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Research paper

Mucosal microbial load in Crohn's disease: A potential predictor of response to faecal microbiota transplantation

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ABSTRACT

Background: The remission of Crohn's disease (CD) can be accomplished by faecal microbiota transplantation (FMT). However, this procedure has a low success rate, which could be attributed to mis-communication between recipient intestinal mucosa and donor microbiota.
Methods: Here we used a human explant tissue model and an *in vivo* mouse model to examine changes in recipient intestinal mucosa upon contact with a faecal suspension (FS) obtained from a healthy donor. CD patients provided resected inflamed and non-inflamed mucosal tissues, whereas control colonic mucosa samples were collected from colorectal cancer patients. For the models, mucosal microbiome composition and tissue response were evaluated.
Findings: We show that cytokine release and tissue damage were significantly greater in inflamed compared to non-inflamed CD tissues. Moreover, mucosal samples harbouring an initial low microbial load presented a shift in composition towards that of the FS, an increase in the relative count of *Faecalibacterium prausnitzii*, and a higher secretion of anti-inflammatory cytokine IL-10 compared to those with a high microbial load.
Interpretation: Our results indicate that FMT during active inflammatory disease can compromise treatment outcome. We recommend the stratification of FMT recipients on the basis of tissue microbial load as a strategy to ensure successful colonization.
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1. Introduction

Crohn's disease (CD), one of the two main forms of inflammatory bowel disease (IBD), is a chronic incurable condition of unknown aetiology whose prevalence is expected to increase exponentially over the next decade, making it a growing healthcare burden [1]. An imbalanced gut microbiota, called dysbiosis, combined with host genetic susceptibility, environmental factors such as smoking, diet and antibiotic use, and dysregulated immune responses contribute to the pathogenesis of CD. Additionally, specific intestinal bacteria may also play a key role in this condition [2].

Environmental and genetic factors lead to an impaired intestinal barrier in CD patients, resulting in the translocation of bacterial antigens and triggering a pro-inflammatory cascade in which mucosal immune cells (*i.e.* macrophages, innate lymphoid cells and T cells) respond to microbial antigens and release pro-inflammatory cytokines [3,4]. The gut mucosa of CD patients is therefore characterized by an increase in pro-inflammatory cytokines, such as tumour

Abbreviations: IBD, Inflammatory Bowel Disease; CD, Crohn's Disease; CM, Healthy Mucosa; HD-FS, Faecal Suspension from a Healthy Donor; HD-ST, Stool from a Healthy Donor; Ni-CD, Non-inflamed Crohn's Disease tissue; I-CD, Inflamed Crohn's Disease tissue; ^{High}CN, High Copy Number; ^{Low}CN, Low Copy Number; qPCR, quantitative Polymerase Chain Reaction; FDR, False Discovery Rate; *n*, Sample Size; PCoA, Principal Coordinate Analysis; rRNA, ribosomal RNA; LDH, Lactate Dehydrogenase

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Research in context

Evidence before this study

Recent findings indicate that remission of IBD can be achieved through multiple whole faecal microbiota transplantations (FMTs) from healthy donors. However, the efficiency of this strategy is lower in IBD than in *Clostridium difficile* infections. This observation is explained in part by the multifactorial nature of IBD. Given these findings, we consider that there is a need to gain further insight into the crosstalk mechanisms between colonic mucosa and faecal microbiota.

Added value of this study

In the present study, we first validated a human gut explant model in order to evaluate the crosstalk between donor microbiota and recipient mucosa during FMT. We confirmed that the inflammatory state of the mucosa determines the outcome of the treatment. We also showed that intestinal mucosa with a low bacterial load is better colonized by the donor microbiota and induces a stronger anti-inflammatory response than mucosa that harbours a high bacterial load. Furthermore, we highlighted that the success of FMT was more dependent on the bacterial load of the recipient mucosa than the composition of the donor faecal suspension. Finally, using an animal model, we observed that a reduction of bacterial load induced by antibiotic treatment not only promoted the colonization of the mucosa by donor microbiota but also triggered an anti-inflammatory cytokine IL-10 response, thereby validating our *in vitro* results.

Implications of all the available evidence

Our results show that the bacterial density of the recipient intestinal mucosa is a critical factor to ensure its colonization by a donor microbiota and to promote an anti-inflammatory response. These data could be useful to stratify CD patients before FMT in order to better identify those who are most likely to respond well to the treatment. Moreover, our findings may pave the way to the development of new therapeutic strategies that reduce bacterial mucosal load prior to FMT.

necrosis factor alpha (TNF-α), interleukin 17 (IL-17AF), which is a T helper 17 cytokine, and IL-12, which drives local T helper 1 and 17 cell responses and suppresses regulatory T cell responses [5,6]. Current therapies for CD, such as corticosteroids, aminosalicylates and immunosuppressive agents, mainly target the patient's immune system to reduce inflammation and induce remission [7]. However, they do not address the modulation of the dysbiotic gut microbiota—which drives pro-inflammatory immune responses—towards a healthier composition.

Faecal Microbiota Transplantation (FMT) involves the transplantation of unaltered faecal microbiota to manipulate the gut microbial communities of experimental models [8,9]. It is also being tested in clinical trials [10]. FMT is a highly effective treatment for *Clostridium difficile* infection [11]. However, the efficacy of this strategy is lower in IBD patients, particularly those with CD [12–16]. This observation could be explained in part by the multifactorial nature of IBD, such as a genetic predisposition towards pro-inflammatory immune responses and intestinal barrier dysfunction [17], which could prevent the transplanted microorganisms from colonizing the recipient gut mucosa. Overall, these studies highlight the need for a better understanding of the mechanisms underlying the crosstalk between the gut mucosa of the CD recipient and the faecal microbiota of the healthy donor.

Few relevant models have been developed to address the early mucosal events during interactions between the donor microbiota and the recipient intestinal mucosa after a FMT procedure. Jalanka et al. showed that a human epithelial culture model treated with a faecal suspension (FS) replicates *in vivo* FMT engraftment [18]. Although this model underlines the usefulness of *in vitro* models to study epithelial cell/microbiota interactions, it does not take into account the immune reactions within the intestinal mucosa. In order to overcome these limitations, an organ culture system was developed to examine the mechanisms that regulate gut homoeostasis and to decipher the microbe-immune crosstalk [19–20]. Indeed, studying this interplay requires a model system that maintains the 3D architecture of the intestinal mucosa and the recognition of bacterial antigens by the resident immune system. The *ex vivo* explant cultures of human intestinal mucosa fulfil these requirements.

Using control mucosa (CM) and CD tissues treated with a FS, here we adapted a gut explant culture to mimic the mucosal response and microbial events that occur after the FMT procedure. Our findings indicate that our model provides a viable approach to study early mucosal immune response and microbial colonization of tissue following exposure to a FS. We observed that FSs obtained from IBD patients promoted less bacterial adhesion and more tissue degradation than a FS obtained from a healthy donor. Finally, using *ex vivo* human explants and an *in vivo* mouse model, we show that a low microbial load in recipient tissues is an unexpected factor that favours the anti-inflammatory response associated with microbial colonization.

2. Materials and methods

2.1. Patients and control subjects

Intestinal samples (ileum from macroscopically inflamed areas and matched adjacent colon when possible) were obtained from 21 CD patients undergoing ileocolectomy or colectomy between 2015 and 2017 (University Hospital of Vall d'Hebron, Barcelona). The main clinical features of the patients and details of their treatments before surgery are given in online Supplementary Table S1. Intestinal samples were also collected from the macroscopically unaffected colons of 45 patients undergoing surgery for colorectal cancer. These samples were taken at least 10 cm from the tumour. Approval for the study was provided by the Ethics Committee of the University Hospital of Vall d'Hebron, and informed patient consent was given in all cases. Biopsies from resected tissues were obtained from 26 CD patients undergoing an ileo-caecal resection at the IBD clinic of the University Hospital of Leuven in Belgium. The study obtained approval from the Ethics Committee of the University Hospital of Leuven, and participants gave written informed consent prior to sample collection.

2.2. Faecal donors

Volunteers (10 healthy subjects, $\geq$ 18 years of age) were screened for faecal donation. One CD and one UC patient were also selected as stool donors. Stool and serology screening was performed for bacterial, parasitic and viral pathogens. Potential donors did not take antibiotics in the eight weeks preceding the screening. Standard microbial screening demonstrated that the selected donor stool for FS preparation was free of *Clostridium difficile*, *Salmonella*, *Shigella*, *Yersinia*, *Aeromonas*, *Campylobacter*, *Vibrio*, *Escherichia coli O157*, *Rotavirus*, *Adenovirus, Atrovirus, Norovirus, and Giardia intestinalis*. Serologic analysis of the healthy donor stool also proved negative for human immunodeficiency virus and for hepatitis A, B and C virus.

2.3. Faecal suspensions

5 g of donor stool was collected and mixed with 50 mL of sterile anaerobic phosphate-buffered saline (PBS). The mixture was emulsified

in an anaerobic chamber. Sterile gauze was used to filter the supernatant. The optical density (OD) of the supernatant was examined at 600 nm to evaluate the cell number. We treated the tissues with two doses of microbial cells. One amount (10^9 cells) was equivalent to that used in several FMT protocols for CD (Supplementary Table S2). A more detailed description can be found in Supplementary Methods.

2.4. Human colonic mucosa explant cultures

Immediately after removal, mucosal tissues were placed in oxygenated Krebs solution at 4 °C supplemented with gentamicin (70 mg/ml, Sigma), penicillin (200 μg/ml, Invitrogen), and streptomycin (200 U/ml, Invitrogen) to eliminate commensal bacteria, and fungizone (1%, Sigma) for 3 h. The tissue was washed twice in RPMI 1640 (Roswell Park Memorial Institute medium) (Invitrogen), and the mucosa was then carefully stripped from the underlying compartment formed by muscularis mucosae and submucosa. Fragments of 4×4 mm^2 were cut out, pinned in 30-mm Sylgard-coated Petri dishes, and maintained in culture for 15 h in 500 μL of RPMI 1640 containing 10% heat-inactivated foetal bovine serum (Gibco) with or without a range of FS concentrations. The explants were maintained at 37 °C in a humid atmosphere of 95% air and 5% carbon dioxide. In each experiment, at least three explants were grown for each condition. The supernatants were centrifuged, and aliquots were stored at −80 °C for further analysis. Some tissue specimens were used for morphological analysis or immunohistochemical analysis, while others were used for 16S sequencing and cytokine analysis. To ensure that the microbiota used to stimulate the tissue sample did not access the colonic lamina propria directly, we measured the relative fluorescence of the supernatants upon contact with FS stained by fluorescein isothiocyanate (FITC) (Supplementary Fig. S5).

2.5. Experimental design of in vivo mouse model

A total of 20 animals were distributed in four experimental groups ($n = 5$; Fig. 5(a)). The four groups of mice were as follows: Control (Ctrl): treated with omeprazole and CitraFleet®; Antibiotics (ATB): treated with antibiotics, omeprazole and CitraFleet®; Faecal Microbiota transplantation (FMT): treated with omeprazole, CitraFleet® and FMT; ATB-FMT: treated with antibiotics, omeprazole, CitraFleet® and FMT. We used antibiotics to decrease the microbial load in the intestinal tissues of mice before FMT. We used omeprazole, which is a proton pump inhibitor, to suppress stomach acid secretion and therefore to increase bacterial survival. Before FMT, CitraFleet® (sodium picosulfate), a stimulant laxative, was also used to remove recipient luminal content. The FS was prepared in exactly the same way as that used for the explant culture experiments and was administrated by oral gavage.

All procedures were conducted in accordance with the European Union Guidelines for Ethical Care of Experimental Animals (EC Directive 86/609/EEC for animal experiments), approved by the Animal Care Committee of the Vall d'Hebron Institut de Recerca (Barcelona, Spain), and conducted in the animal facilities of Vall d'Hebron Institut de Recerca. Further details are described in Supplementary Methods.

2.6. Microbiome sequence analysis

DNA extraction, PCR amplification, and sequencing were performed as previously reported in Lleal et al. [21]. A more detailed description can be found in Supplementary Methods.

Data deposition and accession numbers for raw data

Accession codes to the NCBI: PRJNA422192 and PRJNA526814.

2.7. Microbial load analysis

Extracted genomic DNA was used to amplify the V4 region of the 16S rRNA gene by quantitative real-time PCR (qPCR), using universal primers for counting microbial load and specific primers targeting *Faecalibacterium prausnitzii,* as described in Varela et al. [22]

2.8. Statistics

Statistical analyses were carried out in QIIME [23] (v1.9.1), in R (v3.4.3), and in GraphPad Prism (GraphPad Software Inc, v6.0, La Jolla, CA). The Shapiro-Wilk test was used to check the normality of data distribution. Parametric normally distributed data were compared by means of the Student's *t*-test for paired or unpaired data. Otherwise, the Wilcoxon signed rank test was used for paired data and the Mann-Whitney U test for unpaired data. The Kruskal−Wallis one-way test of variance was used to compare the median number of sequences of the groups at various taxonomic levels. The Friedman test was used for one-way repeated measures of analysis of variance. When possible, the analysis provided false discovery rate (FDR)-corrected *P*-values. FDR < 0.10 was considered significant for all tests.

Further information on the following methods can be found in Supplementary Methods: *Assessment of mucosal damage; Evaluation of the efficiency of the antimicrobial cocktail treatment; Lactate dehydrogenase cytotoxicity assay; Cytokine analysis; Flow cytometry analysis; Tissue processing for Scanning Electron Microscope.*

3. Results

3.1. Validation of explant culture of gut mucosa as an experimental model for faecal microbiota transplantation

Macroscopically inflamed (I-CD; $n = 21$) and non-inflamed (Ni-CD; $n = 20$) tissues were obtained from 21 CD patients. Healthy tissues from colon cancer patients (CRC) were used as control mucosa (CM; $n = 45$). The characteristics of the volunteers can be found in Supplementary Table S1.

After surgical resection, tissues were subjected to optimized culture conditions, as described in Fig. 1(a) and in Supplementary Methods. Briefly, samples were treated for 3 h with an antimicrobial cocktail and then cultured for 15 h with or without FS (Supplementary Fig. S1,2,3).

16S sequence analysis was used to select a healthy donor stool (HD-ST) on the basis of microbial stability (two samples at one month-interval), richness (Chao1 index), diversity (Shannon index) [16] and composition [2]. Recently, we [2] proposed an algorithm that allows discrimination between CD and non-CD faecal samples on the basis of eight microbial genera. Following that algorithm, we considered that the microbiome of donor faecal samples would not contain *Fusobacterium* or *Escherichia* but would contain *Faecalibacterium* or one of the following genera: *Anaerostipes*, *Methanobrevibacter*, *Collinsella*, a genus from Peptostreptococcaceae, or a genus from Christensenellaceae. To this end, we studied the microbiome profiles of stools from 10 healthy volunteers and selected the donor presenting the most stable profile (meaning the highest index of richness and diversity) and a microbial composition that took into account the algorithm proposed by Pascal et al. [2] (Fig. 1(b) and (c)). The HD-ST contained, among others, *Faecalibacterium*, a genus from Christensenellaceae, and *Methanobrevibacter* (Fig. 1(d)). It did not contain *Escherichia* or *Fusobacterium*. Standard microbial screening demonstrated that this stool was free of potential pathogens. Taken together, these conditions met the criteria proposed by Kelly et al. for the selection of a healthy donor [24].

Using a standard protocol [25], a faecal suspension (HD-FS) was obtained from the HD-ST. 16S sequence analysis revealed that the HD-FS showed a similar microbial composition to that of the HD-ST, with the presence of three of the genera cited above as being necessary to correct dysbiosis in CD (*Faecalibacterium*, *Methanobrevibacter*; and a genus from Christensenellaceae) (Fig. 1(c) and (d); Supplementary Table S4).

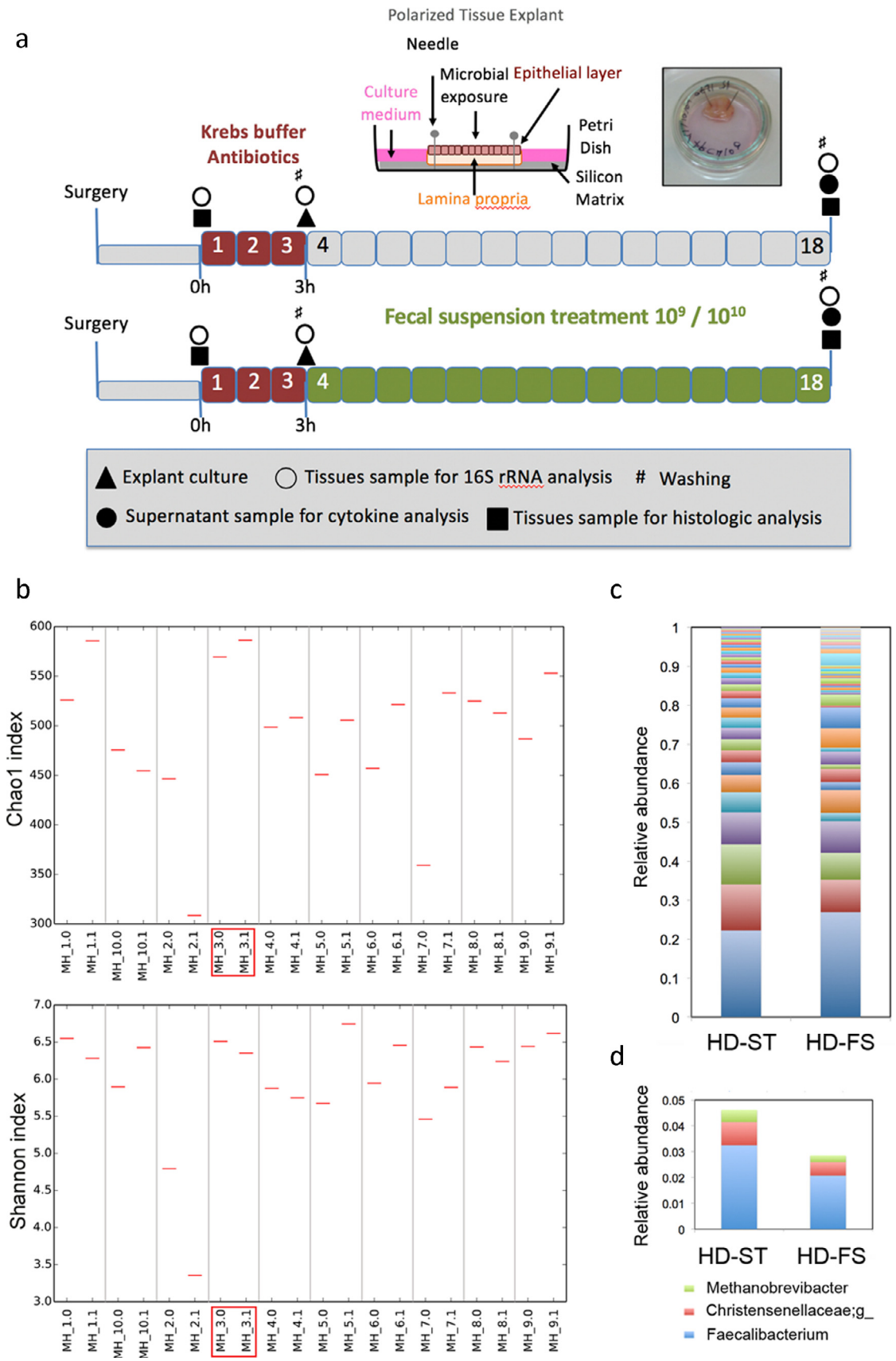

Fig. 1. *Experimental design and screening of donors.* (a) Experimental design of explant culture; (b) faecal samples collected from potential donors at two time points within a 1-month interval ($n = 20$). The subject presenting the highest alpha-diversity indexes (Chao1 and Shannon) for the two time points was chosen as faecal donor. (c) Microbial profile at the genus level of the healthy donor stool (HD-ST) and the faecal suspension (HD-FS) obtained from this stool. Information regarding the name of the genera identified and their relative abundance can be found in Supplementary Table S4. (d) Detection of three of the genera required to correct dysbiosis in CD in both the HD-ST and HD-FS.

To identify the optimal microbial cell number to be in contact with recipient mucosa, we tested two microbial loads for the HD-FS (10^9 and 10^{10} microbial cells) (see the Methods section). All the tissues treated with the HD-FS showed a significant increase in microbial load compared to untreated ones. However, this increase was not significantly different between the two loads tested (10^9 vs. 10^{10} cells) (Fig. 2(a)). Whole microbial quantification by qPCR after a PMA treatment showed that the number of bacteria did not differ between samples treated or not with PMA. This finding therefore demonstrates the viability of the bacteria that colonized the tissue (Supplementary Fig. S3(f)). Moreover, we also showed that the 18-h experiment did not affect the survival of *F. prausnitzii,* a strict anaerobic microorganism (Supplementary Fig. S3(f)).

Alpha-diversity analysis indicated, as expected, that the microbial community of explants treated with 10^9 or 10^{10} microbial cells of FS presented a higher diversity than untreated tissues (Fig. 2(b)). Moreover, an UPGMA clustering method on 16S sequences (Fig. 2(c)) revealed that the microbiome of treated tissues clustered with that of the HD-FS for most samples. This observation indicates a shift towards the microbial community of the donor and strongly suggests that microorganisms from the FS colonized the mucosal explants in our model. The finding that some samples did not cluster with the HD-FS after exposure but rather with the samples before exposure indicates that factors intrinsic to the tissue decreased the efficacy of the colonization.

Haematoxylin and eosin staining revealed that explants treated with 10^{10} cells had significantly higher histological damage scores (Fig. 2(d)) than those treated with 10^9 cells after 15 h of culture, independently of their pathological origin (CM or CD tissues) (Supplementary Table S4). Consistent with the histological score, a dose of 10^9 cells did not modify overall LDH release, another marker of tissue damage, compared to untreated conditions (Fig. 2(e)), whereas exposure to 10^{10} cells significantly increased tissue lysis (Fig. 2(f); Supplementary Fig. S4). In addition, analysis of the presence of bacteria in the explant supernatant demonstrated that the concentration of 10^{10} cells favoured the overgrowth of bacteria from the FS in the culture medium (Supplementary Fig. S5). All together, these results pointed to 10^9 cells as the optimal concentration suitable for the rest of the study.

3.2. Cytokine response of the tissues to the faecal suspension

We next addressed the tissue response to a FS dose of 10^9 cells. To this end, we examined cytokine release of gut mucosal explants into the supernatant after 15 h of culture. Four cytokines were measured, three of which are considered pro-inflammatory (TNF-α, IL-17AF and IL-12) and one anti-inflammatory (IL-10). All the cytokines, except IL-12 (data not shown), were spontaneously released by the three types of tissues (CM, Ni-CD and I-CD) in our culture conditions (Fig. 2(g)). After incubation with the HD-FS, TNF-α and IL-10 release increased significantly in the I-CD tissues, and the release of IL-17AF increased significantly in CM explants. In contrast, none of these cytokines increased in Ni-CD tissues compared with untreated ones.

3.3. Microbiome alteration and cytokine response in inflamed and non-inflamed tissues

We then addressed whether the inflammatory status of the mucosa could affect the tissue response in terms of colonization and cytokine release. We compared the microbial colonization and responses in CM, Ni-CD and I-CD tissues incubated with 10^9 cells. No significant differences in the microbial count of the three groups of tissues were observed, thereby implying that the inflammation status of the tissue is not a critical factor for microbial implantation (Table 1). However, our results revealed that FS-treated I-CD tissues produced significantly more cytokines and were more sensitive to tissue damage and lysis than the other groups (Table 1). This finding could be attributed to the higher degree of epithelium damage and to the greater immune cell infiltration observed in the I-CD group (Fig. 2(f)). Interestingly, a heterogeneous cytokine response to the FS was also observed in the three groups of tissues (Fig. 2(g) and Table 1), independently of the inflammation status. We therefore hypothesized that the initial microbial load or composition of the tissues determines the outcome of microbial colonization and thus cytokine response.

3.4. Microbial copy number in mucosal tissues

We first tested the hypothesis that recipient mucosal microbial load affects colonization efficiency. We compared the microbial load across all the tissue samples available, at baseline and after 18 h of culture without HD-FS. To this end, we quantified the 16S rRNA gene using real-time PCR, providing an approximation of the absolute abundance of microbial cells. No significant differences were observed between the two time points (Fig. 3(a)). However, with regard to the 18 h samples, we detected a bimodal distribution using a logarithm transformation of the abundance data, which provided a local minimum (1.47×10^9) that separated two distinct populations (Fig. 3(b)). The cut-off based on a bimodal distribution observation has been previously used to stratify patients with metabolic syndrome. In those studies, the authors used gene count instead of cell count for stratification purposes [26–28]. We named those samples that carried a 16S copy number lower than the minimum "low copy number" (^{Low}CN) and the others "high copy number" (^{High}CN). The hypothesis of variability in the microbial load was then further tested using the unweighted and weighted UniFrac-based UPGMA clustering method for all tissue sequences before and after exposure to the FS. Interestingly, based only on the microbiome composition and not abundance (unweighted UniFrac), samples that did not cluster close to the FS after exposure were those originally carrying a ^{High}CN of the 16S gene (Fig. 3(c)). Comparison of UniFrac distances revealed that the distances between donor and ^{Low}CN samples were significantly lower than between donor and ^{High}CN samples ($P = 0.018$, Mann–Whitney test, Supplementary Fig. S6).

In contrast to the ^{High}CN explants, all the ^{Low}CN samples showed a significant increase in the copy number of the 16S gene after 15 h of culture with the FS (Fig. 3(d)). Furthermore, there was a significant negative correlation between the copy number before (Spearman test, $r = -0.84$; $P < 0.0001$) or without (Spearman test, $r = -0.81$; $P < 0.0001$) exposure to the FS and the fold change of copy number after contact with the FS (Fig. 3(e)). Moreover, upon FS exposure, ^{Low}CN tissues harboured higher alpha-diversity (Chao1 index) microbiota than ^{High}CN samples (Fig. 3(f)). To evaluate the efficacy of engraftment and based on 16S rRNA sequence data, we used an in-house *R* script to recover the number of shared OTUs between the FS and each tissue sample (see Supplementary methods). Tissue samples with a ^{Low}CN microbial load showed greater numbers of shared OTUs with the FS compared to ^{High}CN microbial load (unpaired *t*-test, $P = 0.003$). This observation suggests that recipient ^{Low}CN tissues captured donor microorganisms more efficiently than ^{High}CN tissues (Fig. 3(g)). To visualize our findings, we generated networks of shared OTUs using the Cytoscape tool [29]. This approach showed shared OTUs between recipient and donor before and after contact with the FS (Fig. 3(h)). These findings suggest that tissue initially harbouring a ^{Low}CN of the 16S gene was more susceptible to colonization by donor microbiota. We also reproduced our results in an anaerobic atmosphere, thereby demonstrating that culture conditions did not influence the colonization results (Supplementary Fig. S7). Finally, to validate our findings, we studied the microbial load of a clinical set of resected tissues from 26 CD patients (52 inflamed and non-inflamed biopsies) who were undergoing ileocaecal resection and were enrolled at the IBD clinic of the University Hospital of Leuven in Belgium. The 16S copy number in the biopsies of the inflamed and non-inflamed regions presented comparable values to those of the explant

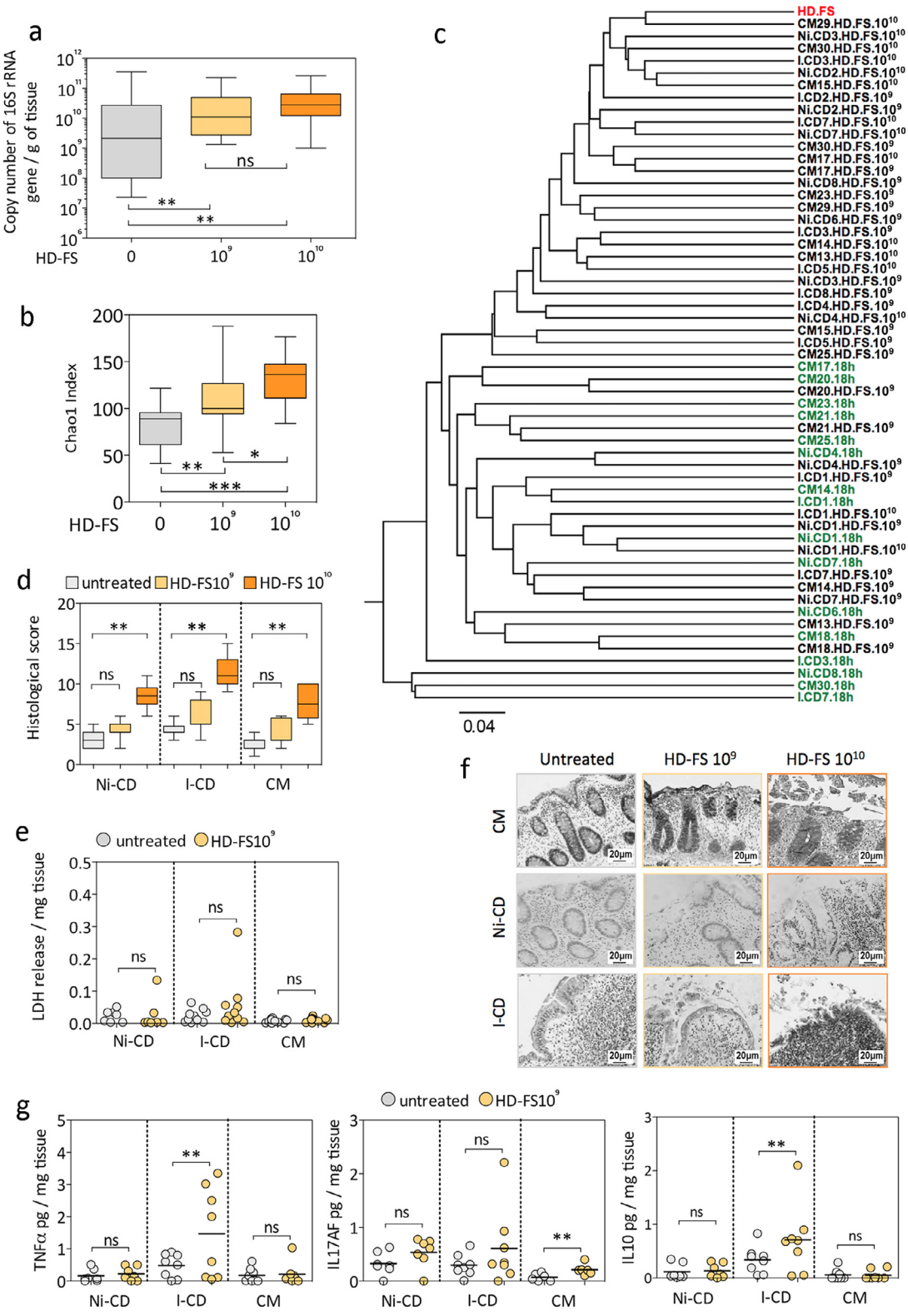

Fig. 2. *Tissue explants of gut mucosa exposed to a faecal suspension.* The tissues from patients with CD ($n = 12$) and from controls (CM; $n = 12$) were cultured for 15 h with two concentrations of a faecal suspension (HD-FS; 10^9, 10^{10}) obtained from a healthy donor. (a) Genomic DNA from the explants was extracted and the copy number of the 16S rRNA was evaluated by real-time PCR (Mann–Whitney test). (b) Microbial richness based on the Chao1 index of the 16S rRNA sequences. (c) Clustering of the mucosal microbiome using an unweighted-UniFrac UPGMA method. HD-FS: faecal suspension from a healthy donor; CM: control mucosa; Ni.CD: non-inflamed part of the mucosal microbiome of patients with CD; I.CD: inflamed part of the mucosal microbiome of patients with CD; 18 h = without exposure to the faecal suspension; HD-FS.10^9 and HS-FS.10^{10}: exposure to the faecal suspension with 10^9 or 10^{10} microbial cells. (d) Histological score histogram of the mucosal explants (Ni-CD, $n = 7$; I-CD, $n = 8$ and CM, $n = 8$) treated with increasing concentrations of HD-FS (10^9, 10^{10}). The Wilcoxon test was applied. (e) Level of LDH released into the explant medium after 15 h of culture with or without HD-FS (10^9 and 10^{10}) (Ni-CD, $n = 7$; I-CD, $n = 8$ and CM, $n = 12$). LDH release measured by optical density at 540 nm. The Wilcoxon test was applied. (f) Morphological assessment (HE staining) of the mucosa explants (Ni-CD, I-CD and CM) after 15 h of culture with or without the HD-FS (10^9 and 10^{10}). Original magnification 200×. (g) Secretion of TNF-α, mature IL-17AF and IL-10 was measured by ELISA in the culture supernatants. Horizontal lines represent mean values. The Wilcoxon test was applied. ns: not significant; $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

Table 1
Microbiome alteration and cytokine response in inflamed and non-inflamed tissues.

	NI.CD HD-FS mean (SD)	I.CD HD-FS mean (SD)	CM HD-FS mean (SD)	NI.CD HD-FS *vs* I.CD HD-FS	NI.CD HD-FS *vs* CM HD-FS	I.CD HD-FS *vs* CM HD-FS
N	*10*	*11*	*12*			
16srRNA gene copy/g tissue	4.5e + 010 (6.4e + 010)	2.9e + 010 (4.8e + 010)	4.4e + 010 (7.3e+010)	*P = 0.53* ns	*P = 0.89* ns	*P = 0.41* ns
α diversity (Chao.1)	55.98 (19.23)	51.17 (24.34)	36.52 (12.91)	*P = 1 ns*	*P = 0.18* ns	*P = 0.61* ns
LDH OD/mg tissue	0.026 (0.048)	0.05 (0.08)	0.01 (0.006)	*P = 0.1* ns	*P = 0.57* ns	*P = 0.04**
Histologic score	6.2 (1.78)	6.14 (2.26)	4.5 (0.84)	*P = 1 ns*	*P = 0 0.16 ns*	*P = 0.18* ns
IL-10 (pg)/mg of tissue	0.13 (0.13)	0.71 (0.64)	0.05 (0.09)	*P = 0.02* *	*P = 0.08 ns*	*P = 0.003* **
TNF-α (pg)/mg of tissue	0.22 (0.21)	1.46 (1.4)	0.20 (0.36)	*P = 0 0.1* ns	*P = 0.51* ns	*P = 0 0.04**
IL-17AF (pg)/mg of tissue	0.53 (0.26)	0.60 (0.70)	0.2 (0.08)	*P = 0.56* ns	*P = 0.03**	*P = 0 0.22 ns*

tissues of the experimental model (Supplementary Fig. S8), which also showed two populations with ^{High}CN and ^{Low}CN.

To understand the potential spatial implication of the microbiota in the ^{High}CN and ^{Low}CN distribution, we examined the mucosal tissues by scanning electron microscopy (SEM). Indeed, ^{Low}CN tissues presented a lower microbial density than ^{High}CN ones (Fig. 3(i)).

3.5. Taxonomic profiling and cytokine response in ^{high}CN and ^{low}CN samples

At the taxonomic level, ^{High}CN and ^{Low}CN CM tissues with or without contact with the FS did not present significant differences. However, CD samples with a ^{High}CN and ^{Low}CN showed significant

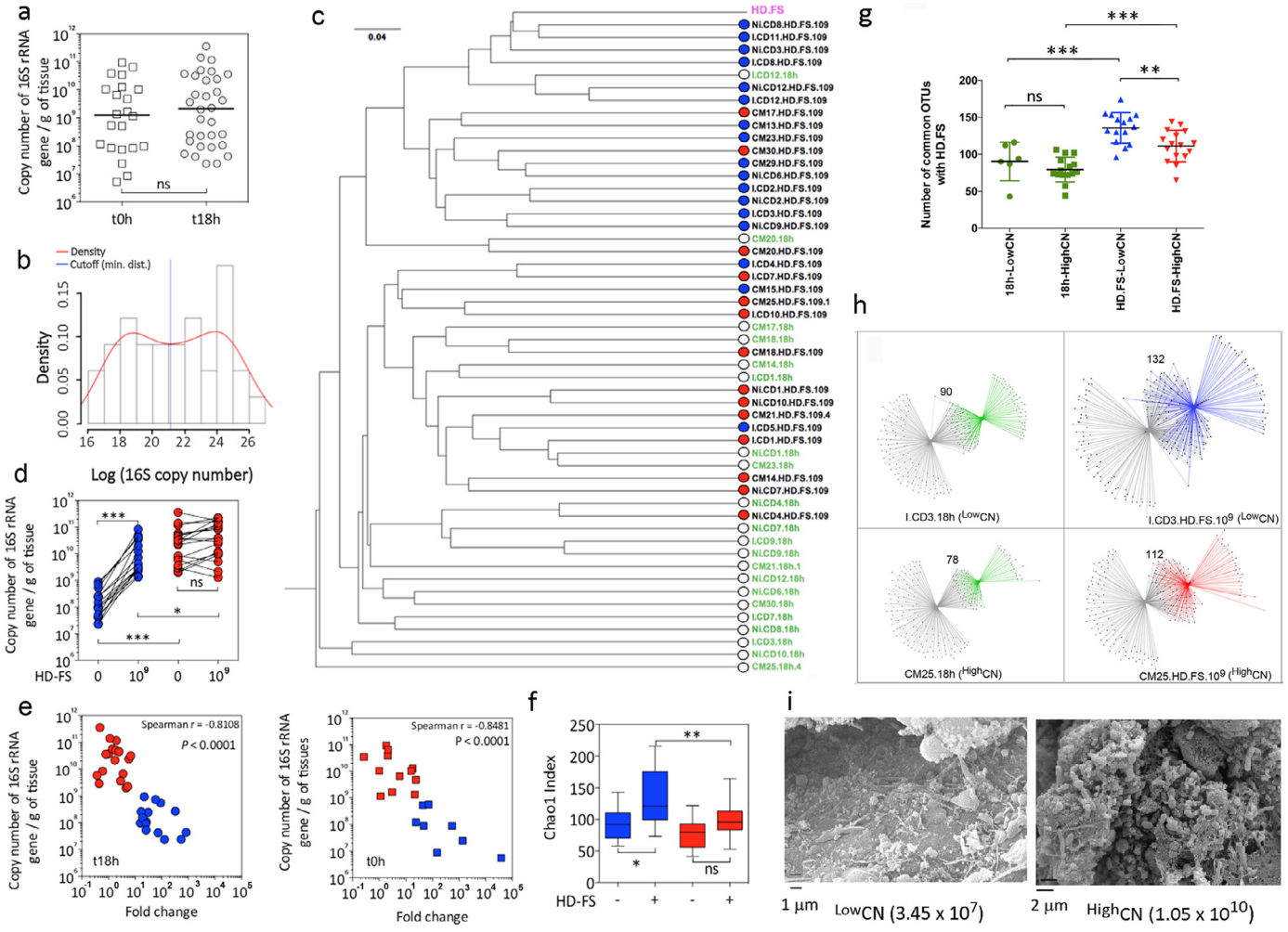

Fig. 3. *Effect of microbial load on donor microbiome colonization.* (a) Quantification of microbes using qPCR of the 16S rRNA gene on all available samples ($n = 54$) at baseline ($n = 22$) and after 18 h ($n = 32$) of antibiotic treatment and culture. (b) Two groups were identified on the basis of the copy number of the 16S gene obtained by qPCR ($n = 32$). "low copy number" (^{Low}CN) refers to $< 1.47 \times 10^9$ and "high copy number" (^{High}CN) $>= 1.47 \times 10^9$. (Mann–Whitney test). (c) Clustering of samples ($n = 53$) using the 16S sequences and an unweighted-UniFrac-based UPGMA method. (d) Copy number evaluated by qPCR in ^{Low}CN ($n = 16$) and ^{High}CN ($n = 16$) mucosal explants cultured for 18 h with or without the HD-FS (10^9). (Mann–Whitney and Wilcoxon tests). (e) Spearman correlation between the 16S copy number and the fold change of copy number in explant tissues before (t0h; $n = 22$) and after (t18h; $n = 32$) culture with the HD-FS (10^9). A smaller basal 16S copy number indicates greater fold change in bacterial copy number. (f) Microbial richness based on the Chao1 index of the 16S sequences. (g) Number of shared OTUs between recipient tissue and donor sample are plotted on the basis of high and low CN and with or without contact with the faecal suspension. (Mann Whitney and unpaired *t* tests). (h) Network plots of shared OTUs are represented as a bipartite graph in which nodes are either OTUs (small) or samples (large), and connecting lines between small and large nodes indicate that the OTU was found in the given sample. Line colour and large nodes indicate the donor sample (grey), the sample before treatment (green), and the sample after contact with the faecal suspension (blue for ^{Low}CN and red for ^{High}CN). The number of shared OTUs between samples is indicated. i, SEM analysis of mucosal samples. ns not significant; $^{**}P < 0.01$; $^{***}P < 0.001$.

differences at the phylum level and after contact with the FS (Supplementary Fig. S9). CD samples with a ^{Low}CN presented a higher relative abundance of Firmicutes ($P = 0.004$; FDR = 0.06) and showed a trend towards a lower relative abundance of Bacteroidetes compared to ^{High}CN samples ($P = 0.03$; FDR = 0.14). After exposure to the FS, CD tissues with a ^{Low}CN showed an increase in Euryarchaeota compared to those with a ^{High}CN ($P = 0.01$; FDR = 0.08). We previously demonstrated that, compared to healthy subjects, CD patients are characterized by a loss of *Methanobrevibacter* [2], a genus belonging to Euryarchaeota, in stool.

Patients with CD have been associated with a loss of *F. prausnitzii* in stool and also in mucosal samples, compared to healthy subjects [30]. Moreover, *F. prausnitzii* has been demonstrated to favour an anti-inflammatory response in CD [31]. qPCR quantification of this species in mucosal tissues revealed that it was significantly less abundant in CD compared to control mucosa (Fisher exact test; Fig. 4(a)). Upon treatment with HD-FS, an increase in the relative copy number of *F. prausnitzii* (Wilcoxon, $P < 0.01$, Fig. 4(b)) was detected; however, this increase was significant only for ^{Low}CN explant tissues (Wilcoxon, $P < 0.01$, Fig. 4(c)).

We also took into account the microbial load status (^{Low}CN and ^{High}CN) of the samples for the study of tissue responses. Analysis of tissue damage and LDH release did not reveal significant differences between these two groups (Supplementary Fig. S10). Regarding cytokine release, ^{Low}CN samples treated with the FS released more IL-10 than untreated explants (Fig. 4(d)), whereas no significant differences were found for IL-17AF. However, ^{High}CN samples were more associated with a pro-inflammatory cytokine ratio (IL-17AF/IL-10) compared to ^{Low}CN samples ($P = 0.045$; Mann Whitney *t*-test). Furthermore, higher bacterial diversity in ^{Low}CN tissues cultured with HD-FS was positively correlated with the release of IL-10 cytokine. In order to assess the implication of particular bacteria, we performed a Spearman correlation test between the microbiome composition of the tissues after contact with the FS and the levels of pro- (IL-17AF and TNF-α) and anti- (IL-10) inflammatory cytokines. *Bacteroides* (rho = 0.69) and *Parabacteroides* (rho = 0.63) were positively correlated with levels of IL-17AF. *Enterococcus faecalis* was positively correlated with the pro-inflammatory ratio of IL-17AF/IL-10 (rho = 0.86).

3.6. Evaluation of faecal suspension from IBD donors

We examined whether the increase in bacterial load was dependent on the donor samples. To this end, we tested additional FSs derived from a CD patient and an ulcerative colitis patient, selected on the basis of their very low microbial diversity compared to the healthy individual used as faecal donor in this study (Supplementary Fig. S11(a)). We observed that bacterial load increased independently of the donor origin (healthy or IBD) but was dependant on the bacterial load of the recipient tissue (Supplementary Fig. S11(b) and (c)). Interestingly, the IBD FSs caused a significantly greater release of LDH, a higher IL-17AF/IL-10 pro-inflammatory ratio, and less capture of OTUs from the donor samples compared to the healthy donor microbiota (Supplementary Fig. S11(d),(e) and (f). This result suggests that donor microbiota affects tissue response. However, this hypothesis requires further testing with more FSs from IBD patients.

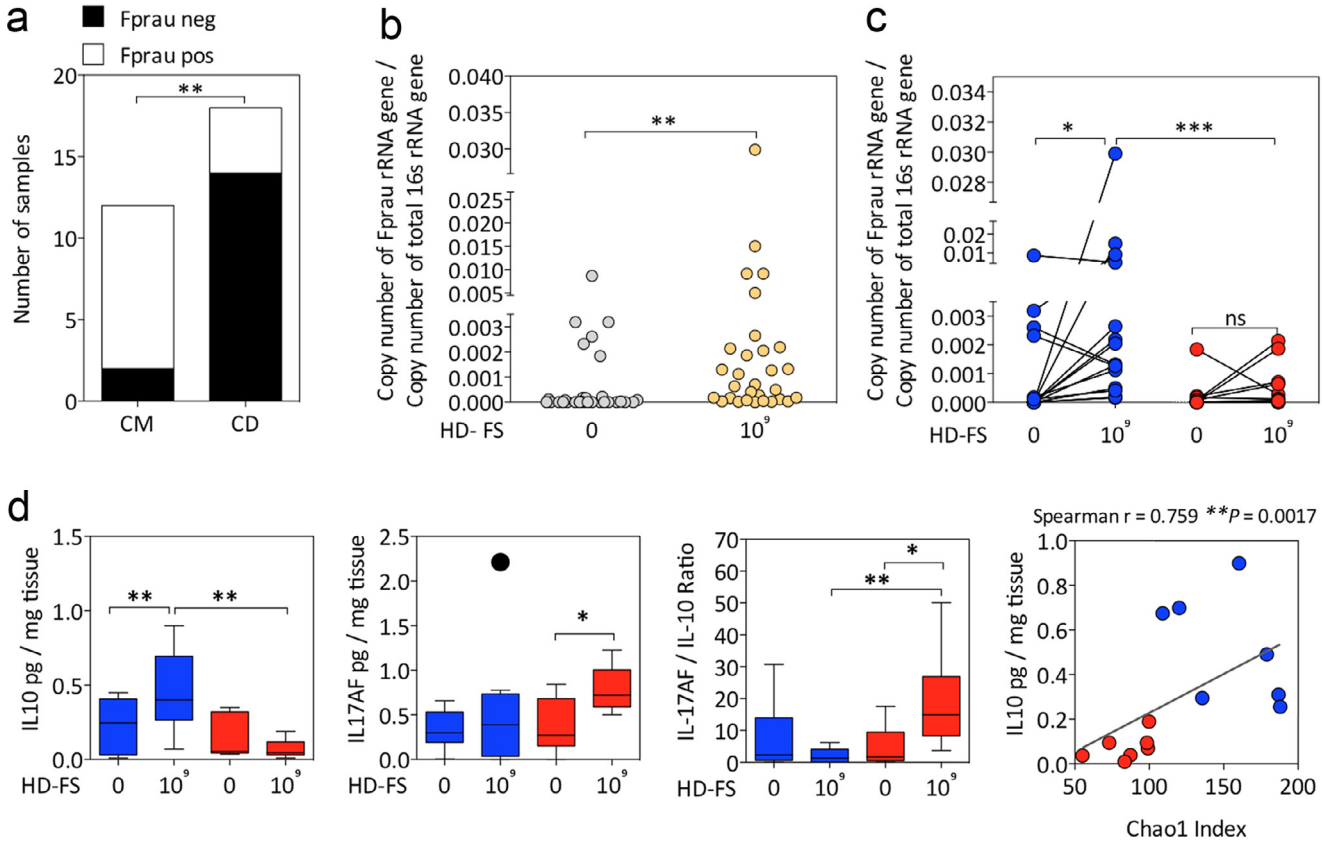

Fig. 4. *Faecalibacterium prausnitzii in tissue explants and cytokine secretion in high and low microbial load samples.* (a) Presence/absence of *F. prausnitzii* as evaluated by qPCR of the V4 region of the 16S rRNA gene in control (CM) ($n = 11$) and Crohn's disease (CD) ($n = 11$) mucosa. (b) Relative load of *F. prausnitzii* at 18 h with and without contact with the HD-FS ($n = 32$; CM and CD). (c) Relative load of *F. prausnitzii* in ^{Low}CN (blue dots) ($n = 7$; CD) and ^{High}CN (red dots) ($n = 7$; CD) mucosal samples with and without contact with the HD-FS. (d) Secretion of IL-10 and IL-17AF was measured by ELISA in the supernatants of Low ($n = 7$; CD) and High ($n = 7$; CD) Copy Number (^{Low}CN and ^{High}CN) mucosal explants cultured for 15 h with or without the HD-FS (10^9). The pro-inflammatory ratio IL-17AF/IL-10 was measured in ^{Low}CN and ^{High}CN mucosal explants treated or not with the HD-FS (10^9). Positive Spearman correlation between IL-10 release and alpha-diversity (Chao1 index). Mann-Whitney and Wilcoxon tests were applied, $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

3.7. In vivo *validation in a mouse model*

To validate our *in vitro* findings, we designed a study using a mouse model, which was approved by the Animal Research Committee of the Vall d'Hebron Institut de Recerca (Barcelona, Spain). We used antibiotics (imipenem and vancomycin) to decrease the microbial load of the intestinal tissues of the mice before FMT (Fig. 5(a)). We used omeprazole, a proton pump inhibitor, to suppress stomach acid secretion and therefore to increase bacterial survival. We also used CitraFleet® (sodium picosulfate), a stimulant laxative, to remove recipient luminal content before FMT [32]. Finally, mice were administered via oral gavage the same FS used in the *in vitro* experiment. Four groups of mice were distributed as follows: Ctrl: treated with omeprazole and CitraFleet®; ATB: treated with antibiotics, omeprazole and CitraFleet®; FMT: treated with omeprazole, CitraFleet® and FMT; ATB-FMT: treated with antibiotics, omeprazole, CitraFleet® and FMT. To validate the effect of antibiotics in reducing bacterial load, we examined bacterial load in the stool of mice that were treated or not with an antibiotic cocktail prior to FMT. The stools of those animals showed a lower bacterial load than those of untreated mice, thereby clearly demonstrating the effectiveness of our treatment (Fig. 5(b)). Our findings also showed that the antibiotic cocktail significantly decreased the microbial load of colonic mucosal tissues compared to controls (Ctrl) (Fig. 5(b)). Then, after FMT, only tissues with

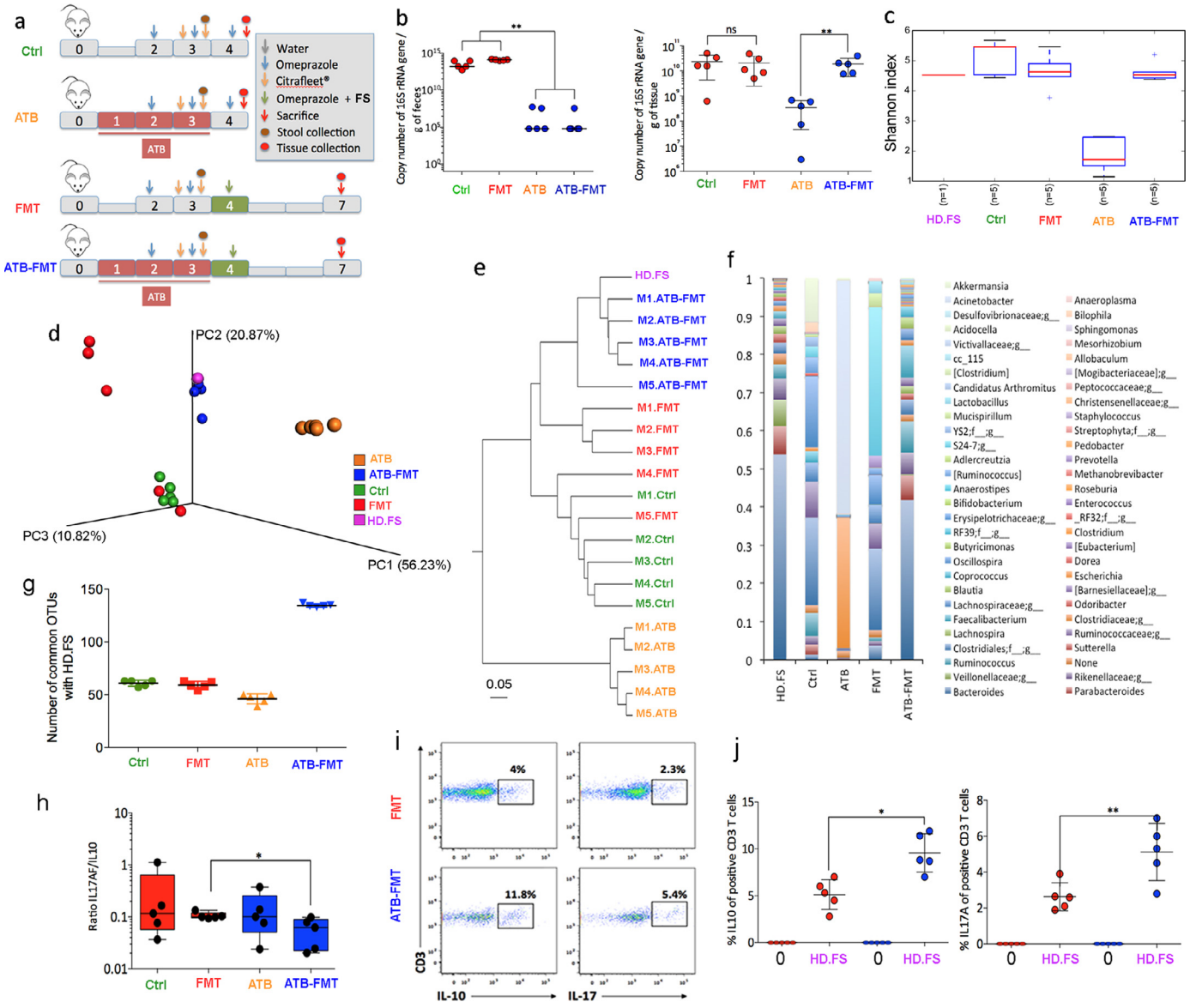

Fig. 5. *Validation of the microbial load hypothesis using a mouse model.* (a) Experimental design; Control group (Ctrl): treated with omeprazole and CitraFleet®; Antibiotics group (ATB): treated with antibiotics, omeprazole and CitraFleet®; faecal microbiota transplantation group (FMT): treated with omeprazole, CitraFleet® and FMT; ATB-FMT group: with antibiotics, omeprazole, CitraFleet® and FMT. (b) Genomic DNA from stool samples (left) at day 3 and from colon samples (right) at days 4 or 7, of the different mouse groups (Ctrl ($n = 5$); FMT ($n = 5$); ATB ($n = 5$); ATB-FMT ($n = 5$)) was extracted and the copy number of the 16S rRNA was evaluated by real-time PCR and normalized by tissue weight. Alpha-diversity (c), and Beta-diversity (d), weighted UniFrac PcoA and (e), weighted UniFrac UPGMA) was assessed on the 16S sequence data of all mice. (f) Taxonomic profiles of the healthy faecal suspension (HD-FS) and all mouse groups were plotted using the mean relative genera abundances. (g) Number of shared OTUs between donor sample and recipient tissues of all mouse groups. (h) Total proteins from colon samples from the different groups of mice (Ctrl ($n = 5$); FMT ($n = 5$); ATB ($n = 5$); ATB-FMT ($n = 5$) were extracted, and IL-10 and IL-17AF concentrations were evaluated by ELISA, normalized by total protein concentration, and the IL-17AF/IL-10 pro-inflammatory ratio was calculated. Representative dot plot (i) and histogram analysis (j) of IL-10 and IL-17A responses of CD3-positive colonic cells from FMT- (red dots) or ATB + FMT- (blue dots) treated mice after 6 h of stimulation by monocytes loaded overnight or not with sonicated FS (1:5). Ratio of IL-17AF and IL-10 FS reactive lymphocytes from colon samples of mice treated with FMT and ATB-FMT. ns: not significant; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

an initial low microbial load after antibiotic treatment showed a significant increase in the number of mucosal bacteria (Fig. 5(b)).

We also assessed the engraftment of the donor microbiota in the recipient colonic tissues by performing 16S rRNA sequencing on genomic DNA extracted from the colonic mucosal tissues of all the mice used in the study ($n = 20$). Alpha-diversity analysis showed that, after antibiotic treatment, recipient tissues gained the same level of diversity as the donor sample (Fig. 5(c)), thereby suggesting that the antibiotic treatment promoted colonization by the donor microbiota. Beta-diversity assessment revealed that only the group of mice that received antibiotic treatment prior to FMT (*i.e.* presenting ^{Low}CN colonic mucosal tissues) clustered with the donor sample (Fig. 5(d) and (e)) and better captured its microbiota (Fig. 5(f) and (g)). These results confirmed that only ^{Low}CN tissues were more predisposed to capture donor microbiota. Furthermore, using the Wilcoxon rank sum test, we showed that antibiotic treatment followed by FMT allowed the capture of several bacterial genera that are not usually encountered in mice, such as *Faecalibacterium*, a well-known anti-inflammatory genus in humans (Supplementary Table S5). Evaluating the expression of mucosal cytokines, we observed a significantly lower IL-17AF/IL-10 ratio in ^{Low}CN animals (antibiotic treated group before FMT; ATB-FMT) compared to ^{High}CN mice (FMT group) (Fig. 5(h)). As shown in Fig. 5(i)) and ((j), colonic T cells produced IL-10 and IL-17A after stimulation by monocytes loaded with the sonicated faecal suspension. Colonic T cells isolated from ATB-FMT animals produced more IL-10 and IL-17A as compared with animals that received FMT alone. These results indicate that our *in vivo* murine model validated the findings in the human explant tissue model at both the microbiome and immune response levels and confirmed that colonization of donor microbiota was facilitated in tissues with a ^{Low}CN—a condition that favoured anti-inflammatory cytokine release (Fig. 6).

4. Discussion

Here we report on the use of human explant tissues and a murine model to study the crosstalk between the enteric immune system and microbiota. Our novel findings show that colonization of donor faecal microbiota was more successful in tissues that had low microbial loads than in those with high microbial loads.

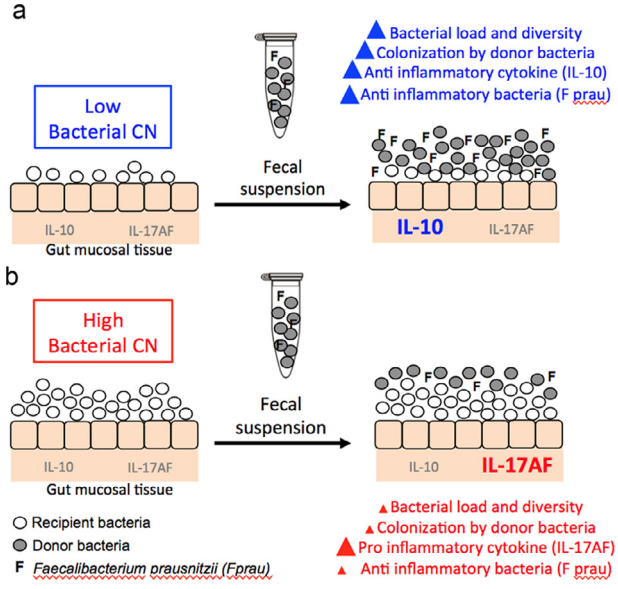

Fig. 6. *Proposed model of gut mucosa stratification to improve microbial colonization and anti-inflammatory response.* (a) After treatment with a faecal suspension (FS), gut mucosa harbouring a low microbial load (^{Low}CN) were colonised by the microbiota from the donor FS, notably by the anti-inflammatory *F. prausnitzii* (F prau), and showed an increase in the release of the anti-inflammatory cytokine IL-10. (b) Gut mucosa harbouring a high microbial load (^{High}CN) were not fully colonized by donor microbiota, in particular *F. prausnitzii*, and showed an increase in the release of the pro-inflammatory cytokine IL-17AF after treatment with the FS.

FMT has been proposed as a potential therapy for patients with *Clostridium difficile* infection when other treatments have failed. However, no such protocol has been proposed for CD, and the results from previous studies have shown mixed results [33,34]. Although many questions remain unanswered regarding the amount of stool to be delivered, the pre-treatment of patients, and patient and donor selection, there is growing interest in using FMT to attenuate inflammatory responses in CD patients, thereby restoring a healthy gut microbiota [35]. Here we used gut mucosal explant cultures to study the responses of the mucosa of CD patients during FMT treatment. Our protocol was designed with the same rigour as that applied for a FMT procedure in human subjects.

FMT failure in CD occurs during the first months after treatment and is associated with non-colonization by the donor stool. Various factors could explain this non-colonization, including donor stool diversity, resident microbiota composition, and host gut mucosal immune response to the donor microbiota. In the light of our results, we propose that the microbial composition of the donor stool as well as a high microbial load of the recipient mucosa, prevent the establishment of a new microbial community.

The choice of donor stool is one of the main concerns for the FMT procedure [36]. In our study, the donor stool was selected on the basis of microbial richness, stability, and composition. Indeed, other authors have shown that the "richer" the donor microbiome, the better the remission score in CD [16]. Also, healthy subjects are associated with a stable microbial community compared to patients with IBS or CD [2,37]. Regarding the composition of the stool, in addition to testing for the presence of common pathogens using traditional screening methods, we also used 16S sequence analysis. Our results show that the method used to prepare the FS was efficient in recovering most of the microbes present in the stool, in particular the genera missing in patients with CD, as previously described by Pascal et al. [2]. However, more comprehensive future studies should be provided for the choice of the donor.

This explant tissue model may be the only paradigm that allows evaluation of an early (18 h) immune response of healthy and inflamed tissues. Our results showed that contact of the inflamed (and not the non-inflamed) regions with the FS triggers a pro-inflammatory TNF-α response. This observation suggests that the use of FMT for patients with active CD may compromise the outcome of this treatment.

One of the limitations of our human explant study is the use of tissues from CRC patients as healthy controls. Indeed, the tissues of these subjects may harbour a significantly different microbiome profile to that of younger adults, as a result of the condition and the greater age of these patients compared to those with CD. However, it was difficult to overcome this limitation as we did not have access to control tissues from younger healthy adults undergoing surgery. Furthermore, we did not observe any association between the copy number status of these tissues and age, gender, site of disease, or medical treatment followed by the patients (Supplementary Fig. S12). Another limitation of our approach is that we performed short cultures of 15 h to avoid excessive destruction of the tissue and also to prevent excessive bacterial growth, notably of aerobic bacteria in the FS. In a similar study using a mouse model, the authors devised an organ culture system that involved a luminal flow to preserve the viability of the tissues and to control perturbations [38]. Our system was limited in time as tissue damage became obvious by 48 h. The short protocol that we developed here is therefore not fully suitable for studying the repopulation of colonic mucosa by a new microbiota. However, this approach did allow us to study the early phenomena occurring during FMT, such as microbe adhesion and tissue reaction to this process.

To overcome this limitation and validate our results *in vivo,* we developed a mouse model for FMT. The collection of colonic biopsies from living mice before FMT to evaluate the gut mucosal bacterial load is not a standardized technique as it is in humans. Therefore, we used a modified version of a previous experimental design [8], in which we modulated the bacterial load prior to FMT using a strong antibiotic cocktail. To this end, we performed FMT one day after the antibiotic treatment ended and not the same day, thereby allowing the elimination of residual antibiotics before the stool transfer. The efficiency of this protocol has been nicely demonstrated in a recent paper published by Khoruts' group [9]. This model confirmed that, *in vivo*, ^{Low}CN mucosal tissues have a greater capacity to increase the bacterial load and to decrease the IL-17AF/IL-10 pro-inflammatory ratio after FMT as compared with ^{High}CN tissues. We also detected an increase in the frequency of CD3+IL-10+ and CD3+IL-17A+ colonic T cells specific to the FS. This observation would point to the induction of an *in vivo* adaptive immune response by the component of the donor microbiota, as recently described by Burrello et al. [39]. In contrast to the IL-17AF/IL-10 colonic cytokine ratio, the CD3+IL-17A+/CD3+IL-10+ colonic lymphocyte ratio did not differ between animals treated or not with antibiotics before FMT. This finding suggests that colonic lymphocytes may not be the only cells involved in the cytokine response to microorganisms after ATB-FMT treatment. Like T lymphocytes, a number of other cell types, including innate lymphoid cells monocytes, macrophages, mast cells, and intestinal epithelial cells, may produce IL-10 or IL-17AF in response to microorganisms [40]. Our validation using an *in vivo* mouse model confirmed that ^{Low}CN mucosal tissues may have a greater capacity than ^{High}CN tissues to capture the donor microbiota and induce an anti-inflammatory IL-10 response.

Our *in vivo* model could be compared with a clinical trial of FMT in ulcerative colitis, which has demonstrated that pre-treatment with antibiotics promotes recolonization and decreases inflammation in patients [41]. However, *in vivo* validation of our findings in humans is not feasible for the time being due to the lack of appropriately designed clinical trials.

Our study is the first to demonstrate that microbes attached to the mucus layer of the recipient play a key role in the outcome of the engraftment of donor microbiota. First, tissues with an originally high number of microbial cells were less prone to capturing the donor microbiome, specifically *F. prausnitzii,* and were associated with the induction of a pro-inflammatory immune response. Sokol et al. [30] demonstrated the anti-inflammatory properties of *F. prausnitzii* in both *in vitro* (cellular models) and *in vivo* [2,4,6-trinitrobenzenesulphonic acid (TNBS)-induced colitis in mice] models. They observed that *F. prausnitzii* has anti-inflammatory effects on cellular and TNBS colitis models, partly due to secreted metabolites able to block NF-kappaB activation and IL-8 production. In addition, these effects have been reproduced in other colitis models [42]. More recently, *F. prausnitzii* has been identified as a major inducer of human CD4CD8$\alpha\alpha$ T cells, a new IL-10+ Foxp3neg regulatory T cell sub population [43,44]. We also demonstrated that these gut-derived Treg cells are reduced in blood samples of patients with IBD, who are known to harbour a lower *F. prausnitzii* count, compared to controls. Finally, we recently demonstrated that *F. prausnitzii* skews human DC to prime IL-10-producing T cells [45]. Those observations suggest that the mechanistic effect of *F. prausnitzii* in humans is associated with the activation of innate and adaptive IL-10-secreting cells. We also found a positive correlation between the IL-17AF / IL-10 pro-inflammatory cytokine ratio and the colonization of explants by *Enterococcus faecalis*. This pathobiont has been described to promote pro-inflammatory responses and to stimulate the development of colitis in a murine mono-colonization model [46]. Overall, these results indicate that patients with a high number of microbial cells in tissues may be less receptive to the modulation of their mucosal microbial composition and anti-inflammatory immune response.

In conclusion, using our model, we have shown that a donor stool weighing approximately 50 g (10^9 concentration) does not compromise the recipient mucosal barrier integrity and is therefore suitable for the preparation of the FS. Donor samples should contain as low a relative abundance as possible of *Bacteroides*, *Parabacteroides* and *Enterococcus faecalis*, which we have demonstrated to be associated with pro-inflammatory cytokine release. IBD donor samples cause greater mucosal barrier degradation, greater IL-17AF/IL-10 cytokine ratio and less capture of the donor microbiota. Human intestinal tissues are colonized by either a high or low microbial load. Furthermore, tissues with a low microbial load, which tend to contain a higher relative abundance of Firmicutes, were more susceptible to colonization by a healthy donor faecal sample, thereby promoting an anti-inflammatory response. Microbial load could be explained by microbial cell density in the mucosal wall. The use of a cleansing method (using Citrafleet®) as in our mice model) and an appropriate antibiotic cocktail to decrease the mucosal microbial load prior to FMT would favour the capture of donor microbiota and thus emerges as an interesting clinical therapeutic strategy. Our findings also indicated that the use of FMT in patients with active disease may not result in successful outcomes of this treatment. Finally, on the basis of our observations, we recommend that future FMT clinical trials evaluate (1) whether CD patients in remission, with a non-inflamed mucosa and a low mucosal bacterial load, would be more eligible; (2) and if reducing bacterial load through antibiotics is an appropriate strategy in CD patients with high mucosal load.

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Declaration of competing interest

No conflict of interest. The sponsor of this study had no role in its design or in the collection, analysis, and interpretation of data.

Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.ebiom.2019.102611.

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