ORIGINAL ARTICLE

Microbial bile salt hydrolases mediate the efficacy of faecal microbiota transplant in the treatment of recurrent Clostridioides difficile infection

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ABSTRACT

Objective Faecal microbiota transplant (FMT) effectively treats recurrent Clostridioides difficile infection (rCDI), but its mechanisms of action remain poorly defined. Certain bile acids affect C. difficile germination or vegetative growth. We hypothesised that loss of gut microbiota-derived bile salt hydrolases (BSHs) predisposes to CDI by perturbing gut bile metabolism, and that BSH restitution is a key mediator of FMT’s efficacy in treating the condition.

Design Using stool collected from patients and donors pre-FMT/post-FMT for rCDI, we performed 16S rRNA gene sequencing, ultra performance liquid chromatography mass spectrometry (UPLC-MS) bile acid profiling, BSH activity measurement, and qPCR of bsh/baiCD genes involved in bile metabolism. Human data were validated in C. difficile batch cultures and a C57BL/6 mouse model of rCDI.

Results From metatransomics, pre-FMT stool demonstrated a reduced proportion of BSH-producing bacterial species compared with donors/post-FMT. Pre-FMT stool was enriched in taurocholic acid (TCA), a potent C. difficile germinant; TCA levels negatively correlated with key bacterial genera containing BSH-producing organisms. Post-FMT samples demonstrated recovered BSH activity and bsh/baiCD gene copy number compared with pretreatment (p<0.05). In batch cultures, supernatant from engineered bsh-expressing E. coli and naturally BSH-producing organisms (Bacteroides ovatus, Collinsella aerofaciens, Bacteroides vulgatus and Blautia obeum) reduced TCA-mediated C. difficile germination relative to culture supernatant of wild-type (BSH-negative) E. coli. C. difficile total viable counts were ~70% reduced in an rCDI mouse model after administration of E. coli expressing highly active BSH relative to mice administered BSH-negative E. coli (p<0.05).

Conclusion Restoration of gut BSH functionality contributes to the efficacy of FMT in treating rCDI.

INTRODUCTION

Clostridioides difficile (previously named Clostridium difficile) infection (CDI) is the major global cause of nosocomial GI infection, with incidence
rates increasing worldwide. One therapeutic strategy for recurrent CDI (rCDI) that has come to prominence is faecal microbiota transplant (FMT). FMT has been demonstrated to be a highly effective therapy for rCDI, with cure rates of >80% in randomised trials, and up to >90% in case series where it was administered colonoscopically. However, FMT is not without drawbacks, including its unpalatability, the theoretical risk of infection transmission and its regulatory complexity.

Understanding the mechanisms underlying the efficacy of FMT in treating CDI may allow formulation of novel, more targeted, anti-CDI therapeutics. In a pilot study, sterile faecal filtrate was shown to be an effective treatment for rCDI in five patients, consistent with bacterially derived proteins, gut metabolites, bacteriophages or other filtrate components mediating the efficacy of FMT in treating this condition, as opposed to intact microorganisms. While the possible contribution of such mediators to FMT are starting to be elucidated, they remain incompletely defined.

One particular area of interest concerns the interaction between the gut microbiota and host bile acid metabolism in rCDI. In vitro, certain bile acids differentially affect the ability of C. difficile to undergo germination and vegetative growth. In particular, the conjugated primary bile acid taurocholic acid (TCA) is a potent trigger of C. difficile germination (with glycine as co-germinant), while the secondary bile acid deoxycholic acid (DCA) markedly inhibits vegetative growth. The transition from conjugated primary bile acids to secondary bile acids in vivo principally involves two enzymatic steps, with both enzymes produced by microbes but not mammals (see online supplementary figure 1). The first step is undertaken by bile salt hydrolases (BSHs), which deconjugate the taurine and glycine groups via a hydrolysis reaction, and consequently reform the unconjugated primary bile acids cholate and chenodeoxycholate. BSHs are widely distributed throughout most major bacterial divisions and archaea species of the gut microbiota, and at least eight different bsh genes exist (see online supplementary figure 2). The second enzymatic step is 7α-dehydroxylation, whereby unconjugated primary bile acids are converted to secondary bile acids, including deoxycholate and lithocholate.

At present, there are only limited data exploring the possible contribution of BSHs to CDI vulnerability, and none investigating the effect of FMT on BSH functionality. Given the key contribution of BSH’s substrate, TCA, to the germination of C. difficile, this is a clear potential mechanistic explanation for the contribution of BSHs to CDI vulnerability, and none investigated the possible contribution of such mediators to FMT are starting to be elucidated.

For the main human data set, stool samples were collected from participants with rCDI (26 participants; samples collected pre-FMT and at 8–12 weeks post-FMT) and their FMT donors (17 participants). rCDI was diagnosed on a combination of clinical and laboratory criteria (see online supplementary methods 1.1). Patient characteristics are described in online supplementary table 1; none of the included patients had IBD. For validation of initial findings, stool samples were also analysed for patients with rCDI from a Canadian randomised controlled trial investigating capsulebased versus colonoscopic FMT as rCDI treatment (18 participants; samples collected pre-FMT and at 1, 4 and 12 weeks post-FMT), together with donors (five participants). FMT slurry was also collected from these donors.

DNA extraction and 16S rRNA gene sequencing
DNA was extracted from 250 mg of stool using a previously described protocol. 16S rRNA gene qPCR data were used to determine total bacterial biomass within each sample (see online supplementary methods 1.2). Sample libraries were prepared following Illumina’s 16S Metagenomic Sequencing Library Preparation Protocol with several modifications. The V1-V2 regions of the 16S rRNA gene were amplified using previously reported primers. The methodology for metatransomic analysis is described in online supplementary methods 1.3. We also predicted the bile metabolising ability of microbial communities using a metagenomic inferential tool, PiphiLLin (see online supplementary methods 1.4).

UPLC-MS profiling of faecal bile acids
The protocol used for initial sample processing and for analysis was as previously described. Methodology for preprocessing and analysis of ultra performance liquid chromatography mass spectrometry (UPLC-MS) bile acid data is described in online supplementary methods 1.5. Integration of metatransomic and UPLC-MS bile profiling data is described in online supplementary methods 1.6.

Abundance and activity of bile metabolising enzymes
qPCR was performed using extracted DNA to quantify gene abundance for (1) specified groups of bsh (using degenerate primer sets previously reported) and (2) batCII (using previously described primers) (see online supplementary methods 1.7). The BSH activity assay was an adaptation of the conventional precipitation-based assay, using a previously described technique.

C. difficile germination batch cultures
These were performed via adaptation of a previously described protocol. C. difficile spore preparation and enumeration is described in online supplementary methods 1.8. A range of different bacterial species established to produce BSH from different BSH groups (and Clostridium scindens, as a known 7α-dehydroxylase-producer) were incubated in sBH (brain heart infusion broth (Sigma-Aldrich), with 5 mg/mL yeast extract (Sigma-Aldrich), and 0.1% w/v L-cysteine (Sigma-Aldrich)), with or without 1% w/v TCA added (see online supplementary methods 1.7). This list also included wild-type E. coli MG1655 (which does not contain bsh genes within its genome), along with two forms of E. coli MG1655 into which bsh genes had been cloned using pBKminiTn7GM2 under the control of the P44 promoter (see online supplementary methods 1.7). C. difficile spores from three different ribotypes (a non-toxigenic ribotype, 010 (strain DS1684), and two toxigenic ribotypes, 011
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012 (strain CD630) and 027 (strain R20291) were resuspended in supernatant in triplicate, and an OD<sub>600</sub> reading taken on a microplate reader at time zero (adjusted to OD<sub>600</sub> of ~0.1 with supernatant/sBHI mix), and again after overnight incubation. An increased OD<sub>600</sub> reading after overnight incubation was interpreted as indicating that spores had undergone germination and had grown as vegetative cells.11 Additionally, UPLC-MS was performed on batch culture supernatants to establish bile acid profiles (see online supplementary methods 1.5.2), and BSH activity assays were performed on spent supernatant from selected batch cultures.

Recurrent C. difficile mouse model

Wild-type C57BL/6 mice (8–10 weeks old; female) were purchased from Envigo (Huntingdon, UK) and acclimatised for 1 week before use. Mice were housed five per cage in individually ventilated cages with autoclaved food (RM1, Special Diet Services, Essex, UK), bedding (Aspen chip two bedding, Northeastern Products Corporation (NEPCO), Warrensburg, New York) and water (provided ad libitum). Mice were subjected to a 12 hours light and 12 hours dark cycle at 20°C–22°C.

We adapted a previously published mouse model of rCDI/FMT (figure 1A). Mice were initially given cefoperazone 0.5 mg/mL (Melford, Ipswich, UK) in their drinking water for 5 days (day −7 to day −2), followed by challenge with 10<sup>7</sup> C. difficile spores by oral gavage on day 0. Mice were then treated with vancomycin 0.4 mg/mL together with streptomycin 5 mg/mL (both Melford, Ipswich, UK) in their drinking water for 3 days (days 4–7), followed by autoclaved antibiotic-free water for the remainder of the experiment. On both days 9 and 10, mice were fed by oral gavage either with ~10<sup>9</sup> colony-forming units (CFUs) of wild-type E. coli (n=5) or E. coli BSH<sub>high</sub> (n=5) (see online supplementary methods 1.7). Serial faecal samples were collected and C. difficile total viable counts (TVCs) quantified until E. coli colonisation began to decline (see online supplementary methods 1.9). Administered E. coli were quantified by plating on MacConkey agar plates supplemented with rifampicin 50 µg/mL (Melford, Ipswich, UK). Mice were not fasted before oral gavages and all interventions were performed during the light cycle.

Statistical analysis
This is summarised in online supplementary methods 1.10.

RESULTS
Successful FMT for rCDI is associated with restoration of BSH-producing gut microbial community members from all BSH groups
We used microbial sequencing to evaluate whether FMT for rCDI was associated within an increase in BSH-producing microbes within the faecal microbiota. Further analysis of metataxonomic

Figure 1  Impact of BSH on a mouse model of rCDI. A mouse model of rCDI/FMT was used that was adapted from a previously described model.26 (A) Protocol for mouse model (*: stool samples taken for C. difficile and/or E. coli counts); (B) Counts of administered E. coli on day 12; (C) Total vegetative counts of C. difficile on day 12. CFU counts as established from plate counts of serial dilutions of faecal supernatant (*, p<0.05, Mann-Whitney U test). BSH, bile salt hydrolases; CFU, colony-forming unit; FMT, faecal microbiota transplant; rCDI, recurrent C. difficile infection.

Gut microbiota data is provided in online supplementary results, figures 3–5 and table 2.

Bacterial species enriched in the faecal microbiota of healthy donors compared with pre-FMT samples were characterised by a range of BSH-producing organisms, including members of group 1 (Bacteroides ovatus, \(q=0.017\); Bacteroides uniformis, \(q=0.007\)), group 2 (Bifidobacterium dentium, \(q=0.014\); Collinsella aerofaciens, \(q=0.009\); Bifidobacterium longum, \(q=0.011\)) and group 3 (Bacteroides vulgatus, \(q=0.003\); Faecalibacterium prausnitzii, \(q=0.003\); Eubacterium rectale, \(q=0.005\); Blautia obeum, \(q=0.014\)) (figure 2A). Similarly, bacterial species enriched in the post-FMT faecal microbiota compared with those pre-FMT also included members of all BSH groups, including group 1 (Bacteroides uniformis, \(q=0.005\); Bacteroides ovatus, \(q=0.009\); Parabacteroides distasonis, \(q=0.003\)), group 2 (Collinsella aerofaciens, \(q=0.006\); Bifidobacterium dentium, \(q=0.029\)) and group 3 (Bacteroides vulgatus, \(q=0.002\); Eubacterium rectale, \(q=0.004\); Blautia obeum, \(q=0.009\); Faecalibacterium prausnitzii, \(q=0.003\)) (figure 2B).

Using Piphillin to infer metagenomic content from metataxonomic data, there were a significantly reduced proportion of sequences predicted to represent both BSH (see online supplementary figure 6A) and 7-\(\alpha\)-dehydroxylase (see online supplementary figure 6B) in pre-FMT samples compared with donors, but a significant increase in both in post-FMT samples compared with pre-FMT (\(q<0.01\), White’s non-parametric test with Benjamini-Hochberg false discovery rate (FDR)).

Successful FMT for rCDI is associated with restoration of normal gut bile acid profiling, including a sustained reduction in TCA

We performed faecal bile acid profiling to assess the effect of FMT for rCDI on key bile acids known to affect the ability of C. difficile to undergo germination or vegetative growth.

On multivariate analysis of UPLC-MS bile acid profiling data, unsupervised principal component analysis demonstrated clustering of donor and post-FMT samples, but clear separation of both groups from pre-FMT samples (figure 3A). Supervised analysis was performed with orthogonal projections to latent structures discriminant analysis (OPLS-DA) to analyse the features responsible for discrimination between donor and pre-FMT groups (see online supplementary figure 7A), and between pre-FMT and post-FMT groups (figure 3B). Discriminatory feature identification was performed from OPLS-DA model data via S-plot, with pre-FMT samples showing an enrichment in primary bile acids (including both conjugated and unconjugated forms) and loss of secondary bile acids as compared with post-FMT and healthy donor samples (figure 3C, see online supplementary figure 7B). OPLS-DA model validation was performed using CV analysis of variance (see online supplementary table 3).

Univariate analysis supported these findings (see online supplementary figure 8). Pre-FMT samples demonstrated enrichment in TCA and loss of DCA compared with healthy donor samples (\(p<0.01\), Mann-Whitney U test), while post-FMT samples were characterised by restoration of both bile acids back to levels comparable to donors (\(p<0.001\), Wilcoxon signed rank-sum test).

Integration of metataxonomic and bile acid profiling data

rCCA modelling was used to integrate metataxonomic and bile acid profiling data. The unit representation plot demonstrated marked separation of pre-FMT and post-FMT samples, but considerable overlap between donor and post-FMT samples (figure 4A). A correlation circle plot demonstrated negative correlations between levels of TCA and the abundance of...
the bacterial genera *Bacteroides* and *Blautia*, both known to include representative BSH-producing organisms that were significantly increased after FMT in our metaxonomic data (Figure 4B). In addition, there was a positive correlation between the genus *Clostridium* cluster XIVa (known to contain 7-α-dehydroxylase producing organisms) and the secondary bile acids DCA and lithocholic acid (Figure 4B).

**Gut bsh gene copy number and BSH enzyme activity is restored by successful FMT for rCDI**

Having established an association between restoration of BSH-producing organisms into the gut microbiota post-FMT and recovery of premorbid stool bile acid profiles, we investigated the effect of FMT for rCDI on BSH expression and activity.
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bsh gene copy number was significantly reduced in pre-FMT samples compared with healthy donors across a range of bsh gene groups (p<0.001, Mann-Whitney U test, figure 5A–C). Successful FMT was associated with significant enrichment in copy number of all bsh gene groups assayed (p<0.05, Wilcoxon signed rank-sum test, figure 5A–C) to levels similar to that of healthy donors. Similarly, copy numbers of the baiCD operon (encoding an enzyme that contributes to 7-α-dehydroxylation29) were significantly lower in pre-FMT samples compared with those of healthy donors (p<0.01), but were also significantly enriched post-FMT (p<0.05, figure 5D). Gene copy number for baiCD in donors and post-FMT was noted to be markedly lower than for each bsh gene assayed for the same participants. Stool BSH enzyme activity showed the same pattern as for bsh gene copy number (figure 5E).

To further explore the timescale of gut bile acid/BSH changes post-FMT, we analysed serial stool samples collected from patients in a randomised trial of colonoscopy versus capsule FMT as treatment for rCDI,15 together with donor samples and FMT slurry itself. This demonstrated that BSH activity was restored (and stool TCA levels greatly reduced) to levels comparable to donors within a week of successful FMT, and was

Figure 5 Effect of FMT on bile-metabolising enzyme gene copy number and BSH enzyme activity. (A) bsh group 1A; (B) bsh group 1B; (C) bsh group 3C; (D) baiCD operon of 7-α-dehydroxylase; (E) BSH enzyme activity within faecal supernatant (*, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001; Mann-Whitney U test for donors versus pre-FMT, Wilcoxon signed rank-sum test for pre-FMT vs post-FMT). BSH, bile salt hydrolases; FMT, faecal microbiota transplant.
BSH groups. For all or post-FMT samples, and which collectively represented most microbiota of pre-FMT patients in comparison to donors and had been shown to be reduced in mean proportion in the gut bated with TCA. The microorganisms selected were those which tant from BSH-expressing microorganisms that had been incu-

C. difficile

significantly reduced germination (p<0.0001) stimulate germination and would not grow.

supernatants with BSH activity would not have TCA available to fore grow, while would have TCA available to stimulate germination and there-

incubated with spent culture supernatants without BSH activity

spores

C. difficile

spent culture supernatant. Using this set-up, spores were incubated in sBHI broth supplemented with the

culture germination experiments.

We prepared spent culture supernatants by incubating bacteria of interest in broth with 1% w/v TCA. After overnight incuba-
tion, cultures were centrifuged and filter-sterilised. C. difficile spores were incubated in sBHI broth supplemented with the spent culture supernatant. Using this set-up, C. difficile spores incubated with spent culture supernatants without BSH activity would have TCA available to stimulate germination and there-

grow, while C. difficile spores incubated with spent culture supernatants with BSH activity would not have TCA available to stimulate germination and would not grow.

Initially, C. difficile spores were incubated with spent superna-
tant from BSH-expressing microorganisms that had been incubated with TCA. The microorganisms selected were those which had been shown to be reduced in mean proportion in the gut microbiota of pre-FMT patients in comparison to donors and/ or post-FMT samples, and which collectively represented most BSH groups. For all C. difficile ribotypes assayed, supernatant from the broth of each of the BSH-producing microbes assayed significantly reduced C. difficile germination (p<0.0001) (figure 6B). As a control, we used spent supernatant from TCA-supplemented broth in which vegetative C. difficile had been cultured (strain DS1864); this failed to affect C. difficile germination.

We assessed whether BSH alone could mediate this inhibition, or if it reflected an alternative aspect of bacterial metabolism within the cultures. For subsequent batch cultures, strains of interest included wild-type E. coli (which lacks a bsh gene), or two forms of E. coli into which bsh genes had been cloned (ie, ‘E. coli BSH<sub>low</sub>’, containing a bsh gene with narrow substrate range against conjugated bile acids; and ‘E. coli BSH<sub>high</sub>’, containing a bsh gene with high glycine and taurine-deconjugating activity) (see online supplementary methods 1.7). While spent supernatant from the culture of wild-type/BSH negative E. coli did not affect the ability of C. difficile to undergo germination, that from both forms of bsh gene-expressing E. coli significantly reduced C. difficile germination across all ribotypes tested (p<0.0001, Student's t-test) (figure 7B). C. difficile germination was significantly lower for all three ribotypes when incubated in TCA-sup-

plemented supernatant from an E. coli BSH<sub>high</sub> batch culture as compared with E. coli BSH<sub>low</sub> (p<0.05).

C. difficile spores were also cultured in spent supernatant from C. scindens which had been incubated with TCA, either by itself or also in co-culture with BSH-expressing E. coli. C. scindens spent supernatant did not affect C. difficile germination ability by itself, but germination was significantly reduced when C. scin-
dens was co-incubated with BSH-expressing E. coli (p<0.01) (figure 7C).

Analysis by UPLC-MS confirmed that TCA-supple-

mented batch culture media—which included supernatant from wild-type/BSH-negative E. coli, vegetative C. difficile and C. scindens culture—had high TCA:CA ratios, consistent with little/absent BSH activity within these batch cultures.

Figure 6  Dynamics of changes in taurocholic acid and BSH activity after FMT for rCDI. Assessed via analysis of taurocholate levels in stool ((A) as assessed by UPLC-MS) and BSH activity in stool and FMT slurry ((B) assessed via precipitation assay). Samples were collected from patients (and their matched donors, along with FMT slurry itself) in a randomised trial of colonoscopy versus capsule FMT as treatment for rCDI<sup>15</sup> (*, p<0.05; **, p<0.01; *** p<0.001; Mann-Whitney U test for donors/FMT slurry versus pre-FMT, Friedman test with Benjamini-Hochberg FDR for pre-FMT vs post-FMT). BSH, bile salt hydrolases; DCA, deoxycholic acid; FMT, faecal microbiota transplant; rCDI, recurrent C. difficile infection; UPLC-MS, ultra performance liquid chromatography mass spectrometry.
(see online supplementary figure 9A). Low TCA:CA ratios were noted for all other batch cultures, indicating the presence of BSH activity. All batch culture experiments in which C. difficile germination was suppressed were characterised by undetectable levels of DCA within supernatant (other than batch cultures containing C. scindens and bsh-expressing E. coli, where DCA was detectable). Sterile-filtered spent supernatant from batch cultures of BSH-producing microorganisms from groups 1, 2 and 3 was found to have BSH activity comparable to that found in healthy human stool (see online supplementary figure 9B).

**DISCUSSION**

In this study, we demonstrate for the first time that a key mechanism underlying the efficacy of FMT in treating rCDI is restoration of gut microbiota BSH functionality. Analysis of human samples illustrate that this function is restored early after FMT and maintained throughout follow-up. In addition, data from batch cultures and mouse modelling show that the BSH-mediated hydrolysis of the major C. difficile germinant, TCA, is sufficient to fully suppress C. difficile germination, and limits further recurrence of disease within the setting of rCDI. Targeted restoration of gut BSH function is a novel therapeutic approach for rCDI that avoids the risks associated with FMT.

The potential role of gut microbiota-host bile acid interactions in CDI pathogenesis has been an area of interest since initial studies in vitro established that various bile acids differentially affected the ability of C. difficile to undergo germination and vegetative growth. \(^{11-13,30-33}\) Furthermore, we have also recently demonstrated that successful FMT for rCDI is associated with stimulation of farnesoid X receptor (FXR) signalling, \(^{34}\) which in itself appears to impact the bile acid milieu and consequently gut microbiota of the small intestine. \(^{35}\) Subsequent studies have demonstrated that the gut of germ-free \(^{36-38}\) and antibiotic-treated mice \(^{36-38}\) –as well as chemostat models of CDI \(^{39,40}\) –have enrichment of stool primary bile acids (particularly conjugated versions) and loss of secondary bile acids, with bile acid homeostasis being restored in patients with rCDI through FMT. \(^{39}\) Exposure of C. difficile spores to the bile acid milieu found in antibiotic-treated mouse caecum \(^{37,38}\) or human stool pre-FMT \(^{41}\) was sufficient to cause spore germination, while that of the non-antibiotic-treated mouse caecum \(^{37,38}\) or human stool post-FMT \(^{41}\) prevented germination and vegetative growth of C. difficile.

Rodent studies have demonstrated that 7-α-dehydroxylase-producing organisms (in particular, C. scindens) partly protected the host against the development of CDI. \(^{42,43}\) However, to date, there is not sufficient evidence to demonstrate that 7-α-dehydroxylase is responsible for the efficacy of FMT for rCDI. For example,
In conclusion, we provide evidence for the first time that microbial BSHs are a key mediator of the efficacy of FMT for rCDI. This adds to other recent data demonstrating that FMT functions by reversing a ‘metabolic dysbiosis’, including through the restoration of the short chain fatty acid valerate, which potently inhibits the growth of *C. difficile* without any apparent adverse effect on gut commensal bacteria.

Furthermore, these data add further to the growing body of evidence demonstrating the central significance of gut microbiota-bile acid interactions in colonisation resistance, and demonstrate that targeted restitution of BSH may be a novel therapy for or preventative strategy against CDI that avoids the risks of antimicrobial resistance.

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**Contributors** BMH and JAKM were involved in all aspects of experimental work and data analysis; IRA, DK and EOP provided samples and contributed to data analysis; AP and EH provided analysis of mass spectrometry data; GFB and DK performed qPCR and data analysis; SAJ and CGMG provided bacteria and aided analysis of batch culture experiments; BMH, JAKM, IG-M and TBC performed the mouse experiments; HRTW, MRT and IJM contributed to study design and provided overall supervision of experimental work and data analysis. All authors contributed to and approved the final manuscript.

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