine and 25% conjugated to taurine. Unconjugated BAs have a pKa value range of 5-6. The addition of an amino acid lowers the pKa value of glycine and taurine conjugated BAs from 5–6 to 4–5 and 1–2, respectively. At physiological pH of human bile (pH 7.0 to. 7.7), bile consists predominantly of conjugated BAs which are almost fully ionised salts (ie. complete separation of positive and negative charged parts) and therefore alternatively known as bile salts or strong BAs [14]. This makes them more water-soluble thus able to fulfil one of their physiologic function and prevents conjugated BA passive re-absorption in the small intestines.

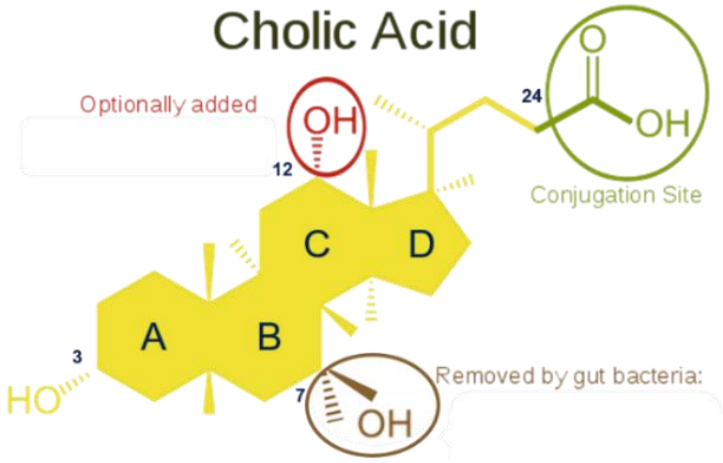


Figure 2.1: Adapted molecular structure of cholic acid annotated to show its relationship to other BAs found in humans [15]

BAs have a number of functions in the body. As amphipathic molecules with hydrophobic and hydrophilic regions, at the right concentration, conjugated BAs form micelles (F**igure 2.2**) which function as biological detergents that play a key role in fat digestion through emulsifying and solubilising lipids in the small intestines [16]. Consequently, BAs are essential for the absorption of dietary lipids including lipoproteins and fat-soluble vitamins such as vitamin D. They also act as a signalling molecule that regulates several important functions including its own expression, immune homeostasis, glucose and lipid metabolism [17], [18]. Due to their amphipathic detergent nature, BAs are toxic to microbes that act as antimicrobial agents through membrane-damage thus help to maintain bacterial homeostasis in the GI tract [19]. Even at sub-micellar concentrations, BAs can alter bacterial membrane lipid composition [20].

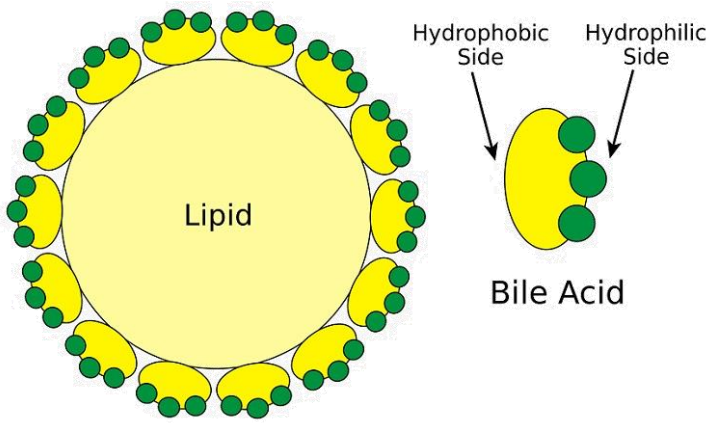


Figure 2.2: Bile acid action on lipids in the gut: Bile acids congregating, at the right concentration, around lipids to separate them into small droplets called micelles [21].

2.2 The Enterohepatic Circulation and Primary Bile Acid Synthesis

BAs are synthesised primarily by the hepatocytes in the liver, but also in the brain and macrophages [22], [23]. On average, human adults secrete between 12-18 g of BAs into the intestine each day from the gallbladder, typically following meals. Yet, normally, at any given time there is between 4–6 g of BAs, which is known as the BAs pool [24]. This is possible due to the enterohepatic circulation (EHC) of BAs (F**igure 2.3**). 95 % of all intestinal BAs are reabsorbed by passive diffusion in the intestines or particularly in the case of conjugated BAs, by active transport in the ileum via apical sodium–bile acid transporter (ASBT). Following reabsorption they are recycled by the liver with the remaining unabsorbed BAs (5 % ~400-800mg) passing through to the colon, and excreted [25].

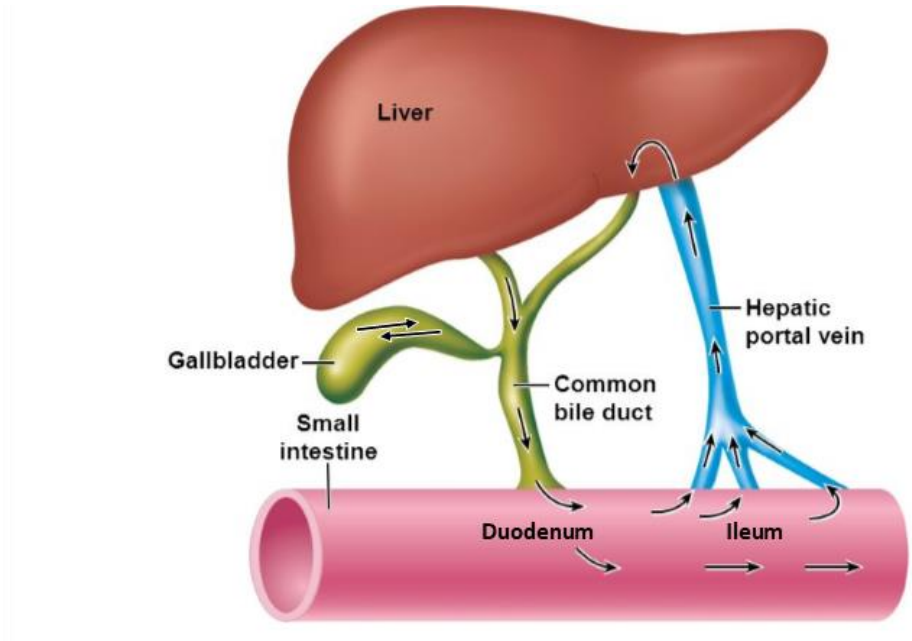


Figure 2.3: Modified enterohepatic circulation of biliary acids from the liver directly into the duodenum (proximal small intestine) or via the gallbladder for storage. Followed by absorption by enterocyte in the ileum (distal small intestine) and transported back to the liver [26].

This occurs through one of two pathways: the classic (neutral) pathway (occurs ~ 90% humans and ~ 75% in mice) or the alternative (acidic) pathway (occurs ~ 10% humans and ~ 25% in mice) (**Figure 2.4**) [27]–[29]. The principal enzyme, cholesterol 7 alpha-hydroxylase (CYP7A1, cytochrome P450 family 7 subfamily A member 1) is required for BA biosynthesis in the classic

pathway. It catalyses the hydroxylation of cholesterol at the 7 position, converting it into 7α-hydroxycholesterol [30]. There are several intermediates in the production of BAs, including C4. Sterol 27-hydroxylase (CYP27A1, cytochrome P450 family 27 subfamily A member 1) is a mitochondrial enzyme that is responsible for facilitating BA synthesis in the acidic pathway. However, unlike in the classic pathway, the hydroxylation take place on the 27 position instead and therefore involves a different enzyme, CYP27A1. In mice this accounts for ~25% of BAs where as in humans it is below ~10% [27], [28].

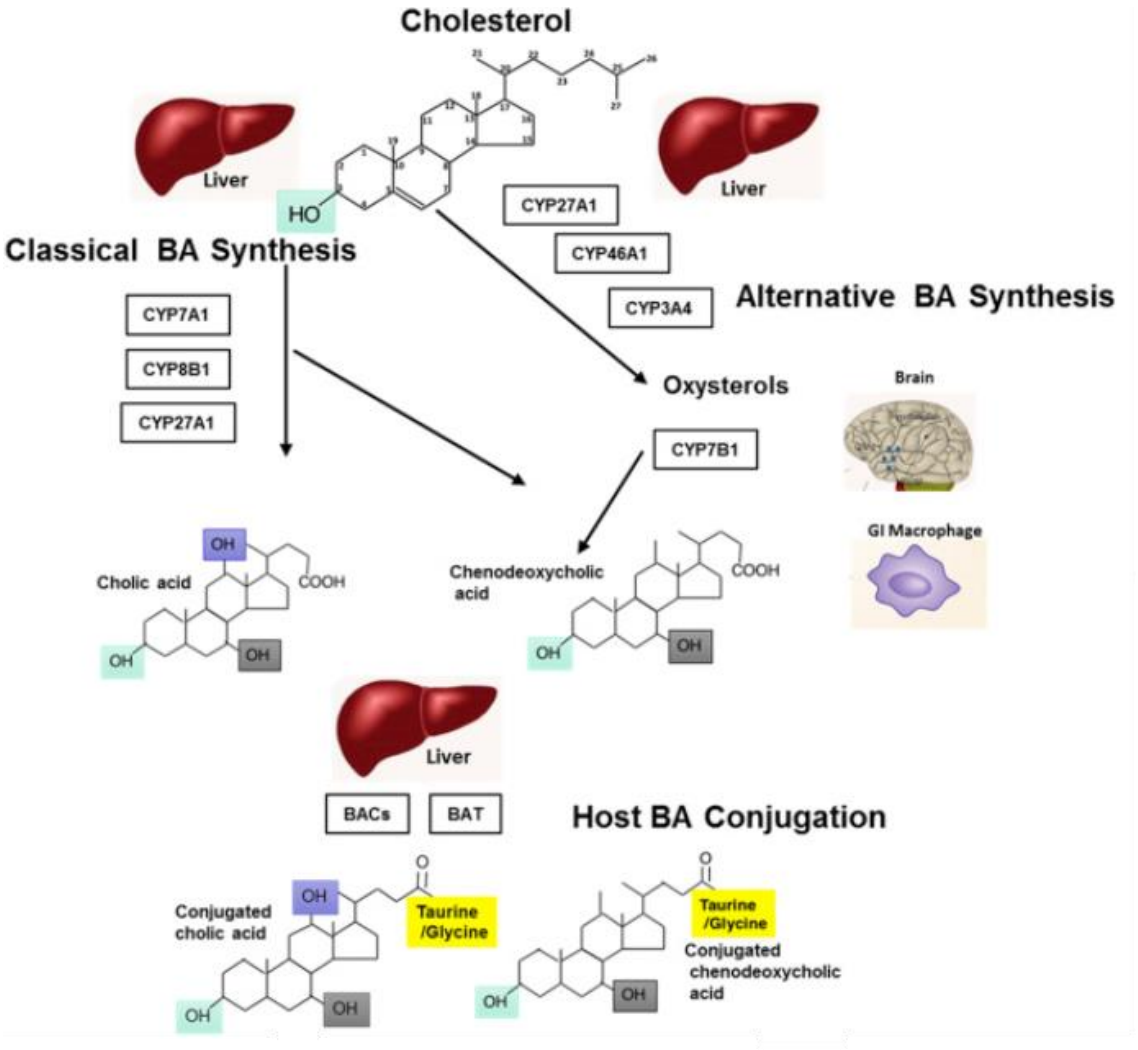


Figure 2.4: Adapted host bile acid metabolism: synthesis from cholesterol in the liver by host Cytochrome P450 enzymes (boxed) through **classical** (in the liver) and **alternative** (in liver, brain and macrophages) bile acid synthetic pathways. Followed by **host bile acid conjugation** a process that occurs in the liver [31]

2.3 Microbiome

The metazoan body hosts several microbiomes; a medley of cross species physicochemical and chemical interactions that have a marked effect on the balance between host health and disease. Culture-independent methods are essential to elucidate the model of the whole microbial community. The techniques and technologies employed for the detection and characterisation of microorganisms have evolved vastly over the past 50 years [32]. The full array of microbiota in or on the human body is known as the human microbiome. The human microbiota is composed of bacteria, archaea, eukaryotes, and viruses. It consists predominantly of bacterial cells (approximately $3.8x10^{13}$) which have a marginally higher order compared to the number of human cells (approximately $3.0x10^{13}$) [33]. Therefore the ratio is almost 1:1 which is quite different to original estimates that the human microbiome contained 10 times as many cells as the host [34].

There are common microorganisms (>95%) and genes associated with certain environments, which are often termed core microbiomes [35]. In humans, these species are often termed biomarkers for human health and disease. The concept of core microbiomes in humans is contentious, with previous work on common enterotypes being widely discredited when applied to larger human cohorts [36], [37]. This is because the diversity of environmental and genetic factors (**Figure 2.5**) impacting the gut can lead to an array of species colonising the gut, with there being no clear definition of what is a healthy gut and what constitutes a core healthy microbiome. It is proposed that an individual's microbiome is as unique to them as their fingerprint; with no two individuals colonised by the same assemblage of microbiota [38], [39].

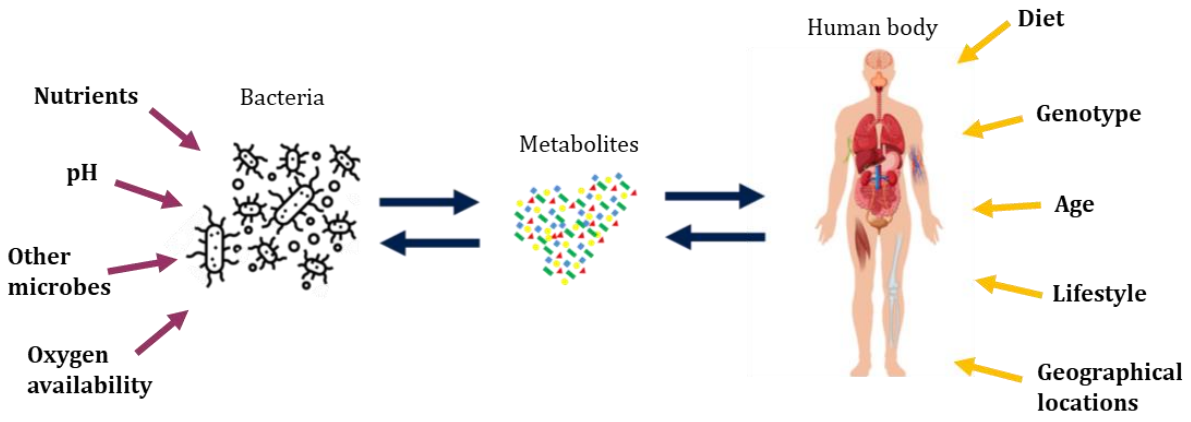


Figure 2.5: Schematic representation of how the microbiome interacts with the human body. Communication between microbiota and human host is mediated by metabolites such as BAs. It includes factors influencing gut microbiome.

2.3.1 Gut microbiome

In humans the lower alimentary canal hosts the gut microbiome. Along the intestinal tract, the microbiota varies according to the anatomical regions and organ they colonise. This is due to variations in pH, O_2 concentration, digesta flow rates (also known as transit time), nutrients and substrate availability [40]. The catalogue produced by large-scale metagenomic projects from faecal samples such as the Human Microbiome Project, shows the human gut contains over 3.3 million microbial genes (> 99% bacterial) [41]. That is 150-fold more than the human genome which has approximately 23,000 genes. As the gut microbiota co-evolved with the host over thousands of years these additional genes often have mutual and added benefit. These genes play a critical role in facilitating host metabolism and regulation of host physiology such as in the removal, synthesis, and absorption of many fundamental nutrients and metabolites, including BAs, lipids, amino acids, vitamins, and short-chain fatty acids. Nonetheless, this can go the other way. Diseases such as inflammatory bowel disease, celiac disease, colorectal cancer and BAD are associated with microbiota dysbiosis [42].

Disease results from microbiota dysbiosis when there is either a depletion or an increase of microbiota derived metabolites. The cause is multifaceted as depicted in **figure 2.5**. These metabolites interact with the host signalling and immune system to contribute to disease. There are three ways in which dysbiosis can trigger chronic sickness; gain of function dysbiosis, loss of function dysbiosis and combination of loss and gain of function dysbiosis [43]. Gain of function dysbiosis results from contracting a pathogen or overgrowth of an opportunistic microbe which often lead to chronic inflammation. For example, organisms such as *Vibrio cholerae* and *Staphylococcus aureus* that cause cholera and skin infections respectively. Loss of function dysbiosis is a consequence of a reduction or complete loss of beneficial bacteria and their function. Chronic disease such as IBS and obesity are thought to result from loss of function dysbiosis. Recurrent *Clostridium difficile* (*C. difficile*) infection results from the final dysbiosis which encompasses both loss and gain of function dysbiosis.

2.4 Secondary Bile Acids

In both the small intestines and particularly in the colon, unabsorbed BAs are deconjugated and further transformed into secondary and additional derivatives of BAs. Bacteria can manipulate BAs at four main sites on the sterol core; C-3, 7, 12, and 24 (**figure 2.1**). C-24 is the site for deconjugation (the removal of the amino acid), C-7 is the site for dehydroxylation (the removal of hydroxyl group) or dehydrogenation (the removal of a hydrogen atom) and C-3, 7 and 12 are

the sites for dehydrogenation. It is important to mention that bacterial dehydrogenation is a reversible process.

2.4.1 Deconjugation

Conversion of conjugated BAs by GI bacteria involves bile salt hydrolase (BSH) enzymes that catalyse the deconjugation of glycine or taurine from the C-24 N-acyl amide bonded to the sterol core [44], [45]. BSHs enzymes found in bacteria have widely different catalytic efficiencies and substrate preferences. Last year Song *et al* found the probiotic *Lactobaclillus* possessed a BSH (they named 'BSH-T3') which showed the highest enzyme activity compared to the other BSH enzymes [46]. There are a number of benefits of BSHs to the bacterium and the host. Deconjugation is believed to detoxify the damaging effects of bile salts [47], [48]. Moreover, deconjugation may provide a source of nutrients by means of obtaining cellular carbon, nitrogen, and sulfur for some bacterial species [49], [50].

2.4.2 Dehydroxylation and Dehydrogenation

The genes that regulate dehydroxylation and dehydrogenation of BAs are found on the bile acid-inducible (*bai*) operon as seen in **figure 2.6**. For example, *baiB* encodes BA CoA ligase which results in a bile acid-CoA conjugate, that is thought to be the first step of dehydroxylation. The gene *baiE* encodes 7α-de-hydratase which removes the 7α hydroxyl group. While *baiA* encodes 3α-hydroxysteroid dehydrogenases.

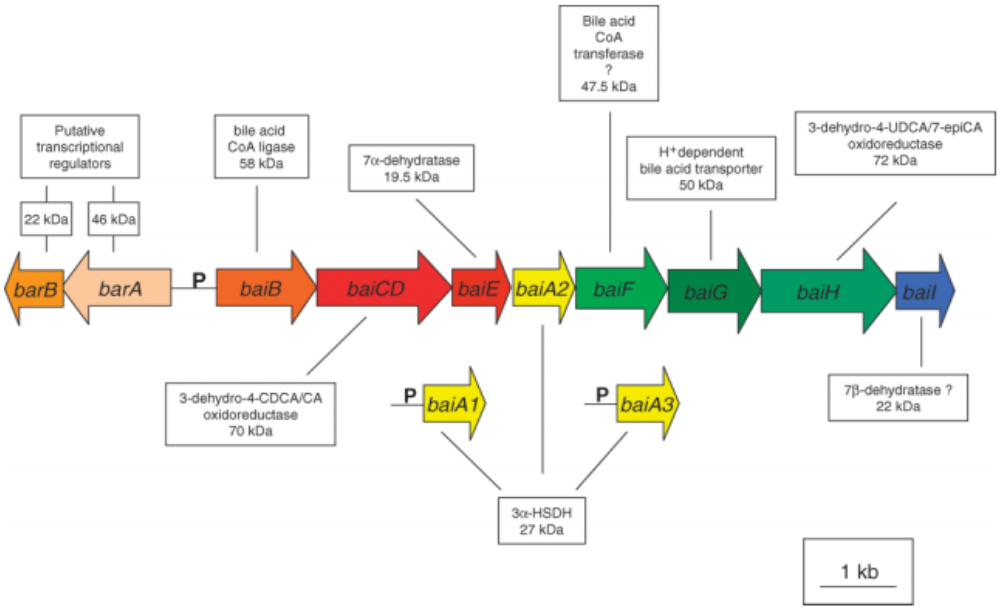


Figure 2.6: Gene organisation of the *bai* 7α/β-dehydroxylation and 3α/β dehydrogenation operons observed in *C. scindens* VPI 12708. 'P' indicates the promoter region. [51]

2.4.3 Dehydroxylation

Bacteria that possess *bai* operons capable of BA 7α-dehydroxylation are not widespread [52]. In humans, this results in deoxycholic acid (DCA; 3α,12α-dihydroxy-5β-cholan-24-oic acid) and lithocholic acid (LCA; 3α-hydroxy-5β-cholan-24-oic acid) from CA and CDCA respectively. These secondary BAs DCA and LCA are predominate in human faeces [51]. Therefore, 7α-dehydroxylation is very quantitatively important in the biotransformation of BA in the human colon. This operon is found predominantly in *Clostridium spp.* [53]. Unlike bile acid oxidation and epimerisation, 7α/β-dehydroxylation appears restricted to free, unconjugated BAs. Therefore removal of the glycine/taurine amino acid via BSH enzymes is an essential prerequisite for 7α/β-dehydroxylation by intestinal bacteria [54], [55]

2.4.4 Dehydrogenation

Dehydrogenation by the enzyme hydroxysteroid dehydrogenase (HSDH) leads to the oxidation, reduction and sometimes the epimerisation of the 3, 7-, and 12-hydroxy groups of BAs. Epimerisation is the reversible change in stereochemistry from α to β configuration (or vice versa) with the generation of a stable carbonyl group BA intermediate (**Figure 2.7**) where 7-keto-lithocholic acid (7keto-LCA) is the intermediate for the oxidation and reduction at C-7. It is difficult to know the degree of α/β-dehydrogenation because of the competing, and reversible α/β-dehydrogenation and α/β-dehydroxylation of BAs. The epimerisation of the 7α-hydoxy group of CDCA into UDCA decreases the hydrophobicity and toxicity of the BA [56]

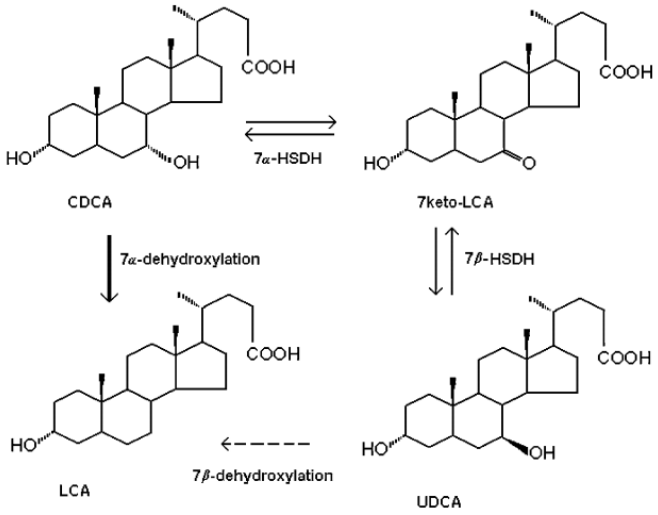


Figure 2.7: Biotransformations of CDCA into UDCA through 7-epimerization via the intermediate 7keto-LCA catalysed by 7α/β-HSDHs. Also shows formation of LCA from CDCA into UDCA performed by 7α/β -dehydroxylation enzymes [57].

2.5 H+ bile acid transporter

7α/β-dehydroxylation activity and therefore the transformation of most primary into secondary BA requires transport of free primary BAs into the bacterial cell, which is carried out by the proton-dependent bile acid transporter *BaiG* (**figure 2.6**) [58]. *BaiG* facilitates the transport of unconjugated primary BAs CA and CDCA but not of secondary BAs DCA and LCA [58].

2.6 Regulation of Bile Acid synthesis

The enterohepatic circulation of bile acids is tightly regulated; FGF19 is a hormone that is produced in the ileum (distal small intestines) and suppresses the expression of the rate-limiting enzymes CYP7A1 and CYP27A1 that are central to BA synthesis (**figure 2.4** and **2.8**). Farnesoid X receptor (FXR) is a bile acid sensor in the liver and intestine which is responsible for the regulation of FGF19 and is primarily expressed by the ileum, liver, adrenal glands, and kidney [59], [60]. BAs, particularly CDCA, are natural agonists for FXR. Consequently, in healthy individuals, high concentrations of BA, activate FXR which upregulates FGF19 and inhibits BA production. It is important to mention that a range of BAs can bind to FXR, with differing binding affinity and the ligand efficacy [61]–[63]. In fact, recent evidence published in early 2020 by Zhao *et al* showed a number of BAs (glycochenodeoxycholic acid (GCDCA), glycoursodeoxycholic acid (GUDCA), glycocholic acid (GCA), ursodeoxycholic acid (UDCA), and 7-ketodeoxycholic acid (7-KDCA)) isolated from human stool could efficiently antagonise CDCA-induced FXR activation *in vitro* [64]. Furthermore, antagonist effects of BAs on FXR have been documented in mice [65]. Fibroblast growth factor 15 (FGF15) is the mouse homologue of FGF19.

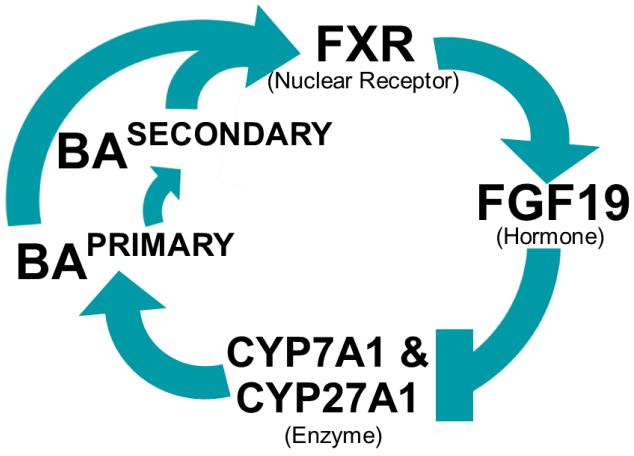


Figure 2.8: BAs negatively regulating their own expression as primary and secondary BAs bind to the nuclear receptor FXR that which transcriptionally activates the hormone FGF19 that inhibits CYP7A1 thus inhibiting BA synthesis.

2.7 Bile Acid Profile Implicated in Bile Acid Diarrhoea

Often BAD patients describe symptoms of diarrhoea that is yellow in colour and greasy in appearance. This is because patients with BAD have shorten colonic transit time that results in higher levels of bile in their stool which contains bilirubin and primary BAs [2], [66]. Sagar *et al* reported an increase in the concentrations of faecal primary BAs, particularly the FXR potent agonist CDCA in BAD patients compared to IBS patients [67]. They also observed elevated serum CDCA in the same sample group [67]. However, as previously mentioned there is insufficient data as very few molecular research studies on BAD patients exist. Studies on diarrhoea predominant IBS patients have also shown similar results [64], [68], [69]. The elevated concentration of primary BAs is a cause and may be an effect of accelerated colonic transit time which results in less available time for BA transformation by gut bacteria. Therefore, this suggests that raised BA synthesis and thus BAD may be the consequence of dysbiosis.

2.8 The Gut Microbiome and Bile Acid Diarrhoea

There have been very few studies on the intestinal microbiota and none on functional analysis of the microbiome in SeHCAT tested BAD patients. Sagar *et al* found individuals with BAD possessed gut dysbiosis with significantly reduced bacterial diversity compared with diarrhoea-predominant IBS (IBS-D). Much of what is known about BAD originates from IBS-D studies. Studies such as Duboc *et al* showed the relative abundance of the faecal microbiota in IBS-D individuals had reduced concentrations of the probiotic *Bifidobacterium* and *Clostridium leptum* bacteria [70]. In addition, they reported an overall decrease in the abundance of bacteria possessing *bsh* gene involved in BA transformation [70]. A recent study published by Zhao *et al* found that individuals suffering from severe IBS-D possessed significantly reduced abundancy of *bsh* genes and an elevated abundancy of genes encoded by the bai operon [64]. This suggests lower transformation activity of the gut microbiota in individuals with IBS-D and possibly BAD.

2.8.1 Manipulating the microbiome

Recent experiments demonstrate that dysbiosis and subsequent disease phenotypes can be rescued by altering the microbiome. Currently, there are three leading therapies: probiotic, live culture that contain beneficial microorganisms which provide health benefits when consumed [71]; prebiotics, a non-digestible carbohydrates that promote the growth of beneficial microorganisms in the GI tract [72]; wide-spectrum antibiotics, which kill pathogenic and quite often beneficial organisms too; faecal microbiota transplantation (FMT), involves the transfer of stool from a healthy human donor, via an endoscopic procedure or enema treatment, into a

recipient thus changing the microbiome. Diet, the environment, stress and drugs such as BA sequestrants can all unsettle this balance, disrupting the diversity of the gut microbiome. Manipulating the microbiome can cause favourable changes in the structure and functions of intestinal microbiota by changing or restoring bacterial diversity temporarily or permanently.

2.9 Conclusion

The human gut, specifically the colon, consists of a complex population of bacteria containing (300–500 different species) which have both positive and negative impacts on health [73], [74]. Taxonomic analysis of microbiota found intestinal dysbiosis with reduced bacterial diversity observed in patients with BAD [67]. The human microbiome is very diverse and individual-specific however, microbiotas that differ in terms of composition share some degree of functional redundancy, producing similar protein or metabolite profiles [75]. Therefore, functional and metabolite analyses are imperative in helping elucidate the virtual organ that is the microbiome in BAD individuals. To date, there have been no metagenomic functional analyses of microbiome in patients diagnosed with BAD. In this studentship, we hypothesise that changes in the microbiome are responsible for type II BAD. We propose that bacterial transformations shape the BA pool available for binding to FXR leading to differing levels of FXR activation and hence reduced FGF19 expression, resulting in over-production of primary BAs and the condition of BAD. Ultimately, the goal of this studentship is to understand the role of the microbiome during BAD and investigate whether FMT is a viable therapeutic option to treat BAD.

2.10 Project Objectives and Hypotheses

Objective 1 (0 – 12 months): **Compare the difference in functional genes and metabolic composition of stool from healthy, IBS and BAD donors.**

Pilot study - The objective of this pilot study is to develop a pipeline for the detection of BA transforming genes (also it aims to work out optimal sequencing depth.)

Main study - **H_1** hypothesis: that healthy control and IBS samples will have higher abundancy of bacteria possessing only BSH genes compared to BAD samples.

Objective 2 (month 12 – 18): **Measure the effects of BAs on expression levels of FGF19 in human cell lines and to examine BA transforming capabilities of bacteria. H_2:** hypothesis: that secondary BAs will antagonise CDCA activation leading to a decrease in fgf19 expression compared to cells incubated with CDCA alone.

Objective 3 (month 18 – 20): **Investigate the effects of FMT, probiotics and antibiotics on GF and ASBT-/- mouse models.** H_3: hypothesis: that BAD can be induced by altering the gut microbiome.

3. Materials and Methods

3.1 Workflow

The following methods and subsequent results report on the pilot study only. Below, **figure 3.1**, depicts the series of steps undertaken during the study in order to observe BA transforming genes present in bacteria and formulate an optimal sequencing depth using three control and two IBS stool samples.

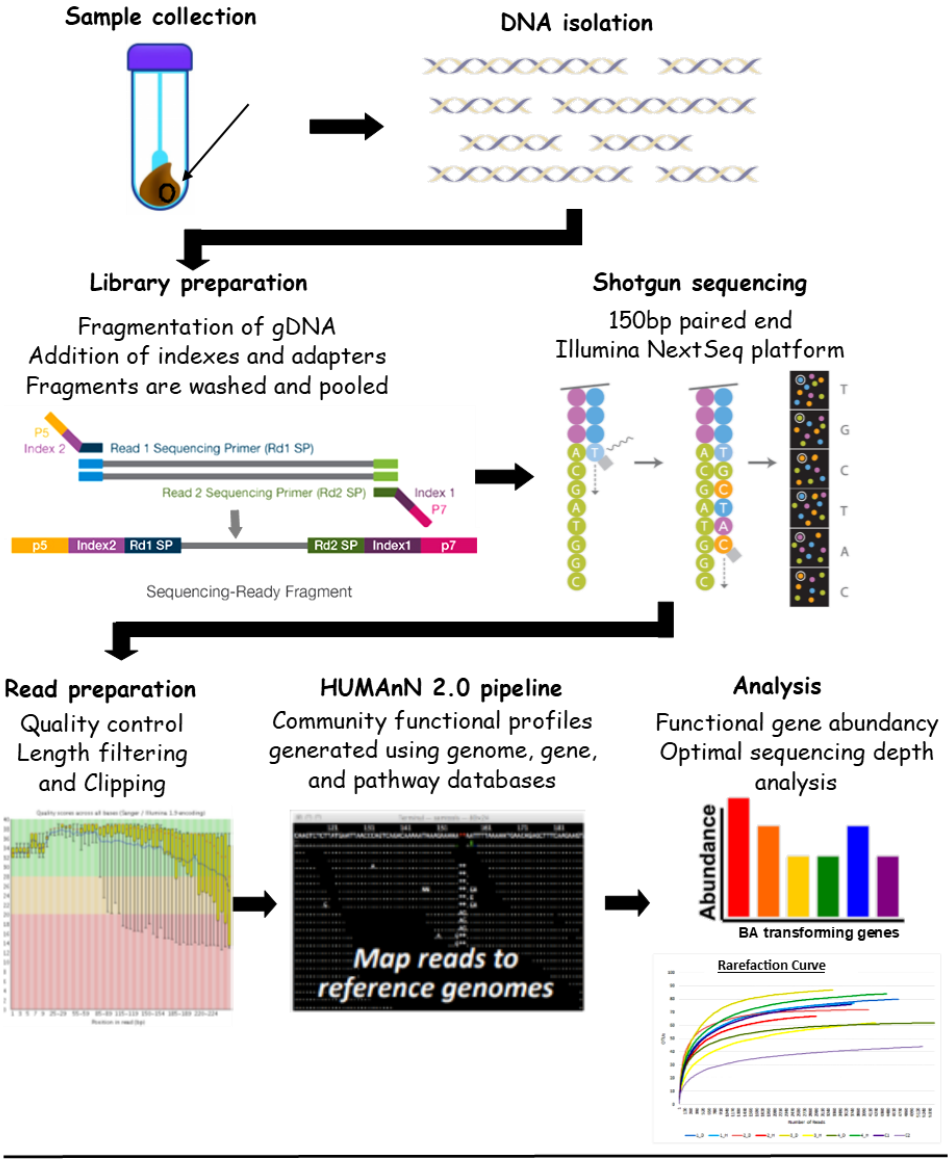


Figure 3.1: Project workflow of pilot study from sample collection to gene analysis of shotgun sequenced data

3.2 Sample Collection

Stool samples were provided in universal 30ml container (with spoon) by prof. Arasaradnam at University Hospitals Coventry & Warwickshire in the department of Gastroenterology. Once collected, samples were stored at - 80°C within hours of excretion. Samples were then couriered to NIBSC on dry ice (-78°C) within a polystyrene box and stored at - 80°C before processing. Prior to aliquoting and subsequent DNA extraction faecal samples were thawed on ice for 30-40 mins.

3.3 Inclusion and Exclusion Criteria

This cross-sectional study included both male and female volunteers. Donors taking gut microbiota altering agents, specifically, probiotics and the use of antibiotics in the last three months were excluded.

3.3.1 Irritable bowel syndrome donors

Donors suffering with chronic (>1 year) IBS were included in this representative subset. As previously mentioned, one third of IBS patients are thought to have BAD [5]. Therefore, ruling out BAD using the SeHCAT scan was a criterion for this patient group.

3.3.2 Healthy control donors

This observational study included samples from healthy participants who were required to meet the following criteria (1) have not been diagnosed with a gut related disease in the last year such as IBS, coeliac disease or IBD (inflammatory bowel disease) and (2) have normal bowel movements.

3.4 Extracting and Sequencing DNA

DNA was purified using DNeasy PowerSoil HTP 96 Kit (Qiagen, Cat No: 12955-4) from 0.2g of stool to for downstream microbiota and functional genetic analysis. Extraction was carried out according to the manufacturer's protocol with the exception of the following. After the addition of solution C1, Powerbead Plate was incubated at 65 °C for 10 mins. In addition, washing with solution C5-D was carried out twice as this was recommended for samples with high humic content. DNA was quantified using Qubit Fluorometric Quantification (ThermoFisher Scientific, Cat No: Q33238) then stored at - 20°C (long term >7 days) and 4 °C (short term =<7 days).

Library preparation for shotgun sequencing was performed using Nextera DNA Flex (renamed to Illumina DNA Prep, Cat No: 20018705) according to the manufacturer's protocol. Sequencing was carried out on a Nextseq 500 platform (Illumina) using the NextSeq 500/550 v2.5 Kits (Illumina, Cat No: 20024908) yielded paired end 150bp reads.

3.5 Functional analysis

Following sequencing, two files for each sample were generated (R1 and R2) corresponding to the paired-end sequencing. Primary quality control checks were executed using the application FastQC on the raw sequence data (www.bioinformatics.babraham.ac.uk/projects/fastqc/). FastQC runs a set of checks to produce a report which allows for quick and easy visualisation to overall sequence quality. Trimmomatic program in a paired-end mode was then used to improve the average read quality [76].

The remaining quality trimmed reads were processed using the pipeline HUMAnN 2.0 (http://huttenhower.sph.harvard.edu/humann2). Default settings were applied, which profiled microbial communities with MetaPhlAn2 (https://huttenhower.sph.harvard.edu/metaphlan) using unique clade-specific marker genes for taxonomic identification; then mapped reads to ChocoPhlAn pangenome database which contains functionally annotated species using Bowtie2 to produce a species-specific list of functional genes. Employing UniRef90 protein reference database, sequences were screened using DIAMOND open-source algorithm that placed reads into protein families and enzyme pathways using PFam and MetCyc respectively. The subsequent protein families files were all normalised to relative abundance and separate sample files combined.

3.6 Optimal sequencing depth

Rarefaction curves compares different samples to provide a graphical representation of richness observed. If or when the rarefaction curve plateaus thus becoming parallel to the x-axis, saturation has been reached and the sampling depth is sufficient. Rarefaction analysis used *R/rarecurve.R* command executed by *vegan* package within R Studio. Extracted DNA in the pilot study were sequenced to a depth between 65-188 million reads per sample (150bp). It was then established at which point new sequences did not result in new observations.

4. Results and Discussion

The aim for the principal study is to compare bacterial genes required for BA transformation across sample groups then to analyse BA composition and correlate this with microbiome data. Initially a pilot study was carried out with the objective of detecting BA transforming genes and working out optimal sequencing depth using three healthy control and two IBS stool samples.

4.1 DNA extraction

After the genomic DNA was extracted, the concentrations were measured for all samples, with results shown in **table 4.1.** The DNA-concentration ranged from 17.1 ng/µl to 41.6 ng/µl. This number reflects all microbial DNA in the 0.2g sample not just the bacterial DNA.

Table 4.1: shows the absolute DNA concentration of all five samples

Sample	**DNA con (ng/µl)**
Control-1	20.5
Control-2	41.6
Control-3	17.1
IBS-1	24.4
IBS-2	23.4

4.2 Sequencing and Quality Control

Overall 710,564,710 sequenced reads from all 5 samples were obtained following 150 bp paired-end sequencing on the Illumina NextSeq platform. Following quality control, this was 355,242,355 reads.

Table 4.2: the number of paired-end reads before and after read clean-up with Trimmomatic of all five samples. M= millions

Sample Name	**Paired-End Reads (M)** Before Trimmomatic	**Paired-End Reads (M)** After Trimmomatic
Control-1	78.02	74.03
Control-2	76.10	72.17
Control-3	94.16	89.34
IBS-1	74.19	70.39
IBS-2	32.82	31.14

A FastQC report was produced before and after Trimmomatic program parameters were applied. The results in **Figure 4.1** show almost all bases line in the green region indicating very good quality base calls were used for analysis.

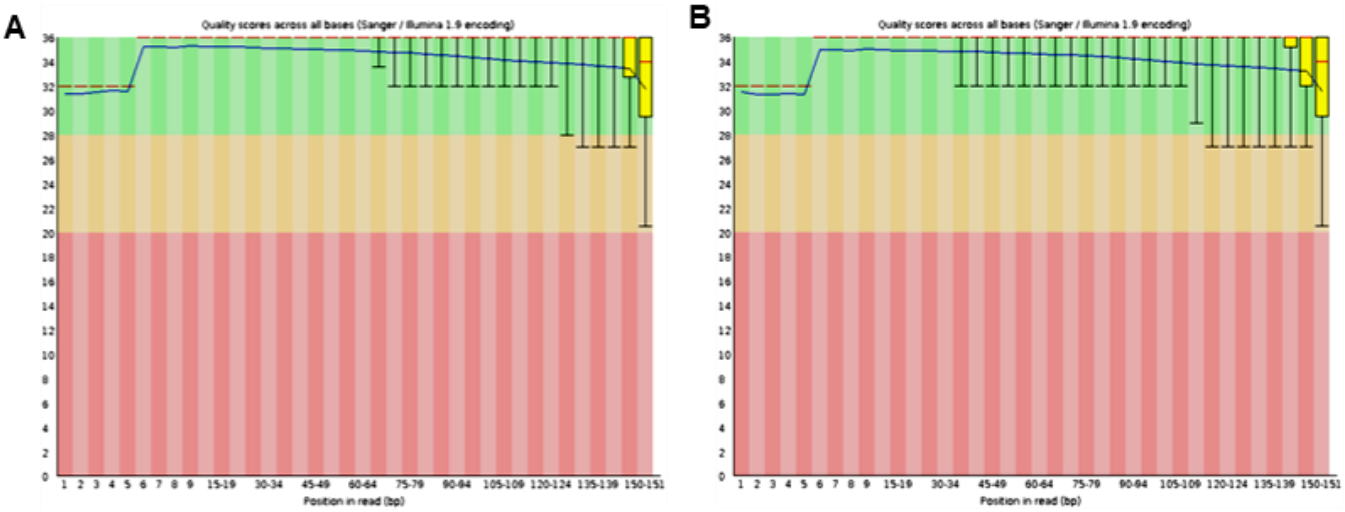


Figure 4.1: shows FastQC per base sequence quality across all bases after quality and length filtering by Trimmomatic. X-axis shows the position in reads in base pair [bp]. Y-axis indicates the quality scores. For each position a Box-Whisker plot is drawn. The central red line represents the median quality score. Yellow boxes represent the interquartile range (25-75%). Upper and lower whiskers represent the quartiles. The blue line represents the mean quality score. Background divides the y-axis in very good quality base calls (green), base calls of reasonable quality (orange) and base calls of poor quality (red), (A) forward, (B) reverse.

4.3 Abundance of Bile Acid Transforming Genes

The first objective was to determine the relative abundancy of *bsh, baiA, baiE, baiF* and sodium symporter transporter genes that determined the donor's microbial potential for secondary BA synthesis between healthy and IBS sample group. Stool from health donors possessed over double the number of secondary BA producing gene reads. With the biggest difference observed in the normalised number of *bsh* reads. Almost 5 times more reads that are potentially involved in deconjugating BAs in healthy (12.19) compared to IBS (2.83) stool samples.

There were approximately 200 hits to BA functional genes of interest from a number of species. After reviewing the literature, it is clear that the ChocoPhlAn pangenome database provided sufficient but not exhausted. Heiken *et al* reported almost 700 bacterial strains and their

corresponding transforming genes [77]. This dataset should be used in combination with ChocoPhlAn to produce a saturated list of functional transforming genes.

Statistical analysis was not performed as donor groups size were too small (three healthy control and two IBS samples). Stool from health donors possessed over double the number of secondary BA producing gene reads. This is consistent with previous studies that show an overall decrease in the abundance of bacteria possessing *bsh* gene involved in BA transformation in IBS-D compared to healthy individuals [70]. Future work will aim to functionally analyse the BAD cohort.

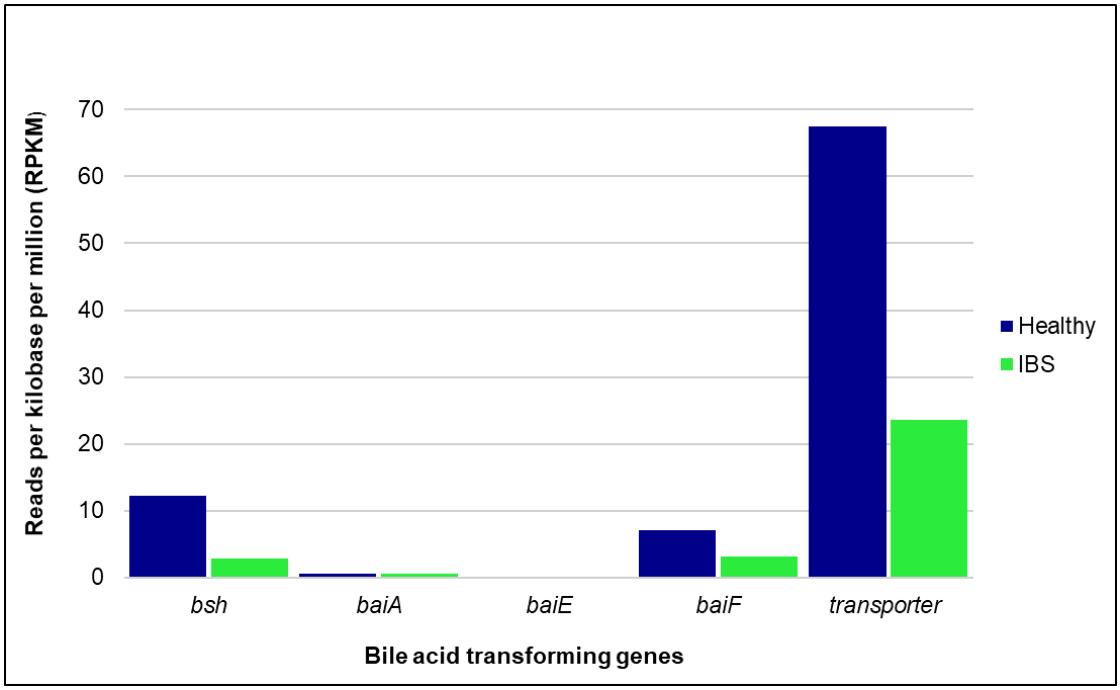


Figure 4.2: Graph showing relative abundances of *bsh, baiA, baiE, baiF* and transporter genes. Average RPKM of genes needed secondary bile acid production

4.4 Optimal sequencing depth

Rarefaction analysis was used to determine the optimum sequencing depth. These curves demonstrated good depth of coverage, as levelling of the curve occurs for all samples at the sequenced depths of ~ 100 million reads. The curves in **figure 4.4** plateau between 25-50 million reads per samples with Control-3 sample showing the highest number of reads and observed pathways. All the curves in the observed pathway plot reach saturation at 40 million reads which is consistent with a study published by Rajan *et al* that suggests sequencing of faecal samples at

a depth of 40 million reads for processing with MetaPhlAn2 [78]. However, this is not the case in the observed genes plot where the number of reads required to end new discoveries is more than 40 million reads. Sequencing depth of all subsequent samples main study should be 45 million reads per samples to saturate the number of new discoveries for both genes and pathways.

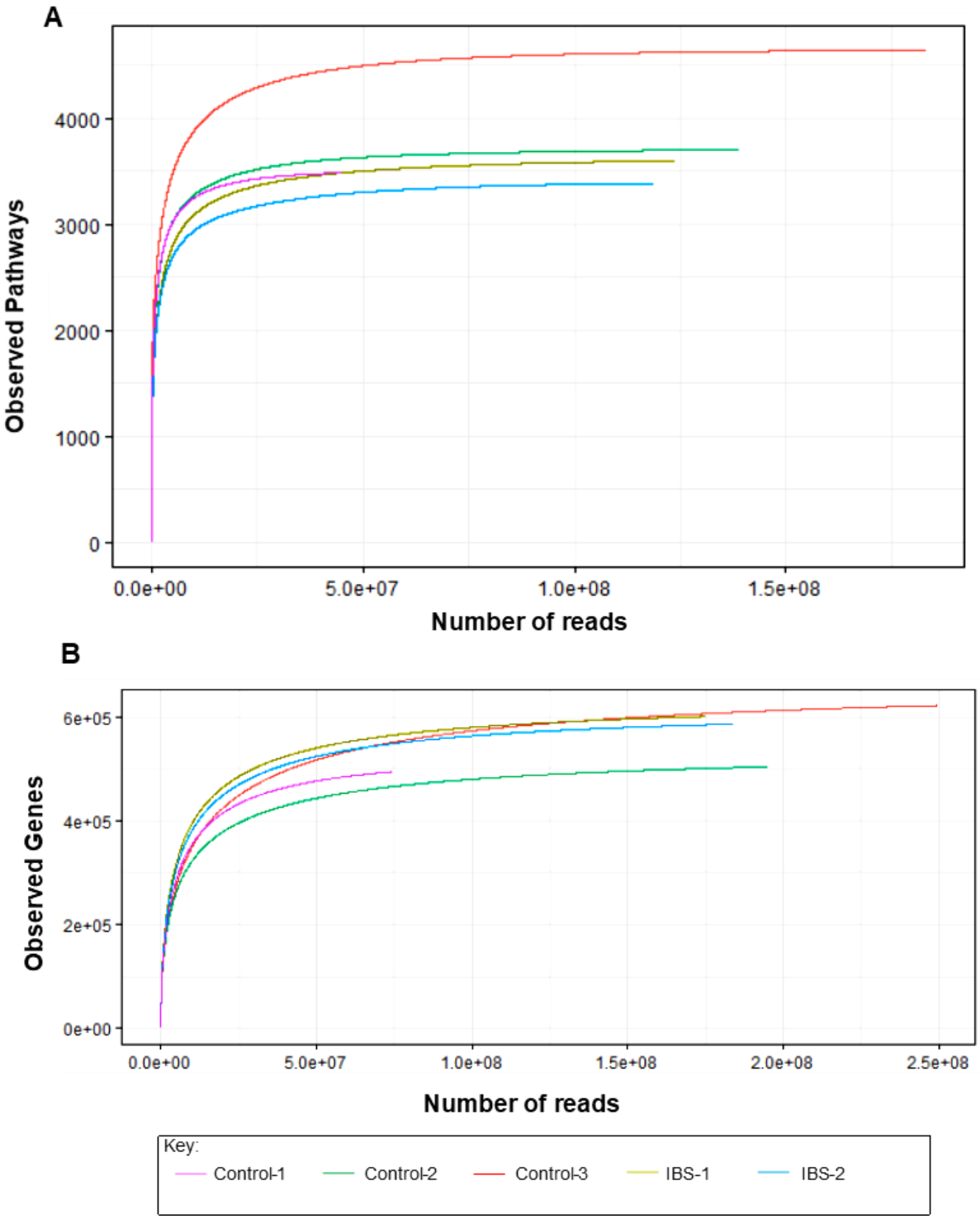


Figure 4.4 Rarefaction curves showing discovery rate of observed (A) pathways and (B) genes. With increasing sequence depth, discovery rates reach saturation.

5. Updated Project plan

5.1 Comparing the difference in functional genes and metabolic composition of stool from healthy, IBS and BAD donors.

Bacterial taxonomic shifts have been observed in the microbiome of patients with BAD [67] and IBS-D [70] when compared with healthy volunteers, however, to date, there have been no metagenomic functional analyses of the microbiome in patients diagnosed with BAD. Synthesis of most commonly found secondary BAs, DCA and LCA) require both BSH enzymes and enzymes expressed from BAI operons. The abundancy of bacteria will be measured that possess only BSH BA transforming genes therefore unable to perform 7α-dehydroxylation and thus incapable of producing secondary BAs. These bacteria will only be able to deconjugate the BAs yielding primary and secondary BAs. BA pool is made up of conjugated, unconjugated, primary, secondary and even tertiary BAs. Here, determining if the microbiome shapes the BA pool and whether this changes across patient groups will be evaluated. The cause of IBS-D is widely unknown, and it is estimated a third of patients have misdiagnosed BAD. Therefore, IBS sufferers as a second patient group, will be included to understand whether the microbiome between BAD and IBS patients is similar.

Faecal samples from BAD patients (~25), healthy donors (~100) and IBS sufferers (~50) have been collected with their corresponding clinical metadata by Prof Arasaradnam at the University of Warwick. Initially, the taxonomic composition of the microbiome and functional bile-acid metabolic potential of the microbiome between patient groups will be compared. Then BA composition will be analysed and correlated with microbiome data. Shotgun sequencing of metagenomic data will be used to analyse both the taxonomic structure of the microbiome, with targeted analysis of BSH gene and BAI genes used to determine the microbial potential for secondary BA synthesis. Profiling of the different BAs in each patient and their relative abundance will be performed using high performance liquid chromatography mass spectrometry (HPLC-MS). Using Song *et al* BSH profile as a reference enzymatic activity will be predicted and measured using a precipitation-based assay [46], [79]. Finally, bacteria potentially involved in BAD will be isolated, flagged through bioinformatics, to use for FMT in *in vivo* study on mouse models. **H_1:** we hypothesise that, healthy control and IBS samples will have higher abundancy of bacteria possessing only BSH genes compared to BAD samples.

5.2 *In vitro* analysis

5.2.1 Measuring the effects of BA on expression levels of FGF19 in human cell lines

Although CDCA is a known primary agonist for FXR, recent evidence has suggested that a number of different bile acid species can be agonists and antagonists for FXR. Bile acid regulation is dependent on binding of the FXR transcriptional activator by BAs. Differences in activation of FXR will directly impact the regulation of FGF19. Patients with type I, II and III BAD are known to have low expression of FGF19. Here, the hypothesis that differences in the bile acid pools caused by microbial bile acid metabolism, influence activation of FXR and hence expression of FGF19 in patients with BAD will be tested (**figure 2.8**).

Here an experiment with the aim of measuring the effects of BAs on *fgf19* expression levels and downstream targets *cyp7a1*, *cyp27a1* and a few other genes involved in BA synthesis in different human cell lines (liver, colon, small intestines and explant cells) will be set up. Primary (CDCA, TCDCA, GCDCA, CA, TCA and GCA) and secondary (LCA, DCA, TDCA, GDCA and UDCA) BA will be added to adhered cells. Following 0, 6, 12, 24 and 48 hours RNA will be extracted, and qPCR performed. **H_2:** we hypothesise that, secondary BAs will antagonise CDCA activation leading to a decrease in fgf19 expression compared to cells incubated with CDCA alone.

5.2.2 Examining BA transforming capabilities of bacteria

The literature strongly suggests that often BA transformation requires two bacteria as both BSH enzymes and enzymes expressed from BAI operons are not present in the same organism. Experimentally (*in vitro*), demonstrating specific cultured bacterial species converting primary BAs verify previously highlighted potential BA transforming bacteria. Last year Heinken *et al* published a manuscript of the two strain-level bacteria and primary BA required for the synthesis of a secondary BA named *in silico* [77].

Using Heinken *et al* publicly available predictions as well as data from microbiome study (**6.1**) the BA transforming capabilities of bacteria will be examined. Bacteria A and B will be combined equimolar and incubated under conditions similar to the human gut environment. A single type of conjugated primary BA will be then added to bacterial cells and further incubated for 0, 6, 12, 24 and 48 hours. Analysis for the detection of secondary BA will be performed using HPLC- MS.

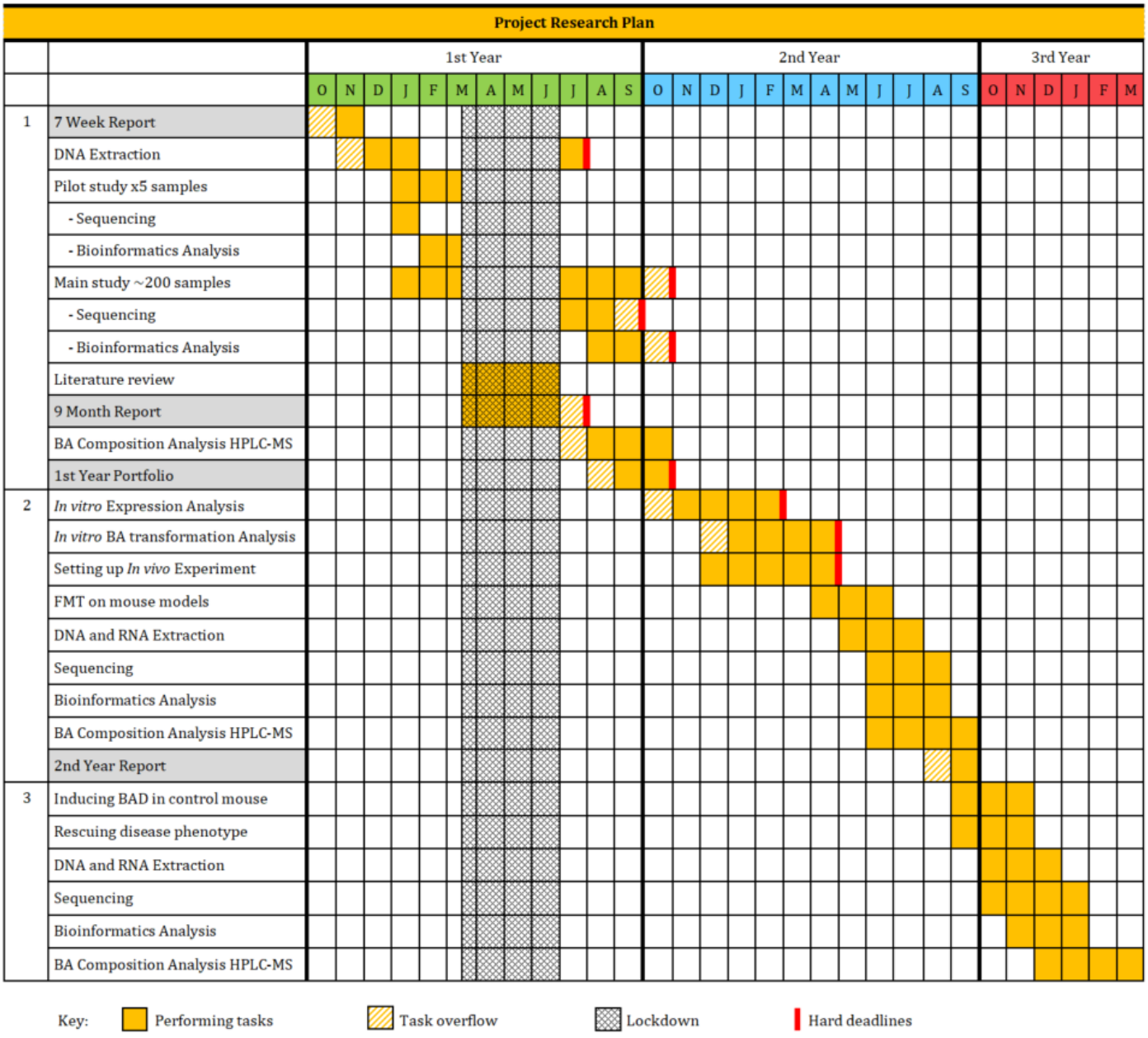
5.3 *In vivo* analysis- investigating the effects of FMT, probiotics and antibiotics on GF and ASBT-/- mouse models.

If a functional consequence of microbiota dysbiosis is elevated levels of BA synthesis due to inability of secondary BAs to sufficiently activate the FXR receptor then BAD can be induced and subsequently the disease phenotype rescued. The microbiome can be manipulated in a number of ways. FMT, probiotics and antibiotics capabilities of eradicating harmful bacteria and spores by displacing them with beneficial bacteria to restore or rescue the phenotype will be investigated. Here, aim to compare the microbiome and symptoms before and after the procedure to determine if altering the microbiome can be used clinically to treat BAD.

The objective is to answer the question 'can manipulating the gut microbiota be used to treat BAD?' using mouse models. The models will include, Germ-free (GF) mice which are microbiologically sterile. GF mice are commonly used in research to study the effects of microbiota on secondary BA synthesis [80]. ASBT-/- mice will be used as they are the only known disease model for BAD [81]. C57/BL6 (WT) mice will be used as a control group. initially, the severity of diarrhoea will be measured on a scale of 1-5 [82]. Mice will be reared for 9 to 16 weeks before experiments are conducted. Initially, severity of diarrhoea will be measured on a scale of 1-5 [82]. Analysis of stool will be performed on the BA pool, BSH activity and the microbiome as described in (**6.1**). Mice will be euthanised and major organs (liver, gall-bladder, small intestine, cecum, colon and blood) involved in enterohepatic circulation dissected [65]. DNA and RNA will be extracted from organs and sera. RT-qPCR will be used for analysing expression of two key regulators of BA metabolism, FGF15 (mouse homologue of FGF19) and FXR [83]. An enzyme-linked immunosorbent assay (ELISA) will be performed on FGF19 in blood sera to compare with expression analysis. HPLC will be carried out on C4 (primary BA precursor) to quantify BA synthesis and diagnose BAD.

Subsequently. FMT, probiotics or antibiotics will be administered on all groups using faeces from diseased and healthy mouse and human samples. Diarrhoea severity, the microbiome, host BA regulation, and the BA pool will be assessed and compared with that observed before FMT to understand whether functional changes of the microbiome result in different levels of BA regulation, changes in the BA pool, and severity of disease. Then isolated bacteria involved in BAD (from both human (**6.1**) and mouse study and transplant them in varying amounts into healthy WT mice will be used to assess their roles in causing or alleviating BAD. $\mathbf{H_3}$: we hypothesise that, BAD can be induced by altering the gut microbiome.

5.4 GANTT chart



The main changes from my original plan are experiments that have been moved back due to COVID-19 pandemic. During the lockdown I was able to complete a literature review, develop my project hypothesis and plan more future experiments. These experiments include the two *in vitro* studies and attempting to include BAD in mice.

5.5 Risk and Mitigation Table

Risk	Level of Risk	Mitigation
No difference can be seen between patient groups	Low	Consider alternative hypotheses
Unable to isolate specific BA transforming bacteria from BAD patients	High	Order type strains from collection based on metagenomic data
Mass spectrometry does not perform well on stool samples	Low	Perform ELISA for 6 significant BAs
Optimising *in vitro* experiment may take a long time	Moderate	Set hard deadlines for experiments and troubleshoot with supervisors
Insufficient material from mice to analyses	Low	Calculate the amount of material needed to be dissected. Leave room for error

5.6 New Budget Plan

Currently in budget however due to the addition of *in vitro* experiments, next year's (highlighted in red) has increased by three thousand pounds. My funding will cover this increase.

Core= NIBSC Core Facility

Budget Plan					
Year 1		Year 2		Year 3	
items	Cost (£)	items	Cost (£)	items	Cost (£)
DNA extraction kit	1604	DNA extraction kit	1604	DNA extraction kit	1604
Sequencing (Core)	Core	RNA extraction kit	1604	RNA extraction kit	1604
UPLC-MS	500	Sequencing (Core)	Core	Sequencing (Core)	Core
qPCR	500	HPLC-MS	500	HPLC-MS	500
Isolation media	500	RT-qPCR	500	RT-qPCR	500
Travel	1000	Cell lines and primers	2000	Animal work	Core
		Bacterial cells	100	Travel	1000
		Animal work	Core		
		Travel	1000		
Total =	**3604**	**=**	**8208**	**=**	**5208**

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