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Original Research

# Antibiotic-induced disruption of the microbiome exacerbates chemotherapy-induced diarrhoea and can be mitigated with autologous faecal microbiota transplantation

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Gastrointestinal toxicity;
Diarrhea;

**Abstract** ***Background:*** Chemotherapy is well documented to disrupt the gut microbiome, leading to poor treatment outcomes and a heightened risk of adverse toxicity. Although strong associations exist between its composition and gastrointestinal toxicity, its causal contribution remains unclear. Our inability to move beyond association has limited the development and implementation of microbial-based therapeutics in chemotherapy adjuncts with no clear rationale of how and when to deliver them.
***Methods/Results:*** Here, we investigate the impact of augmenting the gut microbiome on gastrointestinal toxicity caused by the chemotherapeutic agent, methotrexate (MTX). Faecal microbiome transplantation (FMT) delivered after MTX had no appreciable impact on gastrointestinal toxicity. In contrast, disruption of the microbiome with antibiotics administered before chemotherapy exacerbated gastrointestinal toxicity, impairing mucosal recovery

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Mucosal barrier injury;
Mucositis

($P < 0.0001$) whilst increasing diarrhoea severity ($P = 0.0007$) and treatment-related mortality ($P = 0.0045$). Importantly, these detrimental effects were reversed when the microbiome was restored using autologous FMT ($P = 0.03$), a phenomenon dictated by the uptake and subsequent expansion of Muribaculaceae.

***Conclusions:*** These are the first data to show that clinically impactful symptoms of gastrointestinal toxicity are dictated by the microbiome and provide a clear rationale for how and when to target the microbiome to mitigate the acute and chronic complications caused by disruption of the gastrointestinal microenvironment. Translation of this new knowledge should focus on stabilising and strengthening the gut microbiome before chemotherapy and developing new microbial approaches to accelerate recovery of the mucosa. By controlling the depth and duration of mucosal injury, secondary consequences of gastrointestinal toxicity may be avoided.



## 1. Introduction

Cancer therapy is well recognised for its detrimental effects on the host microbiota, causing profound and often chronic changes in the microbial community of the gastrointestinal tract [1]. Although the exact microbial shifts differ between unique treatment regimens, chemotherapy is typically associated with a loss of key commensals and expansion of enteric pathogens; both of which are exacerbated by prophylactic and empirical antibiotic use [2].

Disruption of the host microbiota is now considered a critical event in the initiation of various treatment complications, many of which are catalysed by breakdown of the mucosal barrier [3,4]. Although mucosal barrier injury (MBI) is initiated by acute cytotoxic injury to the intestinal stem cell niche, it is perpetuated by innate immune responses that are thought to be dictated by host-microbe interactions at the mucosal interface [5]. However, these assumptions are largely based on associations between the gut microbiome composition and gastrointestinal symptoms, with robust causal data lacking [5]. As such, it remains unclear if microbial changes that occur after chemotherapy truly contribute to symptoms or are simply a form of collateral damage resulting from breakdown of the mucosal barrier.

MBI has long been a major clinical obstacle without effective intervention. Acutely, it decreases the absorptive capacity of the intestine and manifests as diarrhoea, causing a debilitating loss in quality of life and thus compliance and dose maintenance [6,7]. In the setting of haematopoietic stem cell transplantation (HSCT), the complications of MBI are particularly profound because of underlying immunosuppression driving immunological complications, including graft versus host disease (GvHD) by permitting translocation of endogenous danger signals that activate donor-derived T cells [8]. This same process underpins the development of bloodstream infection (BSI), with the expansion and subsequent translocation of enteric pathobionts now reported to drive more than 50% of all BSI cases in stem cell transplant recipients [9,10]. Of particular interest, a growing body of evidence suggests that microbial disruption (i.e. caused by antibiotic use) at the time of chemotherapy increases the risk of adverse events linked to MBI, suggesting a causal role for the host microbiome [11].

Faecal microbiome transplantation (FMT) is a powerful tool that can be used fundamentally to explore causal relationships between the host and their resident microbes. It is also recognised for its ability to deliver a diverse and metabolically active microbial community to the host with greater durability compared with commercial probiotics, making it of great interest in supportive cancer care. Its efficacy is well documented in the case of recurrent *Clostridium difficile* infection [12], with experimental efficacy also shown for inflammatory bowel disease [13], highlighting its luminal and mucosal benefits. Within oncology, its use has been met with caution because of the perceived risks of translocation [14]; however, recent reports detail profound benefits in steroid-refractory GvHD [15,16]. Although it has been demonstrated that FMT is able to restore the microbial community after 5-fluorouracil and antibiotics [17], it remains unclear if FMT-induced microbial restoration is able to prevent or minimise MBI and its associated clinical manifestations. The present study therefore aimed to determine the impact of microbial manipulation, using antibiotics and FMT, on MBI and its clinical manifestations using a validated model of methotrexate-induced gastrointestinal toxicity [18,19]. By delivering autologous gut microbes after chemotherapy, we aimed to provide new data to aid in defining the causal contribution of the microbiome to MBI and associated symptoms and identify translational methods aimed at strengthening the host microbiome before treatment.

## 2. Methods

This study is reported in accordance with the ARRIVE guidelines for the robust and reproducible reporting of preclinical research.

### 2.1. Ethical statement, husbandry specifications, and experimental considerations

All experiments were conducted in accordance with ethical guidelines approved by the national Dutch committee: 'Centrale Commissie Dierproeven' and the Institutional Animal Care and Use Committee of the University Medical Centre Groningen (UMCG), University of Groningen, under the licence number 15338-01. All rats were individually housed in conventional, open-top cages at the 'Centrale Dienst Proefdieren' (CDP; Central Animal Facility) at UMCG. Rats were housed under 12-h light/dark cycles with *ad libitum* access to autoclaved AIN93G rodent chow (Research Diet Services, The Netherlands) and sterile water. Sawdust bedding was provided in all cases, as well as a toilet roll for enrichment. All cages were randomly arranged across racks to prevent potential bias. All experiments were performed in nine rats per group, which is sufficiently powered to detect a 25% effect size at power 80% and alpha of 0.05.

### 2.2. Preclinical model of MTX-induced MBI

MBI was induced with the chemotherapeutic drug, methotrexate (MTX), obtained from the Pharmachie Holding B.V. (The Netherlands) administered at 45–60 mg/kg as previously described [19]. Briefly, male Wistar rats (150–180 g; Charles River, Germany) were treated with a single intravenous dose of MTX on Day 0 administered via the penile vein under anaesthetic (3% isoflurane). A volume equivalent dose of 0.9% NaCl was used as vehicle control in all experiments as previously described [20]. All experiments were performed in a blinded manner, with rats given a unique identification number.

The primary endpoint of the study was plasma citrulline, a validated biomarker of small intestinal enterocyte mass and clinically used marker of MBI caused by chemotherapy [21,22]. Plasma citrulline was assessed in repeated blood samples (75 μl) collected from the tail vein into ethylenediaminetetraacetic acid–treated haematocrit capillary tubes. Plasma was isolated from whole blood by centrifugation (4000 g for 10 min), and citrulline quantified using automated ion-exchange column chromatography as previously described [23]. Data are presented as raw citrulline values assessed longitudinally and area under the curve (AUC). AUC was defined as the area below Y = mean baseline citrulline value for all groups and above the individual animal citrulline curve. Accordingly, the more severe the mucositis, the higher the AUC value.

#### 2.2.1. Clinical toxicity assessment

Clinical manifestations of MBI were assessed daily and included body weight, diarrhoea, food intake, water intake, and welfare assessments. Diarrhoea was assessed using a validated grading system where 0 = no diarrhoea, 1 = mild diarrhoea indicated by soft but formed pellets, 2 = moderate diarrhoea indicated by perianal staining, and 3 = severe diarrhoea with extensive staining and leakage [24]. Food and water intake were measured daily by manually weighing the contents of the chow hopper and water bottle. During the experimental period, H.R.W. was responsible for maintaining animal water and chow, eliminating the possibility of animal caretakers to change water bottles or refresh chow.

#### 2.2.2. Histopathology

Routine haematoxylin and eosin (H&E) staining was performed to assess intestinal tissue morphology. Briefly, drop-fixed jejunal tissue was processed and embedded into paraffin wax. Four-micrometre sections were cut on a rotary microtome and mounted onto glass Superfrost slides. H&E staining was performed as per routine protocols, and slides were scanned using the Hamamatsu Photonics Digital Slide Scanner (NanoZoomer S60). Images were evaluated using the NDP.view2 software. Villus height and crypt depth were measured using annotation tools in NDP.view2. Ten well-oriented crypts/villi were measured per slide, and an average calculated per animal.

### 2.3. Antibiotic-induced microbial disruption

Antibiotics (750 mg/l vancomycin, 350 mg/l imipenem, and 1 g/l metronidazole) were administered in drinking water for 1 week to disrupt the intestinal microbiota before MTX treatment. Antibiotics were refreshed twice weekly.

### 2.4. Faecal microbiota transplantation

In all experiments, FMT was performed, prepared using faecal pellets collected during acclimatisation. To eliminate individual variation in the FMT product, faecal pellets were pooled from all rats. Faecal pellets were collected from rats by placing them into individual cages (without bedding) cleaned with 70% ethanol. Immediately after defecation, faecal pellets were collected with sterile forceps and placed directly into deoxygenated amies transport solution with 10% glycerol at a concentration of 1 pellet/ml. Samples were then homogenised by vortexing at maximum speed for 5 min until no visible pellets were present. The faecal homogenate was then aliquoted under anaerobic conditions into individual (daily) portions (7 ml) and stored at −80 °C until the day of administration. An additional FMT was also prepared for quality control (culture analysis of colony forming units (CFUs)). On the day of administration, an aliquot of faecal homogenate was thawed at room temperature under anaerobic

conditions before being centrifuged at 13,000 g for 10 min. The supernatant was removed, and the pellet resuspended in 7 ml of sterile, deoxygenated 1× phosphate-buffered saline (PBS). The sample was then homogenised by vortexing at maximum speed for 1 min and allowed to settle by gravity for 15 min. The supernatant was then administered to rats between 8 am and 10 am via oral gavage (500 μl). Control rats received a volume equivalent dose of 1× PBS. FMT was evaluated as (1) an adjunct to MTX, administered on Days 1–7 after MTX and (2) a restorative approach after antibiotic prophylaxis, administered on Days −3, −2, and −1 before MTX (administered on Day 0). Specific schedules for each experiment are shown in figure panels.

### 2.5. Microbiome analysis

The faecal microbiota composition was assessed using 16S rRNA gene sequencing as previously described [19]. Briefly, DNA was extracted using the double bead-beater procedure and the QIAamp DNA Stool Minikit guidelines. Isolated DNA was quantified using the NanoDrop UV Visible Light Spectrophotometer and the V3–V4 region amplified using polymerase chain reaction (PCR). Each PCR reaction contained 1 μl of 10 μM 341f forward primer (V3F: 5′aatgatacggcgaccaccgagatct 3′) 25 μl Phire HS II Master Mix, 22 μl DNase-free water, 1 μl of 10 μM 806r barcoded reverse primer, and 1 μl DNA template (100 ng/μl) [25]. Amplification was confirmed using gel electrophoresis. Size selection and fragment removal were performed using AMPure XP beads as per manufacturer's guidelines before the final PCR products were normalised to 2 mM and pooled to form a single library, which was stored at −4 °C until sequencing. Sequencing was performed using the MiSeq Benchtop Next Generation Sequencer (Illumina). The paired-end sequencing data received from Illumina software were processed and analysed using the Qiagen CLC Genomics Workbench v12.0 (kindly provided by Qiagen to H.R.W.). De novo operational taxonomic unit (OTU) picking was performed without chimaera filtering using Greengenes (RRID:SCR_002830 version 13.5) as a reference database.

### 2.6. Statistics

All data (excluding 16S) were analysed using GraphPad Prism v8.0 (RRID:SCR_002798). Data were first analysed for normality using the D'Agostino-Pearson Normality test. Parametric data were subsequently analysed using one- or two-way analysis of variance with post-hoc correction for multiple comparisons. For repeated measures, a mixed model with Geisser's greenhouse correction was used to identify significant differences. When normality was not confirmed, a Kruskal–Wallis with Dunn's multiple comparison was used to identify significance. AUC was used to reduce repeated data sets and simultaneously assess the duration and intensity of a parameter. Specific statistical methods are defined in each figure legend. In all cases, $P < 0.05$ was considered statistically significant.

## 3. Results

### 3.1. Methotrexate disrupts the gastrointestinal microenvironment, prompting MBI and pathogen expansion

MTX caused self-limiting MBI indicated in plasma citrulline concentrations (Fig. 1A and B). Maximum MBI was observed 4 days post-MTX treatment; however, it was significantly decreased relative to controls from Day 2 to Day 8 ($P < 0.0001$). AUC for plasma citrulline was significantly increased in MTX-treated rats (44.03 ± 26.44 versus 438.0 ± 51.82; $P < 0.0001$) underpinned by a significant villus atrophy in the jejunum (594.9 ± 18.8 μm versus 327.0 ± 25.3 μm; $P < 0.0001$, Fig. 1C).

MTX also impaired weight gain (ΔBW), with significant differences in ΔBW from Day 2 to Day 10 ($P < 0.0001$; Fig. 1D). This was accompanied by anorexia, with food intake significantly decreased in MTX-treated rats on Day 4 ($P = 0.008$). No changes in water intake were identified (data not shown).

Analysis of the faecal microbiota revealed no significant change in alpha diversity (Shannon's index) after MTX (Fig. 1F). Compositionally, however, there was a profound increase in the relative abundance of Proteobacteria taxa, especially *Enterobacteriales* (Fig. 1G, Fig. S1), with paralleled decreases in *Bifidobacteriales* (Fig. 1H, Fig. S1). More detailed microbiome analyses can be found in supplementary data (Figure Supplementary Fig. S1).

### 3.2. Fecal microbiota transplantation promotes microbial stability during MTX toxicity but does not impact clinical manifestations

After confirmation of microbial disruption in our model, we evaluated the efficacy of autologous FMT to prevent MBI and associated clinical manifestations of MTX treatment. FMT increased both microbial richness and diversity after MTX ($P = 0.03$, $P = 0.04$, respectively; Fig. 2A and B). Although the relative abundance of Proteobacteria taxa was decreased, this failed to reach statistical significance (Fig. 2C). FMT was unable to mitigate MBI, indicated by plasma citrulline (longitudinal dynamics and AUC; Fig. 2D and

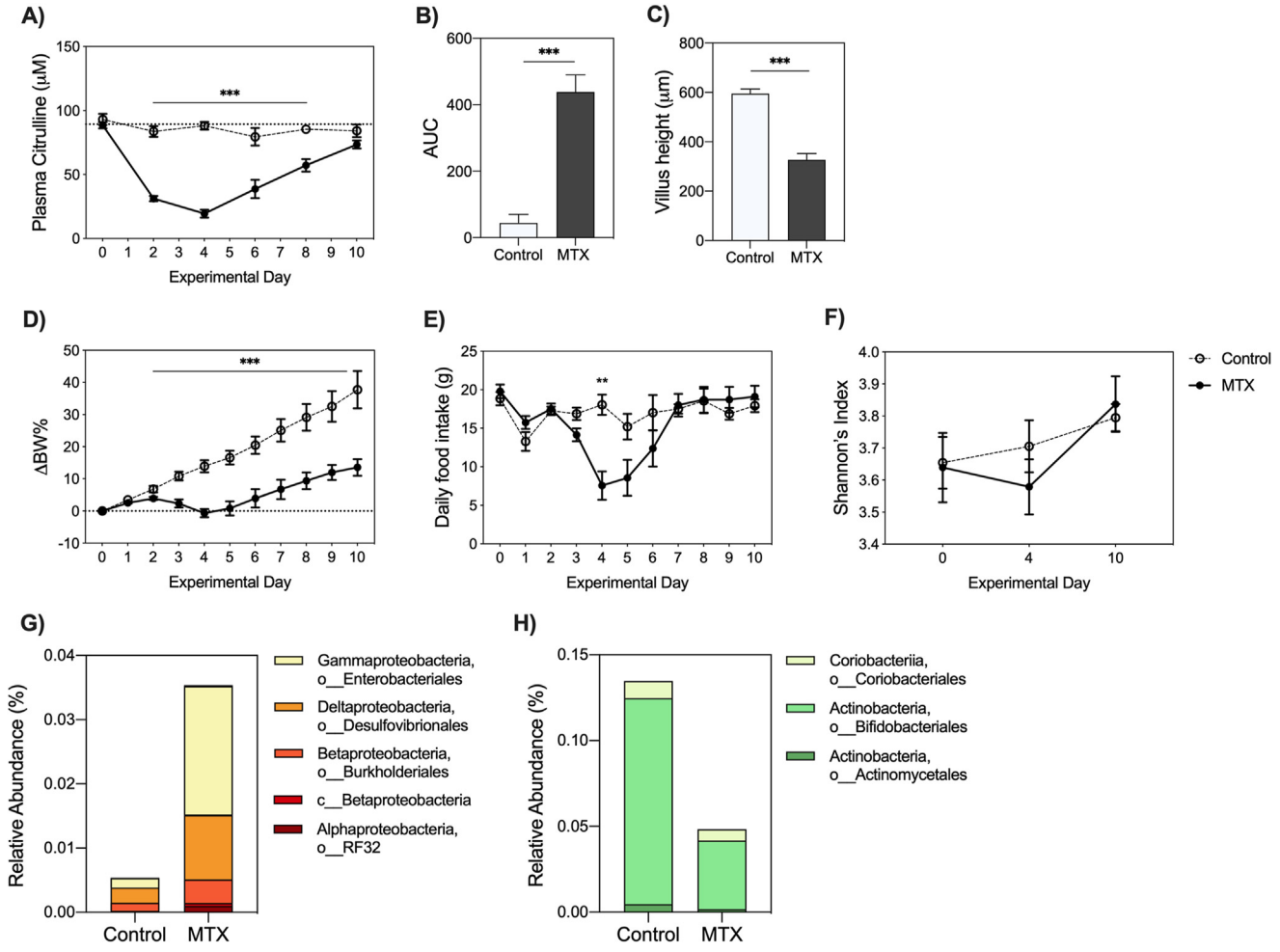


Fig. 1. **MTX disrupts the gastrointestinal microenvironment.** MTX administered intravenously at 45 mg/kg caused significant mucosal injury and microbial changes. A-B show plasma citrulline, a biomarker of mucosal injury where higher AUC indicates more profound MBI. B shows villus height determined from histological analysis of the jejunum. D-E show clinically-relevant symptoms of MBI, change in body weight and food intake, respectively; each of which are decreased with MTX. F-H show 16S rRNA sequencing outputs, including alpha diversity which was unchanged by MTX and compositional changes in key taxa including Proteobacteria and Actinobacteria phyla. Data (A-F) shown as mean ± SEM. Data (G-H) shown as mean relative abundance at day 4. All experiments were conducted in N = 9 rats / group repeated once. * indicates $P<0.05$, ** indicates $P<0.02$, *** indicates $P<0.001$.

E) or ΔBW (Fig. 2F). It did however increase food intake during peak mucosal injury (3.2 ± 0.7 g/day versus 8.5 ± 1.7 g/day; $P = 0.007$; Fig. 2G). There were no significant correlations between peak MBI (defined by citrulline at Day 4) and microbial richness or diversity (Fig. 2H and I). A significant but mild correlation was identified between citrulline and the relative abundance of *Proteobacteria* at Day 4 ($R2 = 0.24$; $P = 0.002$; Fig. 2J).

### 3.3. Antibiotic-induced microbial disruption exacerbates MTX-induced gastrointestinal toxicity by impairing mucosal recovery

To further investigate the impact of the microbiota on MTX-induced MBI, we evaluated the effect of antibiotic-induced microbial disruption (ABX) on MTX toxicity in a high-dose model. MTX was administered at 60 mg/kg (MTX60) to induce a more severe MBI phenotype. ABX, alone or in combination with MTX60, induced a profound decrease in microbial diversity, whereas MTX60 alone caused no significant change in alpha diversity (Fig. 3A). Irrespective of diversity, both MTX60 and ABX+MTX60 caused changes in the phylogenic composition of the microbiota (Fig. 3B) characterised by the expansion of *Proteobacteria*.

ABX significantly increased MTX60-induced mortality ($P = 0.0045$; Fig. 4A). Although acute MBI, indicated by plasma citrulline, was comparable between MTX60 and ABX+MTX60, the ability for the mucosa to recover was significantly impaired in ABX-treated rats (Fig. 4B). This resulted in a significantly increased plasma citrulline AUC in ABX+MTX60 treated rats compared with MTX60 alone (583 ± 21 versus 698 ± 11; $P < 0.0001$; Fig. 4C). This was accompanied

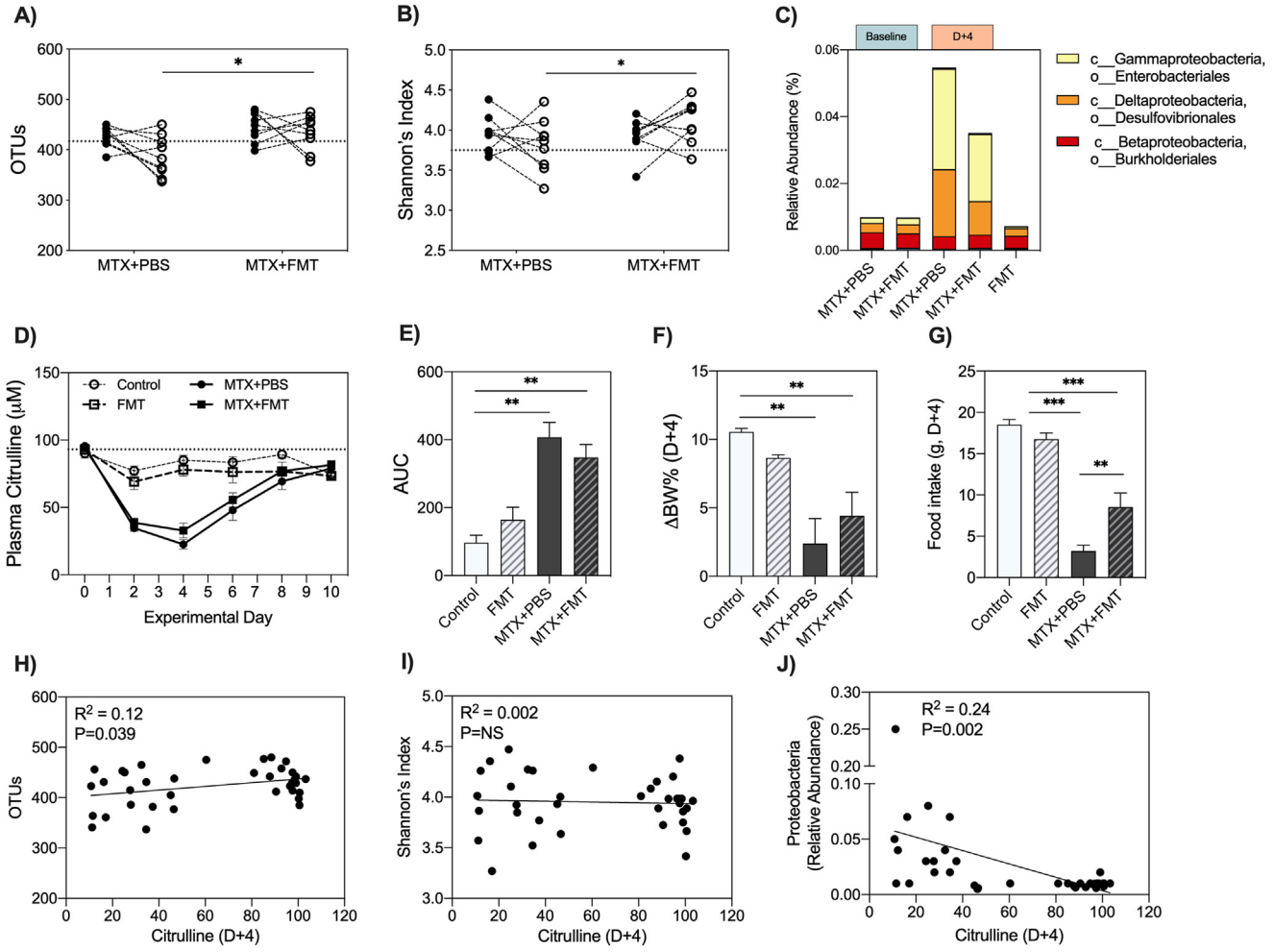


Fig. 2. **Autologous faecal microbiome transplantation protects the microbiome during MTX treatment but does not prevent mucosal injury.** A-B show microbial richness and diversity in MTX and MTX+FMT groups at baseline and peak injury (day 4). D-G show citrulline (raw and AUC) and clinical outcomes across groups. Food intake on day 4 increased in FMT treated animals (G, $^{**}P=0.01$). H-J show correlations between Citrulline on day 4 (peak injury) and microbial read outs. All grouped data shown as mean ± SEM. All experiments were conducted in N=9 rats / group repeated once. * indicates P<0.05, ** indicates P<0.02, *** indicates P<0.001.

by more profound weight loss in rats treated with ABX+MTX60 compared with MTX60 alone (Fig. 4D) irrespective of food intake (Fig. 4E). Histopathological analysis of the jejunum revealed more pronounced villous atrophy in ABX+MTX60 rats compared with all other groups ($P < 0.0001$), including MTX60 ($P = 0.03$; Fig. 4F).

### *3.4. FMT mitigates detrimental effects of ABX on MTX-induced diarrhoea with its efficacy dictated by the colonisation of Muribaculaceae (S24-7)*

Disruption of the microbiome with ABX induced a more pronounced toxicity phenotype. As such, we next aimed to restore the microbiome after ABX using autologous FMT. Given high rates of mortality seen in the MTX60 (high-dose) model, MTX was administered as intravenously at 45 mg/kg (MTX45). At this dose, ABX had no significant impact on mortality (Fig. 5A). However, consistent with the high-dose MTX60 model, ABX slowed mucosal recovery, with a significantly lower citrulline value seen at Day 8 compared with MTX45 only ($P = 0.02$; Fig. 5B). FMT accelerated mucosal recovery, increasing citrulline at Day 8 compared with ABX+MTX45 ($P = 0.04$; Fig. 5B). These subtle differences did not affect the AUC for citrulline (Fig. 5C). Clinically, weight loss and food intake were not affected by FMT, with significant differences only identified between MTX45 and ABX+MTX45 groups (Fig. 5D and E; $P < 0.05$).

Despite only mild effects on MBI, weight, loss and food intake, ABX profoundly exacerbated diarrhoea caused by MTX45 (Fig. 6A–C). Antibiotics induced a severe diarrhoea phenotype compared with MTX45 alone (Day 7, $P = 0.01$; Fig. 6D) and ABX+FMT+MTX45 groups (Day 7, $P = 0.0007$; Fig. 6D). This resulted in a significantly higher

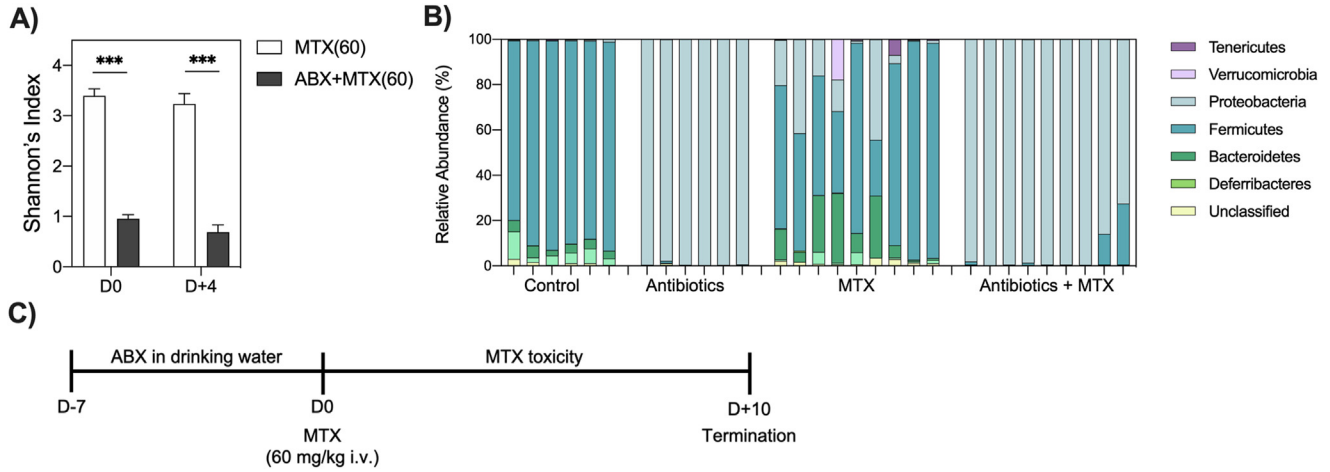


Fig. 3. **ABX and MTX synergistically damage the gastrointestinal microbiota.** A shows alpha diversity (Shannon's Index) at day 0 and +4 after MTX and ABX+MTX. At both time points the diversity of the faecal microbiota was significantly decreased in ABX+MTX rats ($P<0.0001$). B shows composition (taxonomic classification: phylum) of microbiota at day +4, and C shows experimental design. Antibiotics ablated enteric commensals, permitting domination of proteobacteria phyla. Consistent with lower dose model (MTX45), MTX60 caused no change in alpha diversity (A) but altered the abundance of Proteobacteria, Bacteroidetes and Fermicutes (B). All grouped data shown as mean ± SEM. All experiments with MTX treatment were conducted in N = 9 rats / group repeated once; control and antibiotic alone groups had N = 6.

diarrhoea AUC in ABX+MTX45 compared with both MTX45 alone ($P = 0.02$; Fig. 6E) and ABX+FMT+MTX45 ($P = 0.03$; Fig. 6E).

Analysis of individual responses revealed a responder/non-responder effect with significant differences seen in citrulline dynamics ($P < 0.0001$;

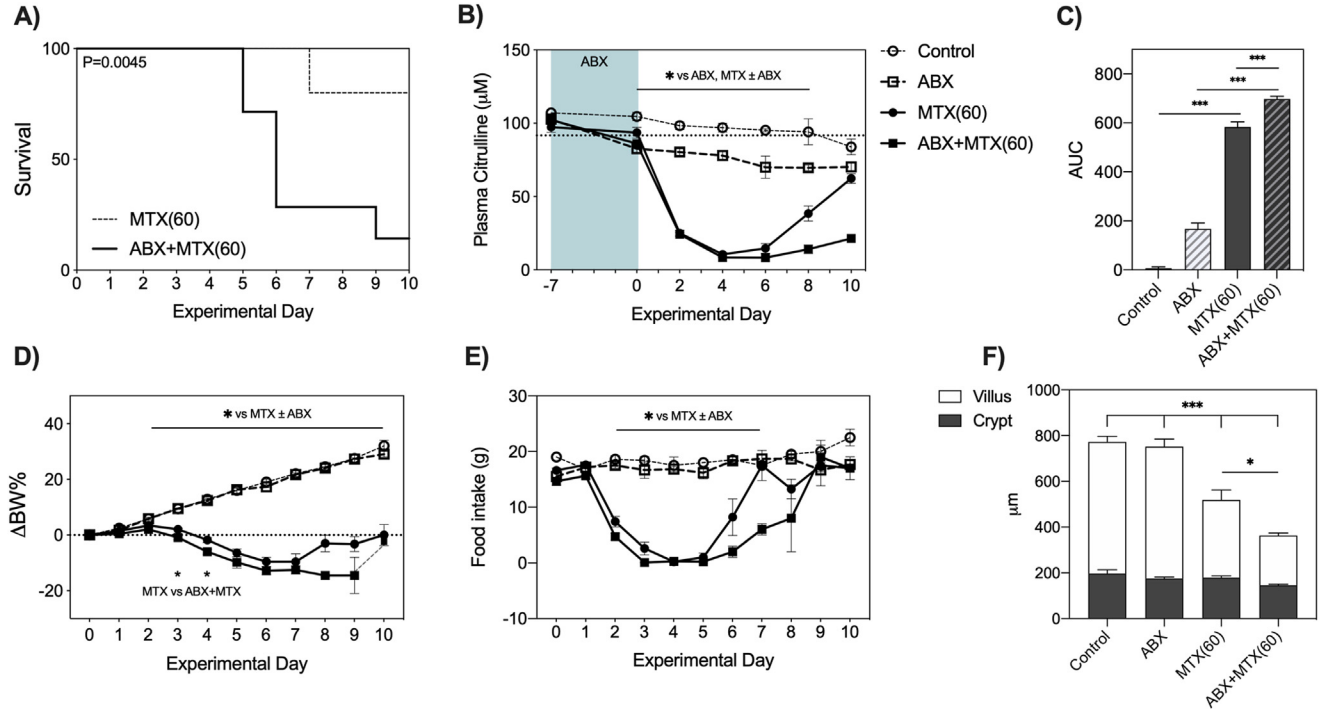


Fig. 4. **ABX prophylaxis impairs mucosal recovery causing a more severe toxicity phenotype.** A shows survival/mortality after MTX±ABX, with antibiotics increasing MTX-induced mortality (P = 0.0045). B-C show plasma citrulline dynamics and AUC indicating impaired mucosal recovery in ABX+MTX. D shows change in body weight after MTX treatment, which was increased in ABX+MTX rats on day +3 (P = 0.039) and +4 (P = 0.006). E shows food intake in all groups, with MTX and ABX+MTX groups showing no differences. F shows histopathological changes in the jejunum at day +4, with ABX exacerbating MTX-induced villous atrophy (P = 0.03). as was histopathological injury in the jejunum (F). All grouped data shown as mean ± SEM. All experiments with MTX treatment were conducted in N = 9 rats / group repeated once; control and antibiotic alone groups had N = 6. * indicates $P<0.05$, ** indicates $P<0.02$, *** indicates $P<0.001$.

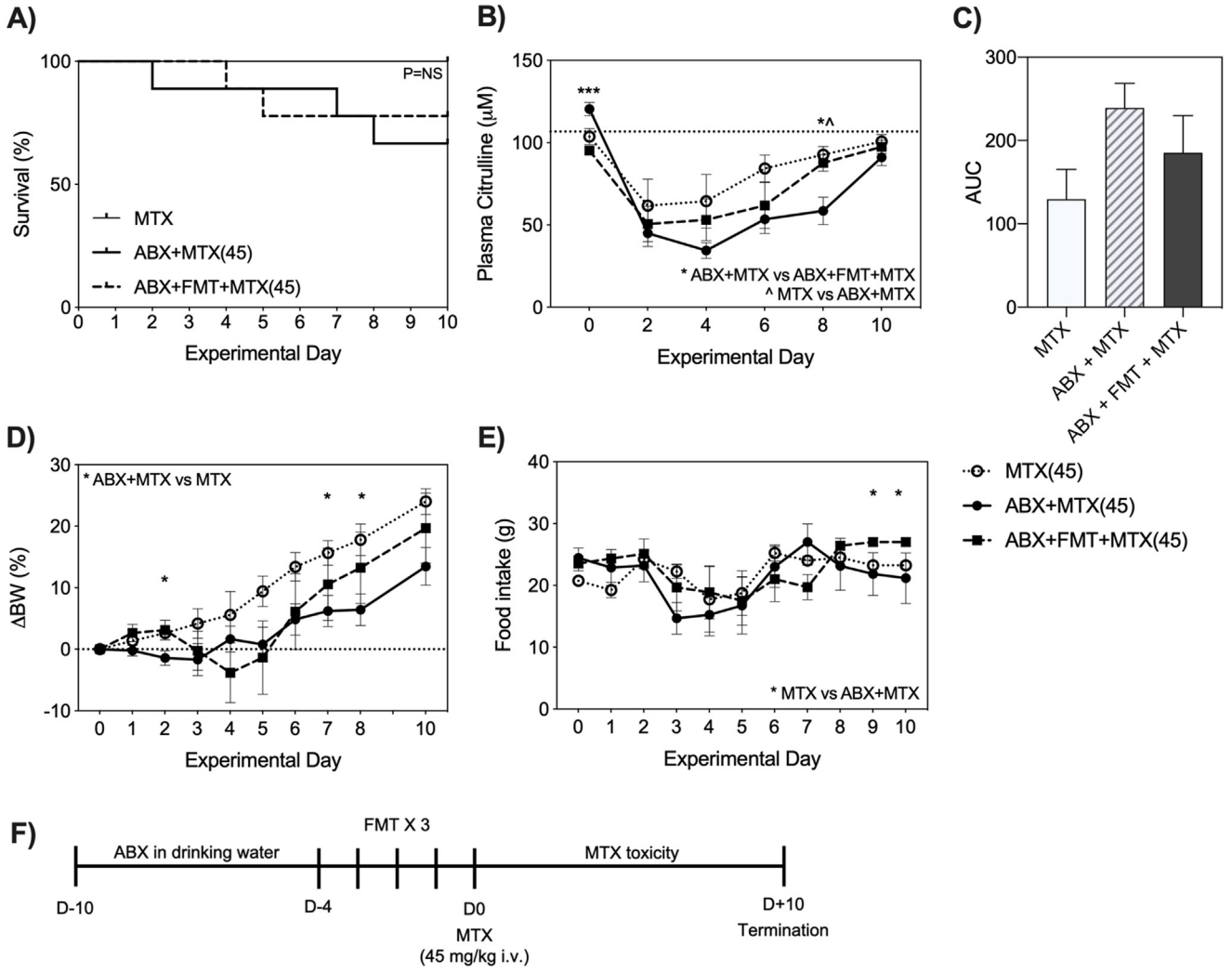


Fig. 5. **Effect of restorative FMT on gastrointestinal toxicity caused by moderate dose MTX (45mg/kg) and ABX.** A shows survival/mortality for MTX vs ABX+MTX vs ABX+FMT+MTX, with no significant difference observed in the low dose model (MTX45). B-C show plasma citrulline dynamics and AUC, which were only mildly influenced by FMT (day +8, P =0.048). D shows change in body weight relative to day 0 in all groups. ABX exacerbated MTX-induced weight loss (P=0.046 day +7, P=0.038 day +8); there were no differences between MTX alone and ABX+FMT+MTX. E shows food intake across the course of the experiment, with no significant effect of FMT observed. F is the experimental design. All grouped data shown as mean ± SEM. All experiments were conducted in N=9 rats / group repeated once. * indicates P<0.05, ** indicates P<0.02, *** indicates P<0.001.

Fig. 7A–C). Responders ('R') were defined as rats maintained stable citrulline values throughout the experimental period (i.e. responded to FMT). Non-responders ('NR') displayed typical citrulline dynamics of MTX treatment characterised by hypocitrullinemia at Day 4 (i.e. did not respond to FMT).

Subgroup analysis of the microbiota composition at Day 0 in ABX+FMT+MTX rats (N = 9) suggests the durability of the FMT dictates response to MTX, with principal component 3 (PCo3) differentiating R versus NR (Fig. 7D). PCo3 correlated with the relative abundance of *Muribaculaceae* (S24-7; Fig. 7E), which was significantly elevated in FMT responders ($P = 0.001$; Fig. 7F).

## 4. Discussion

Disruption of the intestinal microbiome is increasingly associated with unfavourable treatment outcomes in people with cancer, reported to dictate both the efficacy and toxicity of chemotherapy as well as the risk of relapse [1]. Here, we report the first phenotypic analysis of FMT delivered after cytotoxic chemotherapy (MTX) and antibiotics, identifying *Muribaculaceae* (S24-7) as a key regulator of mucosal injury and repair.

MBI (mucositis) in the gastrointestinal tract is a major oncological obstacle because of its acute symptomology and systemic consequences. Although a strong body of data shows microbial injury after chemotherapy drugs, including MTX [26], it remains hotly debated if these

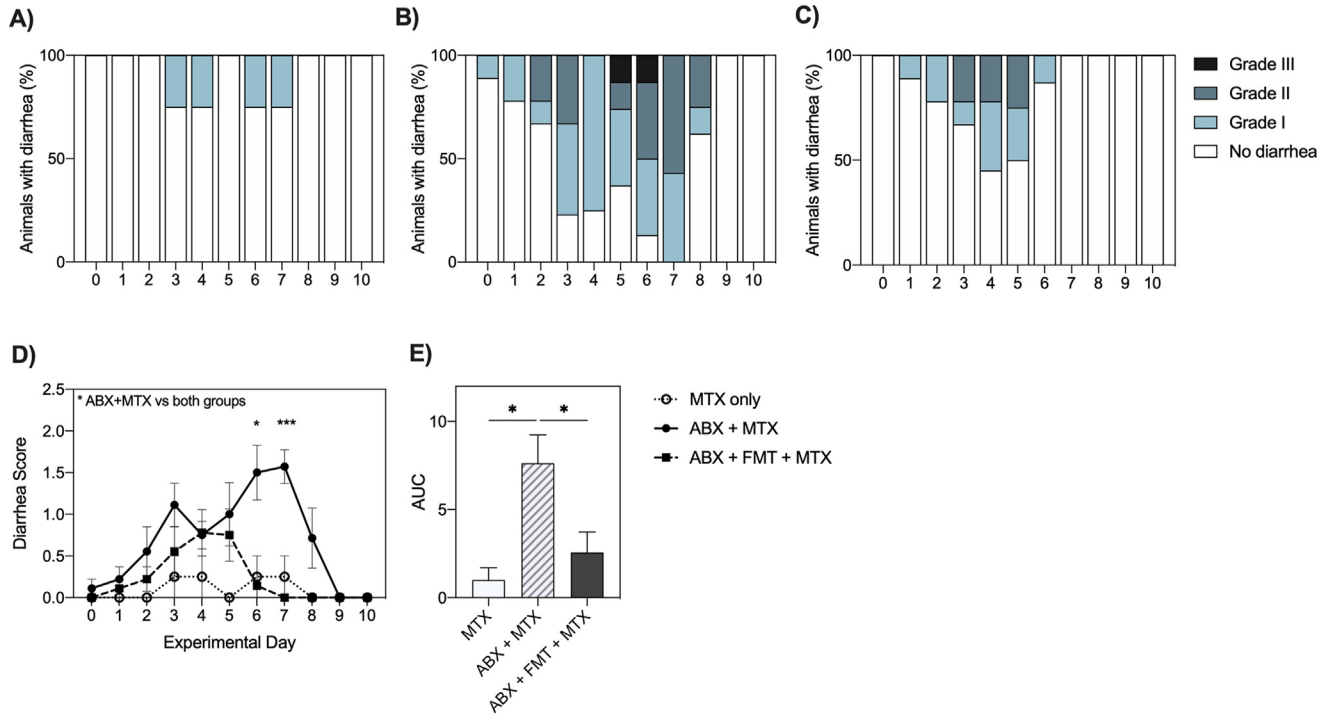


Fig. 6. Pretreatment with antibiotics cause severe diarrhoea when administered in combination with MTX and can be reversed by FMT.

changes are directly mediated by chemotherapy or simply a form of collateral damage resulting from mucosal injury [27]. Our data support the latter, with FMT delivered after MTX failing to appreciably impact mucosal injury (defined by citrulline) and its clinical manifestations (weight loss and anorexia), despite maintaining a more stable microbiome composition defined by 16S sequencing. The lack of FMT efficacy could also be explained by the hypothesis that MTX-induced mucosal injury creates a luminal environment that is inhospitable to microbes introduced via FMT or simply the degree of mucosal injury is too profound for the donated microbes to influence. Each interpretation is based on the concept that microbial disruption occurs secondary to mucosal injury, a hypothesis that is consistent with existing data showing chemotherapeutic drugs, including 5-FU and irinotecan, do not directly interfere with the microbiome [28].

We do however show that disruption of the host microbiome with antibiotics impairs mucosal recovery and exacerbates diarrhoea, with FMT having a beneficial impact. This supports the hypothesis that although microbial disruption occurs after mucosal injury, dysbiosis amplifies mucosal injury and establishes self-perpetuating feedback loops that drive clinical manifestations. This strongly supports interventions targeting the microbiome to minimise the depth and duration of MBI, accelerating recovery and controlling clinical symptoms/consequences.

Although the dynamics of microbial injury after chemotherapy have been the topic of intense investigation, their correlation with treatment outcomes has been variable. In contrast, the composition of the microbiome at baseline (i.e. at the time of chemotherapy) is increasingly linked to how an individual responds [29–31]. This has been most elegantly demonstrated in the case of immunotherapy-induced colitis [32–34]; however, a growing body of evidence anecdotally suggests that similar mechanisms may dictate an individual's risk of mucosal injury caused by chemotherapy [30]. In a recent systemic review of predictors of mucosal injury coordinated by the Multinational Association for Supportive Care in Cancer [11], antibiotic use in the month leading up to cancer therapy was identified as a modulator of mucosal injury [35,36]. Consistent with these findings, antibiotic administration before MTX treatment caused a profound increase in treatment-related mortality and diarrhoea. Underlying this observation was severe mucosal injury, with antibiotics exacerbating villous atrophy and impairing mucosal recovery. In fact, antibiotic-treated rats failed to recover from MTX treatment, with hypocitrullinemia evident at Day 10. Given the large relative expansion in *Proteobacteria*, it may also be the case that expansion of enteric pathogens serves to amplify mucosal injury through interaction with Toll-like receptor 4, an already defined mediator of chemotherapy-induced diarrhoea [24,37]. Further research is required to identify the microbial factors that control mucosa recovery in the context of chemotherapy treatment, with short-chain fatty acids (SCFA) a promising candidate because of their ability to simultaneously promote luminal acidification (thus controlling pathogen expansion) [38] as well as mucosal proliferation and immunology [39]. Of note, SCFAs have been documented to be deficient after chemotherapy and exert

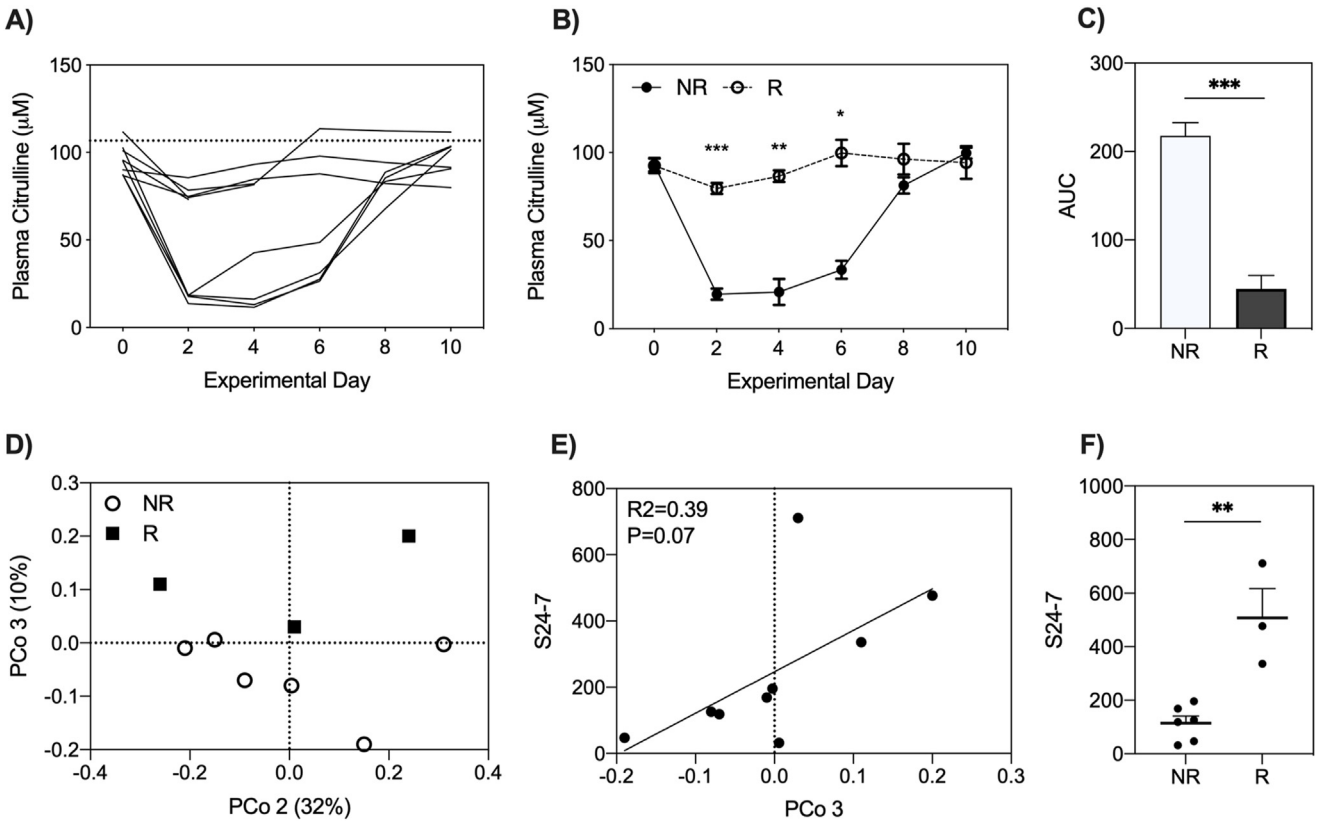


Fig. 7. **Severity of MTX-induced MBI is defined by FMT durability.** A shows plasma citrulline dynamics analysed for N = 9 rats in ABX+FMT+MTX group showing clustering of responders (R; N = 3) and non-responders (NR; N = 6). B-C show sub-group analysis of R vs NR showed significant difference in longitudinal citrulline dynamics and AUC (P = 0.0005). D shows principal coordinate analysis, with PCo3 aligned with responder phenotype and correlating with S24-7 abundance (E). F shows relative abundance of S24-7 (*Muribaculaceae*) between R and NR identified by differential abundance analysis (FDR adjusted P = 0.02). All grouped data shown as mean ± SEM. All analyses were conducted in N = 9 rats / group repeated once. * indicates P<0.05, ** indicates P<0.02, ***indicates P<0.001.

protective effects in chemotherapy- and radiation-induced intestinal injury [40].

The detrimental effects we observed when antibiotics were administered before MTX combined with the growing number of studies associating increased morbidity, mortality, and relapse with antibiotic use [41,42]. This has been explored with the greatest detail in HSCT recipients because of the intensity of chemotherapy they receive and current reliance on antibiotics. Importantly, comparable to our findings, antibiotics and microbial injury have been reported to increase the risk and severity of intestinal GvHD [43–45]. Mechanistically, this is thought to be caused by increased intestinal permeability and mucin degradation – two well-defined aspects of chemotherapy-induced mucotoxicity and diarrhoea [5] – and, as such, may also be mechanisms responsible for our findings.

Microbial injury caused by antibiotic prophylaxis is well documented [41,42]. Although the antibiotic schedule used in our study was not intended to reflect clinical practice, when viewed alongside the growing body of literature, our findings raise important implications for antibiotic stewardship. Specifically, they highlight the need to select appropriate prophylactic antimicrobial drugs that do not damage the microbiome [46]. Ciprofloxacin is reported to have mild effects on the microbiome and may therefore be a potential avenue to restrict antibiotics whilst still providing appropriate infection control [47]. However, in some cases, residual microbial injury may be caused by previous antibiotic exposure, cytotoxic treatment, comorbidities, or other medications. As such, restoring or strengthening the microbiome before treatment represents a potentially important supportive care strategy.

We showed that FMT administered after antibiotics but before MTX was able to restore typical dynamics of mucosal recovery, reiterating our findings that suggest a causal contribution of the microbiome to mucosal healing. However, of particular interest was the clear clustering in response to FMT intervention. FMT uptake is documented to be variable both preclinically and clinically, reflecting donor selection and the preparation/status of the recipient [48]. Our data demonstrated a low response rate to FMT intervention, with only 33% of rats responding to FMT. Subgroup analysis suggests response to FMT intervention is mediated through the

durability of the FMT, specifically colonisation and expansion of *Muribaculaceae* (S24-7). *Muribaculaceae* (S24-7) is a typical rodent bacterial family belonging to the Bacteroidetes phylum already documented to regulate FMT efficacy in rodents [49]. It is recognised to increase in abundance after prebiotic intervention with vitamin $B2^{18}$ and selenium [50], both of which have documented benefits in the prevention of mucosal injury caused by chemotherapy [18,51].

Translating this new knowledge is somewhat challenging, as *Muribaculaceae* is absent in humans. However, it is considered to be a core functional microbe of the rodent gut, found in high abundance under healthy circumstances [52]. As such, our findings may simply reflect that restoration of *Muribaculaceae* is an indicator of successful FMT uptake, and we hypothesise that colonisation of other functional microbes relevant to the human gut (e.g. *Fecalibacterium* or *Blautia*) would likely dictate clinical FMT efficacy. Importantly, it cannot be excluded that the abundance of *Muribaculaceae* was comparable between R and NR before FMT, and such, pre-FMT microbiome composition may also be an important factor dictating its efficacy.

Although our data strongly support adjunctive FMT strategies to improve treatment outcomes, this study is not without its limitations. It must be noted that because of the mild nature of microbial injury seen at 45 mg/kg MTX, a higher dose was used for the subsequent study (MTX60). This, in combination with antibiotics, induced unacceptable levels of mortality, and as such, we reverted to 45 mg/kg for all remaining experiments. Our observations remained largely comparable across both doses, with the exception of citrulline and body weight. As such, we cannot definitively conclude that the exacerbated diarrhoea profile is the result of impaired mucosal recovery in the lower dose model. The heightened diarrhoea may simply reflect antibiotic-induced dysbiosis. However, we did not see substantial diarrhoea in our rats whilst undergoing antibiotic treatment (6/27 developed Grade 1 diarrhoea for an average of 1.16 days), suggesting it is the combination of antibiotics and MTX that is responsible for the exacerbated diarrhoea phenotype driven by impaired mucosal recovery. Nonetheless, given the multifactorial nature of chemotherapy-induced diarrhoea, it is most likely that both the dysbiosis and impaired mucosal recovery caused by antibiotics are driving the diarrhoea profile.

In conclusion, our data underscore the importance of microbial stability during chemotherapy treatment and provide a rationale to re-evaluate the administration of microbial interventions for the control of mucosal injury. We suggest microbial interventions be used before chemotherapy treatment to repopulate or shift the composition of the host microbiome, thus offering a new strategy to improve treatment outcomes. Alternatively, a strong rationale also exists for postchemotherapy intervention aimed at promoting restoration of the mucosal barrier through microbial intervention. This approach would minimise the duration of mucosal injury, decreasing the intensity of symptoms and opportunity for translocation events.

### Authors' contributions

H.R.W. contributed to conceptualisation, data curation, formal analysis, investigation, methodology, visualisation, and writing (draft and reviewing) the article. S.A.J.v.d.A. contributed to conceptualisation, data curation, investigation, and reviewing and editing the article. A.R.D.S.F. contributed to data curation, investigation, and reviewing and editing the article. R.H. contributed to methodology, investigation, and resources. W.J.E.T. contributed to conceptualisation, funding acquisition, project administration, resources, supervision, and reviewing and editing. H.J.M.H. contributed to conceptualisation, funding acquisition, resources, software, supervision, and reviewing and editing the article.

### Conflict of interest statement

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this article.

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### Appendix A. Supplementary data

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