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Assessing the effects of tofacitinib on the gut microbiome in inflammatory bowel disease

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ABSTRACT

Gut microbiota dysbiosis and impaired epithelial barrier function play a key role in inflammatory bowel disease (IBD). Tofacitinib citrate, a Janus kinase (JAK) inhibitor approved for IBD, modulates immune responses via the JAK-STAT (Janus kinase–signal transducer and activator of transcription) pathway, yet its effects on the gut microbiome remain unclear. Here, we employed the short-term colon model (ProDigest, BE) containing human microbiota from three Crohn's Disease donors to assess fermentative and metabolic activities and microbial composition following 48 h of tofacitinib treatment. A Caco-2/THP1 co-culture system was used to assess the impact of tofacitinib on epithelial immunomodulation and barrier integrity. Tofacitinib did not significantly affect microbiota composition and fermentative or metabolic activity. However, it consistently reduced pro-inflammatory chemokines motif chemokine ligand 10 (CXCL10) and monocyte chemoattractant protein-1 (MCP-1), and interleukin-6 (IL-6) in specific donors, indicating targeted immunomodulatory effects. These findings suggest that while tofacitinib may have a minimal impact on microbiota function, it may exert anti-inflammatory effects via microbiota-derived metabolites. The short-term colon model represents a robust platform for investigating microbiome-drug interactions relevant to IBD.

1. Introduction

The gut microbiome, with its 10^{10}–10^{11} bacteria per gram of intestinal content, represents a unique microbial niche that plays a central role in human health (Walker and Hoyles, 2023; Awad et al., 2022). In the gastrointestinal (GI) tract, these microorganisms form spatially organised communities, with those adhering to the gut wall protecting against pathogens, regulating immune responses, and maintaining homeostasis by occupying ecological niches that might otherwise harbour harmful colonisers (McCallum and Tropini, 2023; Donaldson et al., 2016). Interactions between the microbiome and the host significantly influence nutrient absorption, epithelial health, inflammation, energy balance, and even immune function (Nicholson et al., 2012; Asnicar et al., 2021).

Disruption of these host-microbe interactions, commonly termed dysbiosis, is implicated in the pathogenesis of several diseases, including metabolic syndrome (Nicholson et al., 2012), autoimmune diseases (Miyauchi et al., 2023), diabetes (Burcelin et al., 2011), and inflammatory bowel disease (IBD), which encompasses Crohn's disease (CD) and ulcerative colitis (UC) (Khan et al., 2019; Franzosa et al., 2019). IBD is characterised by chronic intestinal inflammation and is associated with reduced microbial diversity: an overgrowth of facultative anaerobes and diminished populations of short-chain fatty acid (SCFA)-producing bacteria like *Faecalibacterium prausnitzii* and *Roseburia hominis* (Morgan et al., 2012; Lloyd-Price et al., 2019). Butyrate, a key SCFA supporting gut epithelial health and immune regulation, is especially reduced in IBD patients (Morgan et al., 2012).

IBD is a chronic, relapsing condition that leads to complications such as progressive bowel damage, hospitalisations, and surgeries, severely impairing patients' quality of life. Early and effective intervention is

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crucial to mitigate these outcomes (McCoubrey et al., 2023; Yeung et al., 2025). Tofacitinib citrate, a Janus kinase (JAK) inhibitor, was the first drug in its class to be approved for the treatment of moderately to severely active UC in 2018 (Palasik and Wang, 2021). By targeting the Janus kinase–signal transducer and activator of transcription (JAK-STAT) signalling pathway, which mediates over 50 cytokines and growth factors, tofacitinib citrate modulates immune responses and inflammation (Palasik and Wang, 2021). However, its effects on the gut microbiota and its metabolites remain largely unexplored. While Texler et al. (Texler et al., 2022) demonstrated its anti-inflammatory effects on monocytes and colonic organoids derived from intestinal epithelial cells (IECs), no studies have evaluated its influence on microbial fermentative processes, metabolite production, or their combined effects on gut wall integrity and inflammatory markers.

To address these gaps, advanced *in vitro* models offer a promising platform for studying microbiota-host interactions and therapeutic interventions (Qi et al., 2023; von Martels et al., 2017; Sangfuang et al., 2025). The short-term colon model (ProDigest, Ghent, Belgium), an adaptation of the Simulator of the Human Microbial Ecosystem (SHIME®), has emerged as a robust tool for such investigations (Van de Wiele et al., 2015). Unlike traditional SHIME® systems, this single bioreactor setup allows frequent sampling from the same reactor providing data on the dynamic evolution of the effects of a single dose of a specific treatment. Moreover, the model integrates a mucus layer through mucus-coated beads consisting of an agar core covered with porcine gastric mucus to cultivate both luminal and mucus-associated microbiota, providing a more realistic simulation of the colonic microbial environment. The short-term colon model was coupled with a co-culture system of Caco-2 epithelial-like cells and THP1-derived immune cells to mimic inflammation-induced barrier dysfunction in a biorelevant context (Satsu et al., 2006). By combining Caco-2 epithelial cells with macrophage-like THP-1 cells, the model recreates the “leaky-gut” phenotype characteristic of IBD, in which cytokines secreted by activated immune cells disrupt epithelial tight junctions.

This study aims to evaluate the impact of tofacitinib on the gut microbiota of three adult IBD donors using the short-term colon model. Specifically, we investigated whether microbial metabolites produced in the presence of tofacitinib positively influence gut epithelial integrity and inflammatory markers. These findings are expected to shed light on the microbiome-mediated effects of tofacitinib and its potential role in the broader context of IBD management.

2. Materials and methods

2.1. Experimental design

The present work was conducted with the advanced model short-term colon bioreactor (ProDigest, Ghent, Belgium). The effects of tofacitinib citrate (Cat no. HY-40354A, MedChemExpress, NJ, USA) were tested in three donors suffering from moderate-severe CD and in triplicate for each donor (Fig. 1). A drug-free negative control was tested for each donor. The final concentration of drug used, 0.017 mg/mL, was calculated based on the maintenance dose of tofacitinib citrate (10 mg) dissolved in 600 mL of colonic fluid, yielding a concentration of 0.0167 mg/mL. This concentration remains well below tofacitinib’s solubility limit of 2.9 mg/mL (XELJANZ /XELJANZ, 2024), ensuring that the drug is fully dissolved and allowing for an accurate assessment of its interaction with the gut microbiota. Changes in pH, gas, SCFAs, ammonium and lactate production at the start of the incubation (0 h) and after 6 h, 24 h and 48 h were analysed for the drug-treated samples and drug-free controls. Samples for 16S rRNA gene sequencing (Section 2.6.1) for both the luminal and mucosal fractions were collected at 48 h.

2.2. Preservation of faecal samples

Stool samples from three Western adult donors (two males and one female) with Crohn’s disease (CD) in remission were collected under ethical approval from the University Hospital Ghent (reference number B670201836585). All procedures were conducted in accordance with relevant guidelines and regulations. Informed consent was obtained from all subjects prior to sample collection. Samples were stored at –80 °C until use. To ensure the anaerobiosis, the faecal slurry was prepared in an anaerobic chamber by homogenising faecal material with an anaerobic buffer (K2HPO4 8.80 g/L; KH2PO4 6.80 g/L; sodium thioglycolate 0.10 g/L; sodium dithionite 0.015 g/L) and a cryoprotectant developed by Hoefman *et al.* (Hoefman et al., 2013). A 7.50 % w/v faecal suspension was made and stored in aliquot at −80 °C before experimentation. The use of cryostocks from the same faecal suspension guaranteed that identical microbial communities were present in each aliquot, providing optimal reproducibility.

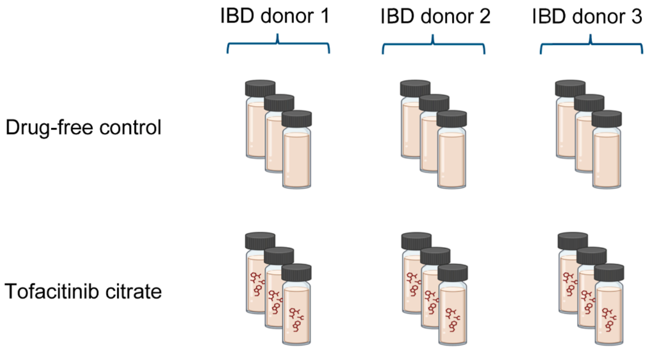

Fig. 1. Experimental design: tofacitinib citrate was tested in the gut microbiota sourced from three IBD donors, and its effects were tested *versus* the same drug-free IBD donors. Each experiment was conducted in triplicates.

2.3. Short-term colonic simulation

The simulation involved incubating a single dose of the drug under conditions that mimic the large intestine, using the bacterial inoculum from the IBD donors. At the start of the experiment, tofacitinib citrate was added to a nutritional medium designed by ProDigest to reflect the colon’s basal nutrients and biorelevant buffering capacity (Ghyselinck et al., 2021). Mucus-coated beads were included to simulate the colon’s mucus layer and to allow the analysis of both the luminal and mucosal microbiome composition at genus level, under both treated and untreated conditions. A faecal inoculum suspension from an IBD donor was added to each reactor ($n = 3$) to a final concentration of 7.5 g/L, serving as the microbial source. A blank was included for each donor, identical to the treated reactors but without the drug. Reactors were incubated for 48 h at 37 °C, with continuous shaking (90 rpm) under anaerobic conditions.

2.4. Metabolite production and fermentative activity

SCFAs and BCFAs quantification was explained by De Boever et al. in their study (De Boever et al., 2000). Briefly, SCFAs, including acetate, propionate, butyrate and the BCFAs isobutyrate, isovalerate and isocaproate, were monitored with gas chromatography (GC) coupled with flame ionisation detection (FID). After the addition of 2-methyl hexanoic acid as an internal standard, a sample (2.0 mL) was extracted with diethyl ether. The extracts were analysed using a GC-2014 gas chromatograph (Shimadzu, ‘s-Hertogenbosch, the Netherlands), equipped with a GC SGE capillary column, 30 *m* × 0.32 mm ID-BP21 × 0.25 μm (Achrom, Machelen, Belgium), a flame ionisation detector and a split injector. The injection volume was 1 μL, and the column temperature profile was set from 110 to 160 °C, rising at 6 °C min^{-1}. The carrier gas was nitrogen, and the temperatures of the injector and detector were both 200 °C. Lactate quantification was performed using a commercially available enzymatic assay kit (R-Biopharm, Darmstadt, Germany) according to the manufacturer's instructions. Ammonium concentrations in the samples were determined using colorimetric analysis with the indophenol blue spectrophotometric methods as previously described (Van de Wiele et al., 2004).

The pH of the incubations was measured as an indirect indicator of bacterial metabolism, reflecting the production of SCFAs, BCFAs, lactate, and NH_4^+. The pH of incubations was assessed with a calibrated pH probe, and gas production was measured in the headspace of vessels using a pressure meter.

2.5. 16S targeted Illumina sequencing for microbial composition

Primers of two hypervariable regions (V3-V4) of the 16S rRNA gene were used. Read assembly and cleanup were derived from the MiSeq platform described by the Schloss lab (Armour et al., 2023). All sequences classified as Eukaryota, Archaea, Chloroplasts and Mitochondria, and those that could not be classified, were removed. Contigs were clustered into OTUs at 97 % sequence similarity, and the most abundant sequence of each OTU was selected as the representative sequence. Reads with a maximum abundance of 5 across samples were removed, as they were supposedly artefacts or bacteria that did not have any biological impact. Samples were analysed with flow cytometry to determine the number of total bacterial cells, thus allowing the conversion of the relative abundances into absolute abundances. Samples were analysed on a BD Accuri C6 Plus Flow Cytometer (BD Biosciences, New Jersey, US).

2.6. *In vitro* Caco-2/THP1-Blue™ co-culture model

The effect of tofacitinib on an inflammatory intestinal epithelium was evaluated with a Caco-2/THP1-Blue™ co-culture model, which has been described in detail by Daguet et al. (Daguet et al., 2016). Briefly, Caco-2-cells were seeded in 24-well semi-permeable plates and cultured at 37 °C for 14 days of growth, until the transepithelial electrical resistance (TEER) was confirmed to be over 300 Ω*cm^2, demonstrating a functional cell monolayer. In tandem, THP1-Blue™ cells were seeded into 24-well inserts and cultured at 37 °C. THP1-Blue™ cells were treated with 100 ng/mL phorbol 12-myristate 13-acetate (PMA) for 48 h to facilitate their proper differentiation into macrophage-like cells. To make the co-culture, the Caco-2-bearing inserts were placed on top of the PMA-differentiated THP1-Blue™ cells for further experiments.

The apical compartment containing Caco-2 cells was filled with sterile-filtered colonic suspensions containing tofacitinib and microbial fermentation products, while the basolateral compartment containing THP1-Blue™ cells was filled with Caco-2 complete medium. Coupling this assay with the colonic suspensions obtained from the short-term colon model allowed for testing the combined effects of tofacitinib and microbial fermentation products on epithelial integrity and immune signalling.

At 24 h, the TEER was measured again, adjusted for the empty insert, and presented as a percentage of the initial value. Sodium butyrate was used as a positive control due to its well-documented gut barrier-protective property. The basolateral supernatant was then discarded, and cells were stimulated with ultrapure lipopolysaccharide (LPS)-containing medium for 6 h. Lipopolysaccharide (LPS) was used to induce inflammation because it elicits a reproducible innate immune activation relevant to IBD pathophysiology.

Basolateral supernatants were collected for cytokine measurement: Interleukin-1 beta (IL-1β), Interleukin-6 (IL-6), Interleukin-8 (IL-8), Interleukin-10 (IL-10), Tumour Necrosis Factor-alpha (TNF-α), C-X-C motif chemokine ligand 10 (CXCL10), Monocyte Chemoattractant Protein-1 (MCP-1) using Luminex® multiplex (ThermoFisher Scientific, MA, USA) and Nuclear Factor kappa-light-chain-enhancer of activated B cells (NF-κB) activity was determined spectrophotometrically as described by Possemiers (Possemiers et al., 2013). The hydrocortisone was selected as a positive control to observe the anti-inflammatory effect within this model. All treatments were conducted in triplicate, and cells were incubated at 37 °C in a humidified air/CO2 (95:5 v/v) atmosphere. Controls consisted of (Walker and Hoyles, 2023) no apical exposure to tofacitinib samples; (Awad et al., 2022) apical exposure to 12 mM sodium butyrate; and (McCallum and Tropini, 2023) addition of hydrocortisone (1 μM) to the LPS medium (data not shown).

2.7. Statistics

To assess whether treatment effects in terms of the fermentation parameters were statistically significant, a paired two-sided *t*-test was used. It evaluated treatment effects across the various donors, using means of technical replicates per donor as input values for the statistical tests (resulting in three biological replicates, *i.e.* one per donor). This considers interindividual differences. Treatment was compared with the control to assess whether it affected metabolite production. The results concerning TEER and immune markers are presented separately for all three individual donors and as the mean of all donors. All colonic incubations were taken as biological replicates ($n = 3$) in the cell assay. To evaluate differences in TEER and immune markers, treatment samples were compared to their non-treated blank controls, and the mean of all donor treatment samples was compared to the mean of the blank controls using multiple paired T-tests.

TreeclimbR analysis was performed to identify the taxa most likely to explain differences between drug-free control and tofacitinib treatment, and the outcome was plotted in a volcano plot (Huang et al., 2021). TreeclimbR was run using treeclimbR v0.1.5 and edgeR v3.42.4. Benjamini-Hochberg multiple testing correction was used, and the significance level was set at 0.05. Statistically significant differences are represented by (*) on the graphs, (*), (**), (***) representing $p < 0.05$, $p < 0.01$, $p < 0.001$, respectively. All statistical analyses were performed using GraphPad Prism (Version 10.2.0; GraphPad Software, MA, USA).

3. Results and discussion

It is recognised that inflammation is associated with a dysbiotic gut microbial community, but it remains unclear whether dysbiosis is the cause or the consequence of inflammation (Dalal and Chang, 2014). Therefore, this work aims to model these two interrelated conditions. On one hand, the use of a gut microbiota sourced from an IBD faecal sample in the short-term colon system allowed for the study of tofacitinib's effects on the microbial community. On the other hand, the Caco-2/THP1-Blue™ co-culture model was employed to mimic the cellular features of IBD and investigate the cellular response to metabolites produced from microbial fermentation (Satsu et al., 2006).

3.1. Tofacitinib's effect on the fermentative activity of the gut microbiota

Monitoring the pH during colonic incubation is a good indicator of SCFA, lactate, and ammonium production. Typically, an initial pH drop occurs due to the formation of SCFAs and lactate (Firrman et al., 2022). This is often followed by a pH increase, resulting from proteolytic fermentation that produces NH_4^+ and the conversion of strong acids into weaker acids through cross-feeding, a process in which these microbes convert acetate and/or lactate (along with other substrates) to the health-related butyrate (Macfarlane et al., 1992; Louis et al., 2010). Fig. 2 shows that excessive pH decreases were not observed and that physiological pH was maintained during incubation. Tofacitinib had little effect on the initial pH and maintained similar pH values over the next 48 h. The error bars on the mean pH reveal higher interindividual variation at 6 h, due to a less pronounced pH decrease in donor 2 compared to the other donors. The mean pH across all treated donors decreased from 6.59 at time 0 h to 6.17 at 6 h, to 6.12 at 24 h, and to 6.17 at 48 h. Overall, the mildly acidic pH caused by tofacitinib treatment in the human colon, despite not being significant, can benefit the

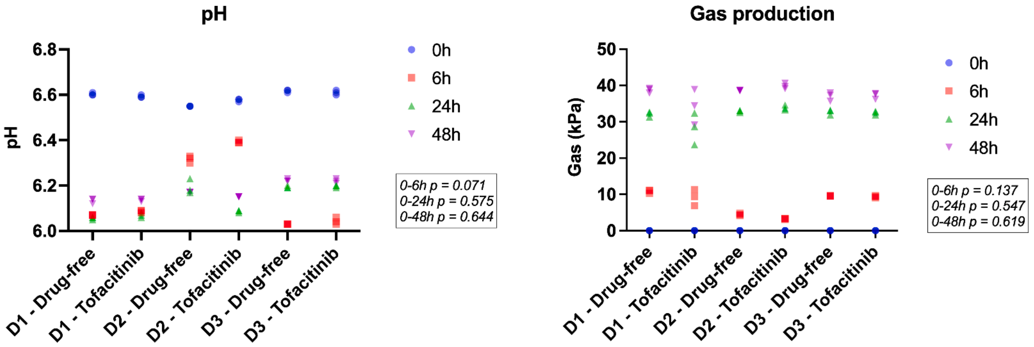

Fig. 2. Changes in pH and gas in kilopascal (kPa) at different timepoints (0 h, 6 h, 24 h and 48 h) of the 48 h incubation for each of the three donors (Donors D1–3). Tofacitinib and negative control (blank) were tested on the faecal microbiome of three adult IBD donors. *P*-values are calculated from the means of the technical replicates of all treated and drug-free donors.

growth of Gram-positive species, which are butyrate-producing bacteria (Duncan et al., 2009).

Gas production is an indicator of microbial activity and thus fermentation rate. Initially, H_2 and CO_2 are produced, which can be subsequently used for CH_4 production, thereby reducing gas volume. H_2 may also be used to reduce sulphate to H_2S, resulting from proteolytic fermentation. N_2, O_2, CO_2, H_2, and CH_4 make up 99 % of intestinal gas volume, with the remaining 1 % comprising NH_3, H_2S, volatile amino acids, and SCFAs. Results on gas production are shown in Fig. 2, which shows lower gas production between 0–6 h in donor 2 as compared to the other donors, together with pH providing evidence for a lower fermentation rate in this donor. Other than that, no significant impact of tofacitinib on gas production was observed ($p > 0.05$ for all donors).

3.2. *Tofacitinib's effect on the metabolic activity of the gut microbiota*

3.2.1. *Saccharolytic markers*

SCFAs are carboxylic acids with aliphatic tails, and the most abundant ones produced by anaerobic fermentation of saccharides in the intestine are acetate, propionate, and butyrate. These acids play a crucial role in maintaining intestinal homeostasis, as they serve as vital fuel for intestinal epithelial cells and are known to strengthen gut barrier function (Parada Venegas et al., 2019). Positive effects, therefore, are associated with increases of acetate, propionate and butyrate. As compared to healthy individuals, the SCFAs bacteria producers are typically reduced in both the mucosa and lumen of IBD patients, with lower abundances of *Bacillota, Roseburia*, and *Faecalibacteria*, and a higher prevalence of *Ruminococcus gnavus, Deltaproteobacteria*, and *Gammaproteobacteria* (Morgan et al., 2012; Knox et al., 2019). The loss of

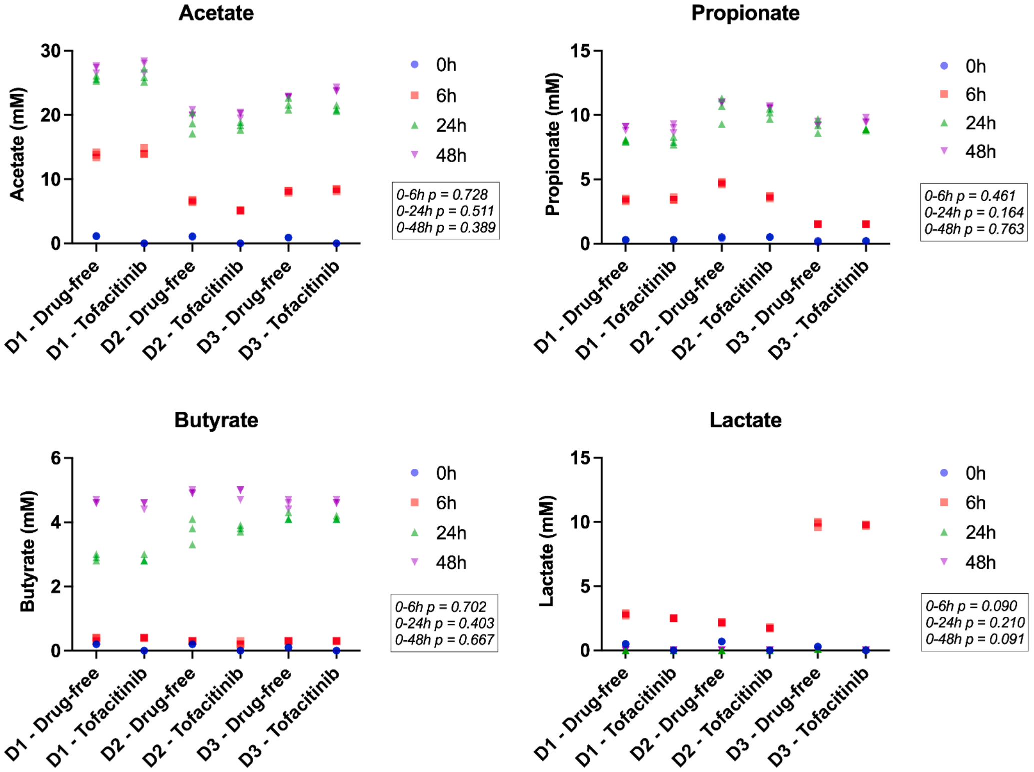

Fig. 3. Acetate, propionate, butyrate, lactate production (mM) during different timeframes (0 h, 6 h, 24 h and 48 h) of the 48 h incubation for each of the three donors (Donors D1–3), in triplicates. Tofacitinib and negative control (drug-free) were tested on the faecal microbiomes of three IBD adult donors. *P*-values are calculated from the means of the technical replicates of all treated donors *vs* drug-free donors.

butyrate-producing bacteria not only reduces luminal anaerobiosis but also exacerbates inflammation by increasing *Enterobacteriaceae*, subsequently decreasing barrier function and impairing mitochondrial function in colonic epithelial cells (Gasaly et al., 2021). From the results shown in Fig. 3, we observed a high level of interindividual variation among the donors, as seen in lower acetate production in donor 2 compared to the other donors. In contrast, similar levels of propionate and butyrate were observed across all donors. Overall, tofacitinib did not significantly affect the production of acetate, propionate, and butyrate, when compared to the corresponding untreated donors.

Lactate, while not a SCFA, is also an important metabolite of the human intestine. Lactate is produced by many species of colonic bacteria, and in subjects with IBD, lactate can build up to elevated levels in the colon (Wang et al., 2020). In contrast, in a healthy colon, lactate is converted by lactate-utilising bacteria into beneficial SCFAs, such as butyrate and propionate, which support host health (Wang et al., 2020). As different microbial species produce and convert lactate, an increase in lactate concentration can result from both an increase in production as well as a decrease in conversion. The impact of tofacitinib on lactate production is shown in Fig. 3. Lactate production was strongly donor-dependent; the microbiota of donor 3 produced much higher concentrations than donors 1 and 2. Moreover, as the lactate production peaked during the first 6 h, and butyrate levels increased from 24 h onwards, it suggested a likely conversion of lactate into butyrate. However, due to interindividual differences, there were no effects on lactate production that were significant across the donors.

3.2.2. Proteolytic markers

Less abundant fatty acids include BCFAs such as isobutyrate, isovalerate, and isocaproate. The production of BCFAs, and to a lesser extent ammonium, results from proteolytic microbial activity. Proteolytic fermentation can also lead to the formation of potentially harmful by-products such as phenols (*e.g.*, p-cresol) and ammonia (Diether and Willing, 2019). Therefore, products that reduce BCFA and ammonium production are considered health-beneficial. The results on BCFA and ammonium production indicate strong interindividual differences, *i.e.* overall low production of BCFA in donors 1 and 3 and higher levels in donor 2 (Fig. 4). Tofacitinib treatment was not associated with changes in the production of BCFA and ammonium across all donors.

Overall, tofacitinib treatment had no significant impact on the metabolic activity of the colonic microbiota, and these results were confirmed by sequencing data, which revealed no significant impact of the drug across the donors.

3.2.3. Tofacitinib effect on microbial composition shifts

The microbial community was analysed in both the lumen and the mucus at 48 h. 16S rRNA-targeted Illumina sequencing was employed to provide proportional abundances of different taxa at various

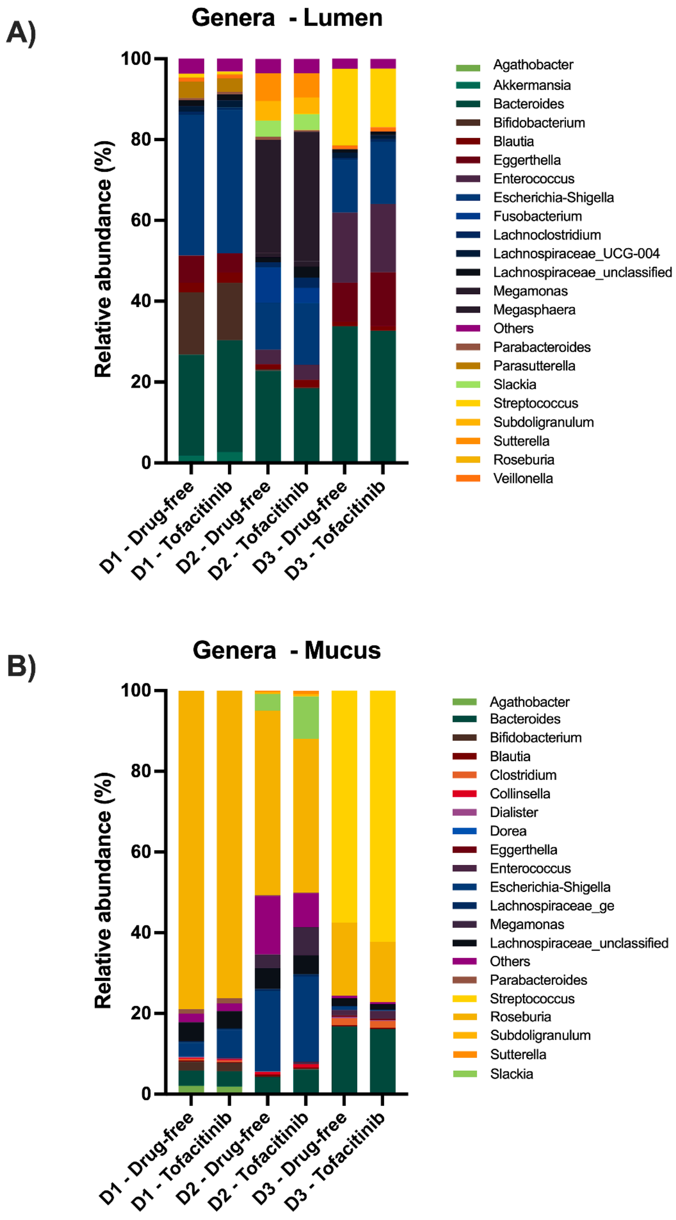

Fig. 5. Relative abundance of the 20 most abundant bacterial genera in lumen (A) and mucus (B) in drug-free control and for tofacitinib treatment with IBD donors D1, D2 and D3. Each colour represents a different genus.

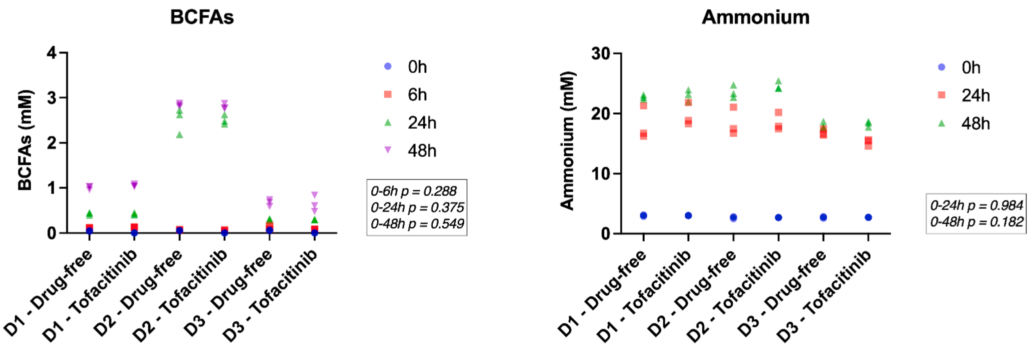

Fig. 4. BCFAs and ammonium production (mM) during different timeframes (0 h, 6 h, 24 h, 48 h for BCFAs and 0 h, 24 h, 48 h for ammonium) of the 48 h incubation for each of the three donors (Donors D1–3, $n = 3$). Tofacitinib and negative control (drug-free) were tested on the faecal microbiomes of three adult IBD donors. *P*-values are calculated from the means of the technical replicates of all treated donors *vs* drug-free donors.

phylogenetic levels (Fig. 5). The three donors exhibited significant variations in luminal microbiota composition, reflecting the highly diverse and dysbiotic patterns commonly observed with IBD patients (Franzosa et al., 2019; Morgan et al., 2012; Hu et al., 2022). Donor 1 was characterised by a higher abundance of *Escherichia-Shigella* and *Bifidobacterium. Escherichia-Shigella* is a commensal genus, although some strains can cause disease when they acquire virulence factors that enable them to evade the host immune system and cause infection (Hu et al., 2022; Phalipon and Sansonetti, 2003). This risk is even higher in individuals with a compromised immune system. Donor 2 exhibited higher abundance of *Fusobacterium* and *Megamonas* compared to the other donors. The presence of *Fusobacterium* has been associated with a pro-tumorigenic microenvironment in the progression of colorectal carcinoma, a long-term complication of IBD (Zepeda-Rivera et al., 2024). The microbiota of donor 3 was richer in *Streptococcus, Eggerthella*, and *Enterococcus spp*. Tofacitinib treatment had a limited impact on the luminal gut microbiota; however, it did increase the abundance of *Sutterellaceae* and reduced that of *Peptostreptococcaceae*. The mucosal microbiota composition of donors 1 and 2 was dominated by *Roseburia*. Similar to what was observed in the lumen, the mucosa of donor 3 was dominated by *Streptococcus*. In mucosal samples, tofacitinib caused a non-significant reduction in the abundance of *Roseburia*. The lower levels of butyrate-producing *Roseburia* in the gut have been linked with a higher risk of IBD (Parada Venegas et al., 2019).

According to Vandeputte *et al.* (Vandeputte et al., 2017), combining high-resolution phylogenetic information obtained from 16S Illumina sequencing with accurate enumeration of cell counts via flow cytometry allows the conversion of relative abundances into absolute abundances (Fig. 6). This calculation confirmed that tofacitinib did not significantly change the number of cells in the sample compared to the respective drug-free donor. However, different results were observed in Favaron et al. work (Favaron et al., 2024), where tofacitinib seemed to increase the number of cells from 2 to 6×10^6 during a 24 h incubation. In that case, *Dorea* was the genus that underwent the most marked increase. However, this positive effect of *Dorea* overgrowth was not observed in the three IBD donors above (Favaron et al., 2024).

These observations suggest that if tofacitinib had some effect on specific bacterial families, its overall impact on gut microbiota composition was limited. Whether these effects were consistent across donors was further assessed with differential abundance analysis (Fig. 7). Low-abundance species, *Bacteroides, Escherichia-Shigella*, and *Alistipes,* were biologically affected, but not significantly ($p > 0.05$), meaning that the effect was not consistent across the donors. Biological significance is achieved if the difference in abundance between treatment and control is at least a fourfold change (Perreau et al., 2023). These findings corroborate with previous research, wherein tofacitinib demonstrated no effect on alpha or beta diversity when studied in a murine animal (Texler et al., 2022). This was true both in the steady state and after dextran sodium sulphate exposure based on 16S rDNA profiling. However, it is important to note that the animal microbiome is different from the human microbiome.

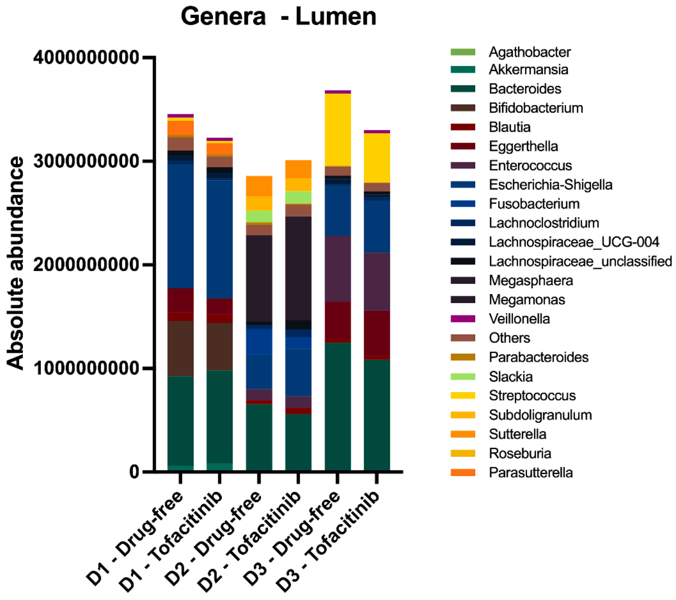

Fig. 6. Absolute bacterial abundances in lumen: bar plot showing the stacked absolute abundances of the 20 most abundant bacterial genera in drug-free control and for tofacitinib treatment with IBD donors D1, D2, and D3. Each colour represents a different genus.

3.3. Tofacitinib's anti-inflammatory effect via the microbiome

In addition to the evaluation of the changes in microbial composition and function, tofacitinib was investigated for any potential anti-inflammatory activity, measuring the gut barrier integrity TEER and the main immune markers, including pro- and anti-inflammatory cytokines and chemokines, in an *in vitro* Caco-2/THP1 co-culture model. This model also shows some features observed in IBD patients and is, therefore, used to test whether tofacitinib can protect the intestinal epithelial barrier integrity and reduce inflammation (Sambuy et al., 2005). Specifically, seeded Caco-2 cells spontaneously differentiate into mature enterocyte-like cells, with the appropriate characteristics of polarisation, the presence of villi, tight junctions, vectorial transport, and the expression of apical brush-border enzymes. THP1 cells are monocytes that, upon treatment with phorbol 12-myristate 13-acetate (PMA), differentiate into macrophage-like cells priming for toll-like receptor (TLR) responses (Sambuy et al., 2005).

3.3.1. Tofacitinib's effect on gut barrier integrity via the microbiome

Tight junction proteins maintain the integrity of adjacent epithelial cells, creating a nearly impermeable barrier to macromolecules. This barrier's "tightness" can be assessed by measuring TEER, where a high TEER indicates a tighter barrier. In the co-culture model, placing Caco-2 cells on top of PMA-activated THP1 cells, which secrete cytokines, disrupts the Caco-2 monolayer. This disruption of tight junctions results in a measurable decrease in TEER, which can potentially be counteracted by drug treatment. In all donors, compared to the drug-free control, tofacitinib treatment showed TEER values comparable to the control, indicating that the treatment did not provide additional protective effects on inflammation-induced intestinal epithelial barrier disruption (Fig. 8).

3.3.2. Tofacitinib modulates immune response

In this experimental setup, colonic suspensions from the incubations in the short-term colon model were applied to the apical side of Caco-2 cells in co-culture. The effects on THP1 cells in the basolateral chamber are mediated indirectly by signals from the Caco-2 cells and transported molecules. This approach allows the evaluation of the effects of both the product and fermentation-derived metabolites produced by the gut microbiota during digestion (Daguet et al., 2016).

After 24 h of apical pre-treatment of the Caco-2/THP1-Blue™ co-cultures with the colonic suspensions from the short-term colon bioreactor, the basolateral supernatant was discarded and the cells were stimulated with LPS. After 6 h of stimulation, the basolateral supernatant was collected to measure NF-κB activity, cytokines and chemokines secreted in the medium (Fig. 9).

The nuclear transcription factor kappaB (NF-κB) activation is markedly induced in IBD patients and through its ability to promote the expression of various pro-inflammatory genes, NF-κB strongly influences the course of the inflammation, specifically the mucosal inflammation (Atreya et al., 2008). Its downregulation would be favourable, however, tofacitinib did not affect NF-κB activity in all donors when compared to

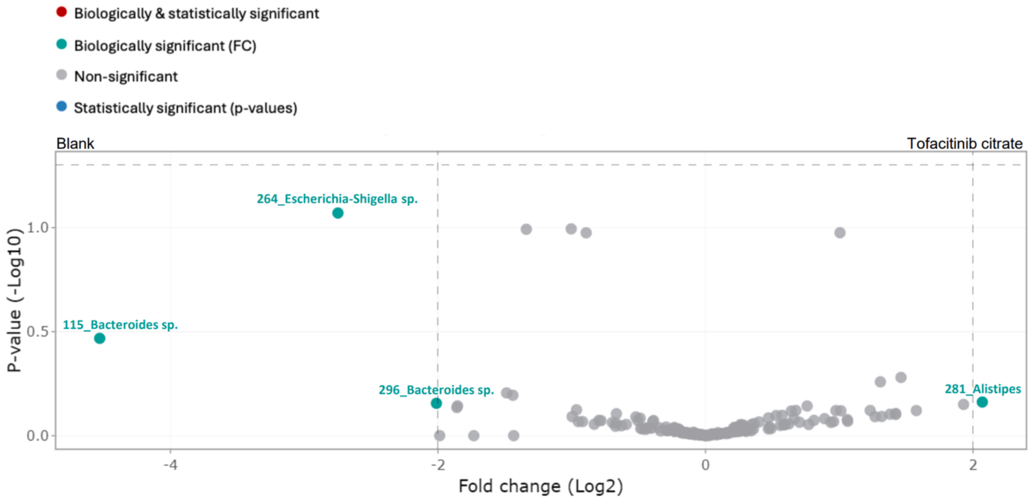

Fig. 7. Volcano plot, obtained through differential abundance analysis (treeclimbR), showing differences in luminal microbial community composition between treated and drug-free donors at 48 h across 3 donors. The obtained scatter plot classifies taxa based on the abundance in compared treatments, including: i) not significant and not biologically relevant (grey), ii) biologically relevant but not statistically significant (green).

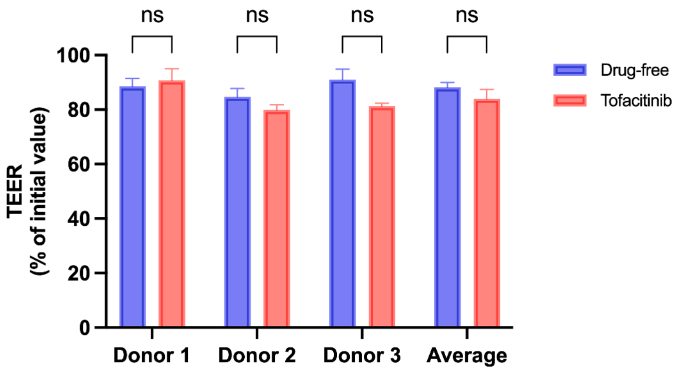

Fig. 8. Effect of tofacitinib colonic suspensions on transepithelial electrical resistance (TEER) of the Caco-2/THP1 co-cultures. TEER was measured 24 h after pre-treatment of the co-cultures, and each 24 h value was normalised to its corresponding 0 h value and is shown as percentage of the initial value (100 %). Data are plotted as mean ± SEM. *ns, non-significant.*

their respective untreated controls ($p = 0.805$, for mean).

IL-6 has a complex biology because it possesses both pro- and anti-inflammatory properties (Scheller et al., 2011). However, in an IBD condition, higher levels of IL-6 would be harmful, as it drives pro-inflammatory effects, leading to tissue damage and promotion of inflammatory cell recruitment (Mudter and Neurath, 2007). Tofacitinib treatment showed a significant decrease in the secretion of IL-6 in donor 2 ($p = 0.009$), while the mean reduction was not significant when pooled with the other donors ($p = 0.245$). It is important to note that IL-6 signals mainly through the JAK-STAT pathway, particularly via activation of STAT3. Because the colonic suspensions used in the co-culture contained both microbial metabolites and residual drug, the observed IL-6 reduction likely reflects the integrated effect of tofacitinib within the microbiota-derived fermentation matrix, encompassing both direct and microbiota-mediated mechanisms. IL-10 is an anti-inflammatory cytokine that suppresses various innate and adaptive immune cells; it activates anti-inflammatory molecules and enhances regulatory T-cell (Treg) function to restore immune homeostasis (Iyer and Cheng, 2012). When these switch-off mechanisms are impaired and immune homeostasis is not achieved, gut pathology and chronic inflammation may result, as in the case of IBD (Iyer and Cheng, 2012). Tofacitinib did not affect the production of IL-10 in any of the three IBD donors ($p = 0.940$, for mean value). No significant differences were observed for IL-8 and IL-1β levels across donors. Both cytokines are key mediators of neutrophil recruitment and epithelial stress; their unchanged levels suggest that under the tested conditions, tofacitinib's anti-inflammatory activity was selective and did not broadly suppress cytokine release.

Tumour necrosis factor α (TNF-α) is a key mediator of intestinal inflammatory processes and is one of the main cytokines involved in the pathogenesis of IBD (Kofla-Dłubacz et al., 2022). Its levels are typically elevated in the serum of IBD patients, and this is why its receptor is a target for IBD and rheumatoid arthritis therapies (*e.g.* infliximab, adalimumab, and other monoclonal antibodies) (Souza et al., 2023). In our study, however, tofacitinib treatment did not lead to any change in TNF-α levels compared to the drug-free control, possibly because the TNF-α signalling is not directly regulated by the JAK-STAT pathway. In addition, microbial products present in the suspension appeared to have no effect on TNF-α secretion under the tested conditions.

The pro-inflammatory chemokines CXCL10 and MCP-1 are both critical in mediating the inflammatory response in IBD, by attracting various immune cells to the gut (Singh et al., 2016). Tofacitinib significantly decreased both CXCL10 ($p = 0.011$) and MCP-1 ($p = 0.035$) levels in all donors compared to untreated samples, indicating pronounced immunosuppressive effects.

Although it did not enhance gut barrier integrity or significantly alter the levels of TNF-α, IL-8, and IL-1β, tofacitinib's ability to decrease CXCL10, MCP-1, and IL-6 secretion (in specific donors) highlights its potential to modulate the immune response. This data aligns well with Texler et al. (Texler et al., 2022) findings, where they have shown that tofacitinib exerted anti-inflammatory effects on monocytes through the suppression of IL-6, CXCL10, TNF-α, and induction of IL-10, at a drug concentration of 500 nM. Moreover, they tested tofacitinib's effect on colonic organoids prepared from intestinal epithelial cells (IECs) of UC patients, and demonstrated that it significantly lowers values of TNF-α and IL-8 production compared to untreated models (Texler et al., 2022).

While this study provides valuable insights on the microbiome-mediated effects of tofacitinib in an IBD context, some limitations should be acknowledged. Firstly, the composition and metabolic activity of the gut microbiota in IBD patients is characterised by great interindividual variability (Khan et al., 2019; Franzosa et al., 2019; Lavelle and Sokol, 2020). Moreover, given that both UC and CD are classified as IBD pathologies and that the microbiota profile in UC patients is different to CD patients (Mills et al., 2022; Ungaro et al., 2014), the small donor pool used herein limits the general applicability of these findings. Therefore, future studies should consider larger and more diverse donor cohorts, including individuals with UC, to better capture the heterogeneity of IBD

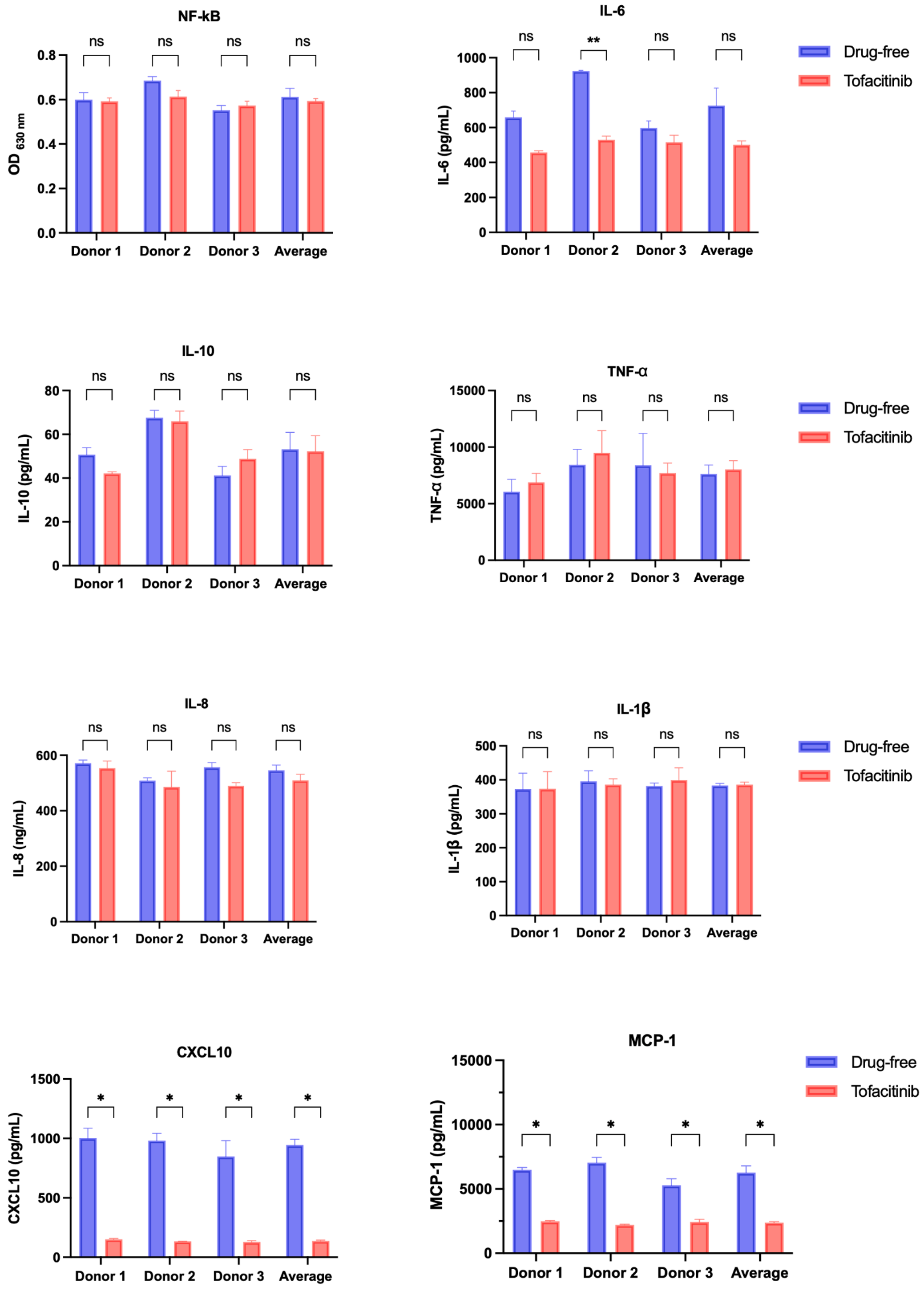

Fig. 9. Effect of tofacitinib on the activation of NF-κB and secretion of IL-6, IL-10, TNF-α, IL-8, IL-1β, CXCL10 and MCP-1. Cytokine levels were measured 6 h after LPS treatment on the basolateral side of the Caco-2/THP1-Blue co-cultures after pre-treatment of the apical side for 24 h with colonic suspensions. Data are plotted as mean ± SEM. Statistical significance: $*p < 0.05$, $**p < 0.01$, $**p < 0.001$ *(paired two-sided t-test vs. untreated control).*

patient populations.

The concentration of tofacitinib used in this study was 0.017 mg/mL, calculated based on the standard maintenance dose of tofacitinib citrate (10 mg) dissolved in 600 mL, which reflects the average volume of colonic fluid. This yielded an estimated concentration of 0.0167 mg/mL. However, this value remains an approximation and does not account for the *in vivo* pharmacokinetics of tofacitinib, including absorption, distribution, and metabolic clearance. As such, the actual concentration of the drug that reaches the colon and interacts with the gut microbiota may be significantly different. A more accurate estimation of colonic drug concentrations would require direct *in vivo* sampling of colonic fluid or the application of modelling approaches. Notably, Favaron et al. have recently demonstrated that physiologically-based pharmacokinetic (PBPK) modelling enables compartment-specific predictions of drug levels throughout the GI tract, including the colon, where microbial density is highest (Favaron et al., 2024). Such modelling techniques could be useful in refining the concentration of tofacitinib and assessing its effects on the IBD microbiome composition and functioning.

The colonic suspensions applied to the co-culture system contained both microbial metabolites and residual dissolved drug, forming what we term the *fermentation matrix*. This approach was selected to preserve the complexity of the luminal environment, where epithelial cells are simultaneously exposed to microbial fermentation products and any unabsorbed drug. Such integration enables evaluation of the combined microbiota–drug effects on barrier function and immune responses, providing physiologically relevant insights that would not be captured by testing the drug or microbial supernatant alone. Nevertheless, this design does not allow for full mechanistic separation of drug-driven and microbiota-driven effects. Given that tofacitinib is chemically stable in the presence of human gut microbiota (Favaron et al., 2024), the observed immunomodulatory effects likely reflect a combined influence of postbiotic metabolites and residual drug. Future studies should consider separating microbial supernatants from the tofacitinib or include specific readouts of JAK-STAT pathway activation to better clarify the origin of the observed effects. Additionally, to strengthen the robustness of the conclusions, future investigations should incorporate larger and more diverse microbiome cohorts, including individuals with active (non-remission) Crohn's disease and ulcerative colitis. Furthermore, replication and validation in more comprehensive study designs, supported by scalable methodologies, are essential to facilitate the translation of this therapeutic strategy from pilot-scale research to broader clinical application. Beyond these methodological considerations, this work contributes to a growing body of research exploring how JAK inhibitors interact with the gut microbiome. By integrating microbial fermentation with an epithelial–immune co-culture system, our study extends this field by assessing tofacitinib's effects in a biorelevant, microbiota-conditioned context, highlighting the importance of microbiome-mediated pharmacology in the next generation of IBD therapeutics.

4. Conclusion

This study aimed to investigate the impact of tofacitinib citrate on the gut microbiota composition and functionality in IBD patients, as well as its immunomodulatory effects using a Caco-2/THP1 co-culture model. The main finding of this study is the immunomodulatory potential of tofacitinib in an IBD context, particularly through its capacity to reduce key pro-inflammatory mediators such as CXCL10, MCP-1 and IL-6. While no additional effect on intestinal epithelial barrier integrity was observed in the short-term colon setup, these findings do not exclude that tofacitinib may exert a barrier-protective role *in vivo*. Indeed, recent evidence suggests that tofacitinib may help restore mucosal integrity and tight junction function in IBD patients. Importantly, our findings suggest that tofacitinib may act through microbiota-mediated immunomodulation. These insights reinforce the importance of considering the gut microbiome in the evaluation of GI drugs. Integrating microbiome research into drug development may help uncover additional mechanisms of action and support more effective, personalised treatment strategies for IBD.

Data availability

The 16S rRNA sequencing data generated during this study have been deposited in the NCBI Sequence Read Archive (SRA) under BioProject accession number PRJNA1260775. These data are publicly available. The metabolite profiles and *in vitro* Caco-2/THP1-Blue™ co-culture assay data are available from the corresponding author upon reasonable request.

CRediT authorship contribution statement

Alessia Favaron: Writing – original draft, Visualization, Validation, Methodology, Data curation, Conceptualization. **Nannapat Sangfuang:** Writing – review & editing, Visualization, Formal analysis. **Laura E. McCoubrey:** Writing – review & editing, Methodology, Conceptualization. **Atheer Awad:** Writing – review & editing, Funding acquisition, Conceptualization. **Jonas Ghyselinck:** Methodology, Investigation, Formal analysis, Data curation. **Massimo Marzorati:** Project administration, Methodology. **Lynn Verstrepen:** Writing – review & editing, Resources, Methodology. **Julie De Munck:** Resources, Methodology, Formal analysis. **Jelle De Medts:** Validation, Project administration, Methodology. **Abdul W. Basit:** Writing – review & editing, Supervision, Resources, Funding acquisition. **Mine Orlu:** Writing – review & editing, Supervision, Resources, Funding acquisition.

Declaration of competing interest

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 paper. This work was conducted before Laura E. McCoubrey joined GSK, and as such, this work does not reflect the views of GSK.

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