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Exploring the interactions of JAK inhibitor and S1P receptor modulator drugs with the human gut microbiome: Implications for colonic drug delivery and inflammatory bowel disease

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ABSTRACT

The gut microbiota is a complex ecosystem, home to hundreds of bacterial species and a vast repository of enzymes capable of metabolising a wide range of pharmaceuticals. Several drugs have been shown to affect negatively the composition and function of the gut microbial ecosystem. Janus Kinase (JAK) inhibitors and Sphingosine-1-phosphate (S1P) receptor modulators are drugs recently approved for inflammatory bowel disease through an immediate release formulation and would potentially benefit from colonic targeted delivery to enhance the local drug concentration at the diseased site. However, their impact on the human gut microbiota and susceptibility to bacterial metabolism remain unexplored. With the use of calorimetric, optical density measurements, and metagenomics next-generation sequencing, we show that JAK inhibitors (tofacitinib citrate, baricitinib, filgotinib) have a minor impact on the composition of the human gut microbiota, while ozanimod exerts a significant antimicrobial effect, leading to a prevalence of the *Enterococcus* genus and a markedly different metabolic landscape when compared to the untreated microbiota. Moreover, ozanimod, unlike the JAK inhibitors, is the only drug subject to enzymatic degradation by the human gut microbiota sourced from six healthy donors. Overall, given the crucial role of the gut microbiome in health, screening assays to investigate the interaction of drugs with the microbiota should be encouraged for the pharmaceutical industry as a standard in the drug discovery and development process.

1. Introduction

In the last 20 years, we have witnessed remarkable discoveries on the human gut microbiota (GM). Research has highlighted a wide array of functions of the GM encompassing nutrient metabolism, immune system regulation, maintenance of the gut barrier, digestion, metabolism and the synthesis of essential compounds such as short-chain fatty acids (Bull and Part, 2014; Jandhyala et al., 2015; Louis and Flint, 2017). Moreover, studies have revealed correlations between alterations in the gut microbial composition and diseases affecting various systems and organs, such as cardiovascular and metabolic disorders, autoimmune diseases, and psychiatric conditions (Lim, 2022; Fan and Pedersen, 2021; Simpson et al., 2023; Miyauchi et al., 2023; Chamtouri et al., 2023; Valles--Colomer et al., 2019). The gut microbiota, with its 10^{10}–10^{11} bacteria per gram of intestinal content (Walker and Hoyles, 2023), represents a unique microbial niche. In the same way that diet, stress, physical activity, psychosocial factors, and sleep patterns play a role in shaping the composition of the gut microbiome (Wilson et al., 2020; Lopera-Maya et al., 2022; Ghosh et al., 2022), medicines, particularly those designed with antimicrobial properties can rapidly deplete specific populations of gut bacteria (Maier et al., 2018; Maier and Typas, 2017). It is widely recognised that the use of antibiotics leads to a rapid reduction in bacterial diversity and shift in relative abundance, resulting in a state of so-called dysbiosis that can negatively affect the host health (Fishbein et al., 2023). However, besides antimicrobials, approximately 25 % of non-antibiotic drugs intended for human administration have been found to inhibit the growth of at least one bacterial species, and this percentage is likely to be higher, given the vast number of microbes residing in the human gut and the number of drugs that have not yet been tested for anti-microbiome activity (Maier et al., 2018).

Research has only recently begun to uncover the bidirectional interaction between our resident microbes and medications: the great

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density of microbes in the intestinal tract can be affected by drugs, but these microbes can also enzymatically biotransform a drug's structure, causing alteration in its bioavailability, bioactivity, or potential toxicity - a phenomenon referred to as pharmacomicrobiomics (Zimmermann et al., 2019; Javdan et al., 2020). The gut microbiota is rich in hydrolase and reductase enzymes which mainly produce non-polar low--molecular-weight metabolites (Sun et al., 2019). It is inevitable that orally delivered drugs, especially those that are not completely absorbed from the small intestine or those displaying enterohepatic circulation, are in contact with the gastrointestinal tract bacteria (Sousa et al., 2008).

Despite the substantial implications of drug-microbiota interactions, biotransformation of drugs by the gut microbiota or the impact that drugs may have on gut microbial growth and functions is not routinely screened for in pharmaceutical development or clinical practice. This is particularly relevant to modified-release drug formulations, which are designed to release the drug along the length of the gastrointestinal (GI) tract, particularly in the large intestine, where the microbiota density is the highest (McCoubrey et al., 2023). Currently, regulatory bodies are not prioritising the importance of the gut microbiome's interactions with drugs (Villanueva, 2021). However, this is likely to change as researchers continue analysing these complex interactions, both with new *in vitro* tools (Tannergren et al., 2014), and machine learning models (McCoubrey et al., 2022; LE McCoubrey et al., 2021a; LE McCoubrey et al., 2021b).

Tofacitinib citrate, baricitinib, and filgotinib are Janus Kinase (JAK) inhibitor drugs; small molecules that are delivered orally through immediate-release formulations (Shawky et al., 2022). By binding to the JAK enzymes, JAK inhibitors reduce the transcription of pro-inflammatory cytokines, reducing the inflammatory response. JAK inhibitors are approved for a wide range of inflammatory diseases such as rheumatoid arthritis (RA), psoriatic arthritis, atopic dermatitis, and inflammatory bowel disease (IBD) (Shawky et al., 2022; Dudek et al., 2021). Specifically, tofacitinib is classified as a BCS Class III drug, with rapid absorption, around 74 % of approximate bioavailability, for a 3 h half-life (Anon., LE European Medicines Agency, 2024). Baricitinib falls under BCS Class III with a bioavailability of approximately 79 % and a half-life of about 8.6 h (Anon., LE European Medicines Agency 2024). Filgotinib is classified as BCS Class II, demonstrating rapid absorption with a C_{max} reached within 1–3 h and a half-life of approximately 5–10 h (Namour et al., 2022). Tofacitinib and filgotinib are approved for the treatment of ulcerative colitis (UC) and filgotinib has successfully completed a Phase 3 study for Crohn's disease. However, systemic exposure to JAK inhibitors can cause severe side effects, especially thromboembolic events, which led to The Food and Drug Administration (FDA) designating a black box warning in the United States (FDA, 2021; Initial safety trial results find increased risk of serious heart-related problems and cancer with arthritis and ulcerative colitis medicine Xeljanz, 2024). Sphingosine 1-phosphate (S1P) receptor modulators are another drug class recently developed for the treatment of inflammatory conditions. Ozanimod is an S1P receptor modulator approved for the treatment of moderately to severely active UC and relapsing multiple sclerosis and is currently undergoing a phase 3 trial for moderately to severely active Crohn's disease (Surapaneni et al., 2021; Paik, 2022; Feagan et al., 2022). The absorption of ozanimod is slow, with a peak plasma concentration (C_{max}) achieved at 6–8 h after oral administration, and a large volume of distribution (Surapaneni et al., 2021).

Given that JAK inhibitors and S1P receptor modulators are established treatments for IBD conditions, their efficacy and safety profiles could be promoted by targeted drug delivery to the colon. This strategy aims to concentrate the drug at the disease site while reducing its systemic exposure. An example of this approach can be seen in the work of Yadav et al. (2022), who investigated the colonic delivery of tofacitinib citrate in a rat model with lipopolysaccharide-induced intestinal inflammation. Their findings indicated a significant enhancement in the safety and effectiveness of the therapy following colonic delivery. Therefore, it is reasonable to anticipate similar favourable outcomes when employing colonic delivery for other anti-inflammatory JAK inhibitors and ozanimod.

However, before any modified or colonic drug delivery system of JAK inhibitors and ozanimod is developed, there is a need to understand the fundamentals behind the potential interaction between the compounds and the gut microbiota. In this respect, this study aims to explore whether the gut microbiota sourced from healthy individuals is responsible for the biotransformation of tofacitinib citrate, baricitinib, filgotinib, and ozanimod and whether these drugs deplete any bacterial species within the gut microbiota. As a secondary objective, this study focused on exploring the appropriateness of conventional *in vitro* approaches, namely the isothermal calorimetry technique and optical density, to investigate the capacity of medications to impact the growth of the gut microbiota.

2. Materials

Tofacitinib citrate (Cat no HY-40354A) and baricitinib (Cat no HY-15,315) were obtained from MedChemExpress USA. Filgotinib (Cat no A14232) and ozanimod (Cat no A15819) were obtained from Adooq Bioscience LLC. Bryant and Burkey (BB) broth medium was obtained from Sigma Aldrich, UK. ¼ Ringer's tablets, and triethylamine were purchased from Merck Life Science (Gillingham, UK). Peptone water and yeast extract were obtained from Oxoid Limited (Basingstoke, UK). Sodium chloride and dipotassium hydrogen phosphate were obtained from Fisher Chemical (Loughborough, UK). Magnesium sulphate heptahydrate and calcium chloride hexahydrate were obtained from VWR (Lutterworth, UK). Sodium bicarbonate was from Sigma Aldrich, while hemin, l-cysteine HCl, vitamin K and Resazurin were obtained from Sigma Life Sciences (Dorset, UK). Bile salts and tween 80 were from Fluka Analytical and Sigma Aldrich, UK. All other chemicals were of high-performance liquid chromatography (HPLC)-grade and used as received.

3. Methods

3.1. Cultivation of the microbiome samples

Six healthy humans (3 males and 3 females) donated their stools to Intract Pharma Limited. Ethical approval to collect human feces was obtained from the UCL Biobank Ethical Review Committee at Royal Free London NHS Foundation Trust (reference no NC2017.010). Fecal material was mixed and diluted with basal medium at a ratio of 1 part fecal matter to 3 parts basal medium to achieve a 25% w/w fecal slurry, as per the methodology from Yadav et al. (2013). Specifically, the basal media was made as follows. Peptone water (2 g) and yeast extract (2 g) were weighed into a glass flask containing distilled water (800 mL). Calcium chloride hexahydrate (0.01 g), dipotassium hydrogen orthophosphate (0.04 g), magnesium sulphate heptahydrate (0.06 g), sodium chloride (0.1 g), tween 80 (2 mL), l-cysteine (0.5 g) and bile salts (0.5 g) were dissolved in the solution. Into this, hemin (0.005 g in two drops of 1 M sodium hydroxide (NaOH)), 0.025 % resazurin in deionised water solution (4 mL), and vitamin K (10 μL) were added under stirring. Upon dissolution, sodium bicarbonate (2 g) and distilled water were added to make the final volume of 1 liter. The slurry was mixed manually to break fecal pellets and extract fecal bacteria and enzymes. The slurry was then sieved through a 250 μm pore size SefarNitex TM sieve.

From the fecal slurry, derived microbial culture suspensions were made. 1 mL of the slurry was transferred in sterile 100 mL BB broth. After 24 h incubation, 1 mL of culture was transferred into a new sterile 100 mL BB broth. Inside the anaerobic chamber, cultures were mixed with sterile 40 % glycerol in ¼ Ringer's solution at a ratio of 1:1, and 1.5 mL aliquots were transferred into cryovials and frozen at −80 °C. The enumeration of the microbes in each gut microbiota sample (donor 1 to 6) was observed by plating the final cultures onto Bryant and Burkey

Agar medium and incubating them in anaerobic conditions at 37 °C for 48 h.

3.2. Antimicrobial activity of drugs

To study the effect of the drugs on the growth of the bacteria, three in vitro techniques were employed: isothermal calorimetry, optical density, and next-generation sequencing metagenomics. 30 μM was selected as a relevant concentration to test the drugs' antimicrobial activity, following the Maier et al. estimations (Maier et al., 2018) which calculated that >70 % of orally delivered drugs reach the colonic region at the concentration of 20 μM.

3.2.1. Isothermal calorimetry

A Thermal Activity Monitor (TAM, 2277, TA Instruments Ltd., UK) was used to measure the microbial growth within the medium, as the heat generated from the metabolic processes of microorganisms can be used as a direct indicator of their growth (O'Neill and Gaisford, 2011). A volume of 2.8 μL of the microbial culture suspension (as explained in Section 3.1) was pipetted aseptically into 2.8 mL BB broth, inside glass vials pre-warmed to 37 °C ($n = 3$). The inoculated vials were sealed hermetically and placed inside the TAM. The temperature of the instrument was set at 37 °C to mimic *in vivo* conditions. All loaded samples were allowed to equilibrate thermally at the intermediate position for 30 min before measurement. Data was collected every 10 seconds using the software package, Digitam 4.1. All experiments were set up inside an anaerobic chamber (Whitley A20 Workstation, Don Whitley Scientific, UK). Each drug investigated (tofacitinib citrate, baricitinib, filgotinib, and ozanimod) was dissolved in BB broth at a concentration of 30 μM. The growth of the gut microbiota sourced from a male donor in the presence of 30 μM of drug was measured via TAM (n = 3 for each drug) during a 24-hour experiment. Growth curves obtained in the presence of the drug were compared with growth curves obtained in pure microbial growth medium (n = 3). The area under the curve (AUC), the time taken to reach peak power (T_{max}), and the maximum power (P_{max}) were used as comparative features. A parametric unpaired *t*-test was used to assess whether differences between microbial growth in the presence of drugs, compared with the absence of drugs, were significant ($p < 0.05$). Where a drug significantly reduced or increased AUC, T_{max}, or P_{max}, then the drug was determined as inhibiting or promoting microbial growth. Conversely, if the drug did not significantly alter AUC, T_{max}, or P_{max}, then they were deemed as neutral: neither inhibiting nor promoting microbial growth. All analyses were done in GraphPad Prism 10.2.

TAM was also used to monitor the effect of DMSO on the growth of the same male donor microbiota. DMSO at the concentration of 1 % v/v was used to increase drugs' solubility in the BB medium. To evaluate its antimicrobial effect, DMSO was diluted at the same concentration into the broth medium and compared with the control curves, in triplicate, of the same bacterial sample without the addition of DMSO (Supplementary data).

3.2.2. Optical density measurements

The Biochrom WPA CO8000 Cell density meter (Scientific Laboratory Supplies Ltd, UK) was used at 600 nm wavelength to monitor the effects of the drugs on the gut microbiota samples. Each drug was incorporated into BB broth at a concentration of 30 μM. The growth of the microbial culture suspension sourced from a male donor (2.8 μL in a final volume of 2.8 mL) in the presence of 30 μM drug was measured via cell density meter ($n = 3$ for each drug). The optical density (OD) of the cultures was measured in 3 mL cuvettes. OD values obtained in the presence of the drug were compared with those obtained in pure microbial growth medium (n = 3). OD reads were collected at 0, 1, 2, 3, 4, 6, 7, and 24 h. A two-way ANOVA test, with GraphPad Prism 10.2., was used to assess whether differences between microbial growth in the presence of drugs, compared with the absence of drugs, were significant ($p < 0.05$), at the two time points 7 h and 24 h.

3.2.3. Metagenomics

The microbial culture suspension sourced from one male donor and treated with JAK inhibitors and ozanimod were sent to Eurofins Genomics (Germany), for analysis in their INVIEW Metagenome Advance platform. Briefly, each sequence read of samples was inspected for their base quality, low-quality bases were removed and only mate pairs (forward and reverse read) were used for the next analysis step. The host sequences were then removed from the sample genes. Clean samples were analysed for taxonomic profiling, resistance profiling and functional profiling.

3.2.3.1. Taxonomic profiling. Taxonomic profiling was conducted using MetaPhlAn (Truong et al., 2015), which profiles microbial communities at the species level from metagenomic shotgun sequencing data. This tool utilises unique markers identified from approximately 17,000 reference genomes, including bacterial, archaeal, viral, and eukaryotic genomes, ensuring high specificity in microbial detection. For unclassified reads, KrakenUniq was employed (Breitwieser et al., 2018), which classifies reads based on k-mers. KrakenUniq matches each k-mer to the lowest common ancestor in a precomputed database and counts unique k-mers per taxon to enhance accuracy and reduce false positives. The tool constructs a weighted classification tree for each read, classifying it based on the path with the highest cumulative weight. To quantify and visualise the microbial diversity and abundance from our sequencing data, the raw read counts and their corresponding percentages obtained from the taxonomic profiling were transferred to GraphPad 10.2. for graphical representation. Bar plots were generated to display the distribution of both genera and species across different samples, providing a clear view of the microbial landscape within each sample group. This approach facilitated an intuitive comparison of microbial diversity and abundance across different treatment conditions.

3.2.3.2. Functional profiling. The HMP Unified Metabolic Analysis Network (HUMAnN) profiles the abundance of microbial metabolic pathways and other molecular functions from metagenomic sequencing data. This tool allowed for the annotation of the genes in each sample as GO (Gene Ontology) with correspondent total counts per million (CPM), and in further molecular function [MF], biological process [BP] and cellular components [CC] categories.

The Shannon diversity index was employed to assess biodiversity within microbial communities. For this analysis, raw read counts for genera and species, along with CPM for each GO term, were obtained from control and drug-treated samples, including tofacitinib citrate, baricitinib, filgotinib, and ozanimod. Each dataset was first normalised to convert counts to proportions of the total counts in each sample. This normalisation process allowed for the comparison of diversity across samples with different sequencing depths or total read counts. The Shannon index graphs were plotted in GraphPad 10.2.

Bray-Curtis dissimilarity indices were calculated to evaluate the similarity between microbial communities in the control and drug-treated samples. For this analysis, normalised data for genera, species, and gene ontology (GO) terms from each sample group were used. To visualise these differences, Multidimensional Scaling (MDS) plots were generated with Scikit-learn (Version 1.1.3) Python package.

To provide a detailed account of the distribution and overlap of metabolic pathways affected by each treatment, an UpSet plot was constructed using the UpSetPlot (Version 0.9.0) Python package to analyse the intersections of Biological Process [BP] terms across control and drug-treated samples.

For the heatmap analysis, the workflow was methodically structured to highlight specific metabolic pathways influenced by drug treatments. Initially, all BP terms associated with each drug and GM control sample were compiled. Subsequently, an enrichment analysis was performed using the ReviGO online software (Supek et al., 2011) to identify and prioritise significant GO terms. Selected terms were those involved in

critical metabolic categories such as carbohydrate and energy metabolism, lipids, amino acids and peptides, nucleotides and nucleic acids, secondary metabolites, cofactors and vitamins, short-chain fatty acids (SCFAs), and tryptophan (Supplementary Table 1). Data normalisation was conducted by row to ensure comparability across terms. Finally, the normalised data was visualised through a heatmap plotted in GraphPad, providing a clear representation of the metabolic impacts induced by each drug on the gut microbiome, and facilitating a nuanced understanding of drug-microbiome interactions.

3.2.3.3. Resistance profiling. Resistance profiling examined the diversity of antimicrobial resistance (AMR) genes within metagenomes. This analysis was performed using the GROOT software, which utilises a variation graph for gene sets and a locality-sensitive hashing scheme to classify metagenomic sequences rapidly. This approach allowed for the precise reconstruction of complete gene sequences by aligning reads against the graph and scoring their similarity. The reference database for this analysis included over 6000 well-curated ARGs from public repositories.

All the metagenomics analysis was carried out on Python (Version 3.10.4).

A detailed protocol on INVIEW Metagenome Advance analysis (Eurofins) can be found here: https://eurofinsgenomics.eu/media/1610467/ef-demo_metagenome_analysis_report.pdf.

3.3. Stability of drugs

3.3.1. Experimental evaluation of drugs' stability

For the experimental evaluation of their stability, the four drugs were incubated with the bacterial static culture of donors 1 to 6 for 24 h in an anaerobic chamber at 37 °C and 70 % relative humidity. JAK inhibitors and ozanimod at a concentration of 10 mM in DMSO were diluted in 4 mL of BB medium to obtain a 100 μM solution of drugs in 1 % DMSO. Drugs' solutions were combined with 8.3 % of microbial culture suspensions from each donor (1 to 6). During incubation, samples were agitated at 100 rpm on a horizontal shaker (VXR basic Vibrax®, Leicestershire, UK). Aliquots of the reaction mixture were removed for analysis at 0, 1, 2, 4, 6, and 24 h. Withdrawn aliquots for each time point were immediately combined with quencher solution (methanol) at a ratio of 1:2 to halt microbiota activity. Samples were then centrifuged at 14,000 rpm for 10 min at 4 °C. The supernatant was collected and analysed using HPLC. As a negative control, JAK inhibitors and ozanimod were incubated in BB medium only, in the absence of the microbiota. A percentage lower than 80 % of free drug remaining in each gut microbiota sample after 24 h was set as the cut-off value for drug depletion. A multiple unpaired *t*-test, with GraphPad Prism 10.2., was used to compare means between drug stability in multiple donors of independent observations ($p < 0.05$).

The same protocol was used to test the susceptibility of metronidazole to microbial degradation. Specifically, metronidazole is a model drug substrate of the enzyme nitroreductase, previously used to test bacterial enzymatic degradation in faecal slurries (Vertzoni et al., 2018; Karatza et al., 2017). Metronidazole was tested in the 25% w/w faecal slurries prepared as per the methodology from Yadav et al. (Yadav et al., 2013), and in the correspondent derived microbiota samples as used to test the stability of JAK inhibitors and ozanimod. Metronidazole at the final concentration of 100 μM, was solubilised in BB broth, and combined with 8.3 % of faecal slurries and gut microbial samples from each donor (1 to 6). Samples were treated as above. The 100 μM concentration was chosen as a relevant concentration to test drug stability as it is higher than the 20 μM predicted by Maier et al. (Maier et al., 2018) and because it allowed for an accurate detection of drugs' depletion over time.

3.3.2. High-performance liquid chromatography

Chromatography analyses were run on HPLC Agilent 1260 Infinity II. Chromatographic methods were different for the JAK inhibitors, ozanimod, and metronidazole.

Tofacitinib citrate, baricitinib and filgotinib: An isocratic mobile phase of 50 % water and 50 % methanol was used. An injection volume of 20 μl was pumped through a Rastak C18 column (150 × 4.6 mm, 5 μM) in reverse mode at 1.0 mL/min. The detection wavelength was 254 nm, and the temperature was not controlled.

Ozanimod: A gradient elution method was used, wherein the mobile phase constituted 0.1 % ammonia in water (A), and acetonitrile (ACN) (B). An injection of 20 μl was pumped through a column ACE C18 (4.6 × 50 mm, 3 μm) at 1.0 mL/min. The run was maintained at the controlled temperature of 30 ± 0.8 °C, and with a detection wavelength of 210 nm. For each HPLC run results were inspected for the absence of any peaks related to the bacterial sample of growth medium that could overlap with the drug peak.

Metronidazole: A reversed phase Rastak C18 column (150 × 4.6 mm, 5 μM) at the flow rate of 0.5 mL/min was used. Mobile phase was composed of acetonitrile, water, and formic acid (10:90:0.1 v/v/v). Absorption was measured at 315 nm and injection volume was 20 μl. The temperature was not controlled.

All results were inspected for the absence of any peaks related to the bacterial sample of the growth medium that could interfere with the drug peak.

4. Results and discussion

4.1. Ozanimod, but not JAK inhibitors, exhibits antimicrobial activity

4.1.1. Isothermal microcalorimetry and optical density

Isothermal microcalorimetry and optical density were employed to assess the susceptibility of a gut microbiota sample to the co-presence of 30 μM JAK inhibitors and the S1P receptor modulator ozanimod, dissolved in BB growth medium. Specifically, as microbial fermentation processes generate heat, the isothermal microcalorimeter registers the energy released or absorbed by the sample, which corresponds to the net metabolic activity of the bacteria and is expressed as a power-time curve (O'Neill and Gaisford, 2011; Braissant et al., 2013). Since many drugs and excipients can alter bacterial growth and act as metabolic modifiers, changes in the power output of the sample will be reflected in the curve outlines, as shown in Fig. 1. The AUC represents the average growth curve of the gut microbiota when exposed to the drugs dissolved in the growth medium. For each experiment, bacterial growth in the presence of the drugs tofacitinib citrate, baricitinib, filgotinib, and ozanimod, is compared with a control curve without the drug through the measurement of the AUC from 0 to 24 h, and through the comparison of T_{max} and P_{max} of the curve.

The GM sample used for both TAM and OD experiments was inoculated at the initial bacteria concentration of approximately 10^5 colonies forming units per millilitre (CFU/mL). The minimum energy level that TAM can detect corresponds to that produced by 10^6 CFU/mL of bacteria. In Fig. 1 we can observe a power signal from the beginning of the recording, indicating that during the 30-minute acclimatisation phase before initiating energy monitoring, the bacteria undergo exponential growth with a growth rate constant of 10. The lower the CFU present in a sample, the longer the time required to monitor a power signal, as illustrated in Supplementary Data, Fig. 1.

Overall, JAK inhibitors did not affect the growth of the GM: in Fig. 1A,B,C, the values of AUC, Tmax, and Pmax did not exhibit statistically significant differences between the gut microbiota exposed to the JAK inhibitor drugs and the corresponding reference samples without the drugs, meaning that the initial number of CFU in the starting sample is not significantly affected. No significant difference was seen between the AUC of the growth curve of the GM with tofacitinib citrate and the control ($p = 0.6190$) (Fig. 1A). When baricitinib was added to the

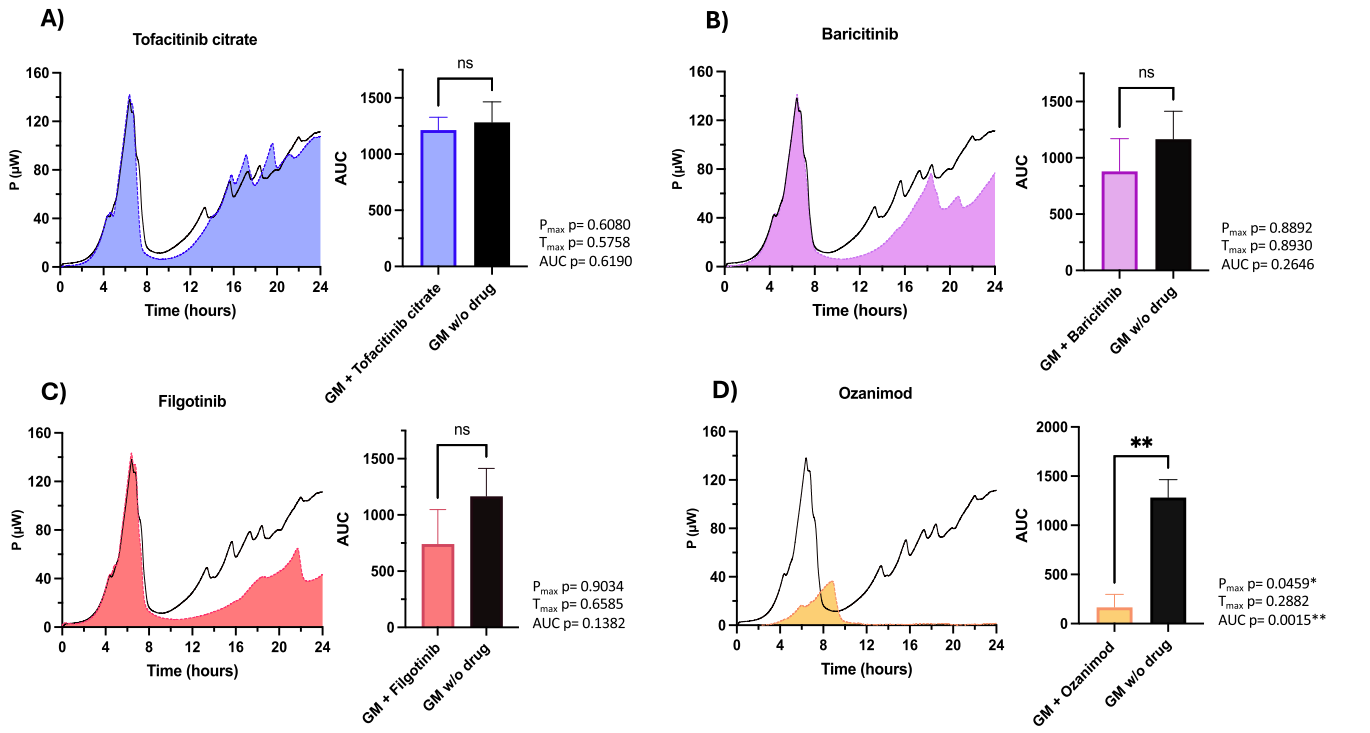

Fig. 1. Thermal Activity Monitor (TAM) graphs of the growth of the gut microbiota sample, with and without the drug, in 24 h experiment; with respective statistical *t*-tests of the relative AUC (histogram), Pmax, Tmax. A) Tofacitinib citrate: the blue curve represents the growth of the bacteria in the presence of the drug ($n = 3$), vs the control sample without the drug ($n = 3$). B) Baricitinib: the pink curve represents the growth of the bacteria in the presence of the drug ($n = 3$), vs the control sample without the drug ($n = 3$). C) Filgotinib: the red curve represents the growth of the bacteria in the presence of the drug ($n = 3$), vs the control sample without the drug ($n = 3$). D) Ozanimod: the yellow curve represents the growth of the bacteria in the presence of the drug ($n = 3$), vs the control sample without the drug ($n = 3$).

medium, there was a slight reduction in the growth of the bacterial sample, although an unpaired *t*-test did not show this difference to be significant (for AUC, $p = 0.2646$) (Fig. 1B). Filgotinib showed a noticeable reduction in the growth of the gut microbiota sample after the 8-hour time point, but again this was not statistically significant (Fig. 1C).

Interestingly, in contrast to the JAK inhibitors examined in this study, ozanimod demonstrated a clear antimicrobial activity on the human gut microbiota. This is evident from the significant difference in the area under the power-time curve compared with the control sample without the drug, as confirmed by an unpaired *t*-test with a p-value of 0.0015. Furthermore, based on the power signals observed in Fig. 1D, it can be concluded that ozanimod led to a reduction of approximately 10^2 CFU/mL in microbial counts, an effect becoming detectable around four hours post-inoculation, highlighting the sensitivity of TAM in capturing delayed microbial responses to drug exposure (Supplementary Data, Fig. 1).

In a study conducted by Braissant et al. (2013), it was shown that the AUC of the isothermal curve measured with TAM exhibited a direct proportionality to the optical density of bacterial growth. This observation implies a close relationship between heat production and the growth of the bacteria: upon integration of the data, patterns were seen that resemble the conventional OD growth curve of bacteria. Fig. 2 illustrates the impact of the four tested drugs on the cumulative heat expressed in joules (J), generated by the same gut microbiota sample. This includes an initial lag phase, followed by an exponential growth phase, and eventually a stationary phase (plateau).

Optical density measurements are used to determine the number of bacterial cells in a culture, employing a photometer that detects the light that microbial cells scatter. OD depends on the concentration of cells in the sample, making it capable of registering fluctuations in bacterial growth in response to the addition of various xenobiotics, including

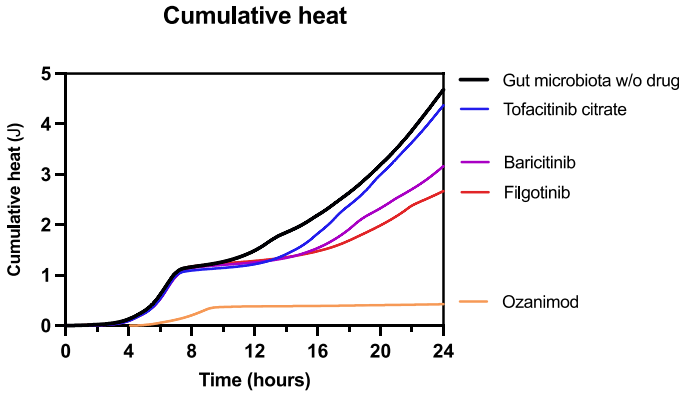

Fig. 2. Cumulative heat (expressed in joules) of the TAM curves of the bacterial growth from TAM experiments.

drugs and excipients. In this study, the OD of both the samples containing the drug ($n = 3$) and the control ($n = 3$) were measured to assess whether the drug decreased or increased the absorbance of the suspension, thus inhibiting or stimulating the growth of microbial cells. The JAK inhibitors did not appear to influence the number of bacterial cells, consistent with the findings obtained with TAM. No statistically significant differences were observed at 7 h and 24 h. In the case of baricitinib and filgotinib, a minor effect on bacterial growth was noted using calorimetry, but this effect was not mirrored by the optical density measurements. One possible explanation for this discrepancy lies in the principles underlying these techniques. TAM is based on the heat generated by the bacteria as a result of their metabolic activity, while OD relies on the count of cells, whether they are alive or dead, within the sample at a given time. Hence, it is plausible that baricitinib and

filgotinib may slow down the bacteria's metabolism, leading to a reduced release of energy, without necessarily affecting the overall number of bacteria growing in the samples, as indicated by the OD experiments. This suggests that drugs impact microbial metabolism more than growth, highlighting the importance of using multiple methods to fully understand drug effects on microbiota. In the case of ozanimod, however, the same outcome observed with isothermal calorimetry was confirmed by OD measurements. From 7 h, the number of bacteria present was significantly lower in the test sample with the drug ($p < 0.0001$) when compared to the reference sample without the drug, indicating a possible antimicrobial activity of ozanimod.

Despite the small set of drugs used in this study, TAM and OD are demonstrated to be a valuable tool for the initial screening of the antimicrobial activity of drugs on communities of selected bacteria, or gut microbiota samples. For instance, they offer the advantages of registering microorganisms' growth in a simplistic, quick, non-destructive and low-cost way. TAM is characterised by great sensitivity, reproducibility, and baseline stability, and the colour of the sample does not interfere with the analysis. However, since the data it collects is the sum of all the biological, chemical, or physical processes taking place in a sample, OD complements TAM by measuring the total concentration of cells in a bacterial culture. To determine whether TAM and OD are dependable methods for assessing the effects of xenobiotics on the survival and reproduction of a complex human gut microbiota sample, metagenomic next-generation sequencing (NGS) provides a detailed understanding of how the overall composition and function of the gut microbiota changes when the drug is present in the broth media.

4.1.2. Metagenomics

The same GM sample utilised in the aforementioned studies involving TAM and OD was subsequently sent to Eurofins Genomics (Germany) for INVIEW Metagenomic Advanced analysis. The GM control sample, untreated with drugs, was compared to the identical GM sample treated with tofacitinib citrate, baricitinib, filgotinib, and ozanimod. Analysis of (i) taxonomic profiling (ii) functional profiling, and (iii) anti-bacterial resistance profiling was conducted. The growth medium of choice for this study was BB broth which was shown to capture the majority of the bacterial diversity in fresh feces after a 24 h incubation (Tao et al., 2023), without uncontrolled expansion of the facultative anaerobes, *Enterobacteriaceae*, at the expense of the obligate anaerobes, *Ruminococcaceae*, which can be the case of some other culturing media (Javdan et al., 2020). In healthy humans, the colon is generally characterised by a high abundance of Bacteroidetes (*Bacteroidaceae* and *Enterobacteriaceae* families), Proteobacteria and Firmicutes (*Lachnospiraceae, Enterococcaceae* and *Ruminococcaceae* families) (McCallum and Tropini, 2023; Donaldson et al., 2016), although variations in compositions between individuals are substantial. The GM sample used to study the antimicrobial effect of JAK inhibitors and ozanimod had a low variability in phyla with 96.0 % *Firmicutes*, 2.5 % Proteobacteria, 1.5 % Actinobacteria and <0.1 % Bacteroidetes. Therefore, the predominance of Firmicutes represented a limitation in our study since the effect of JAK inhibitors and ozanimod could be observed mainly in this phylum.

4.1.2.1. Taxonomic profiling. Taxonomic profiling enabled the examination of microbial community composition (bacteria, archaea, eukaryotes, and viruses) through metagenomic shotgun sequencing data with species-level resolution. The total reads were purged of host-related sequences, constituting 0.07–0.08 % of the total raw sequences. The final number of high-quality reads, classified using MetaPhlAn (Truong

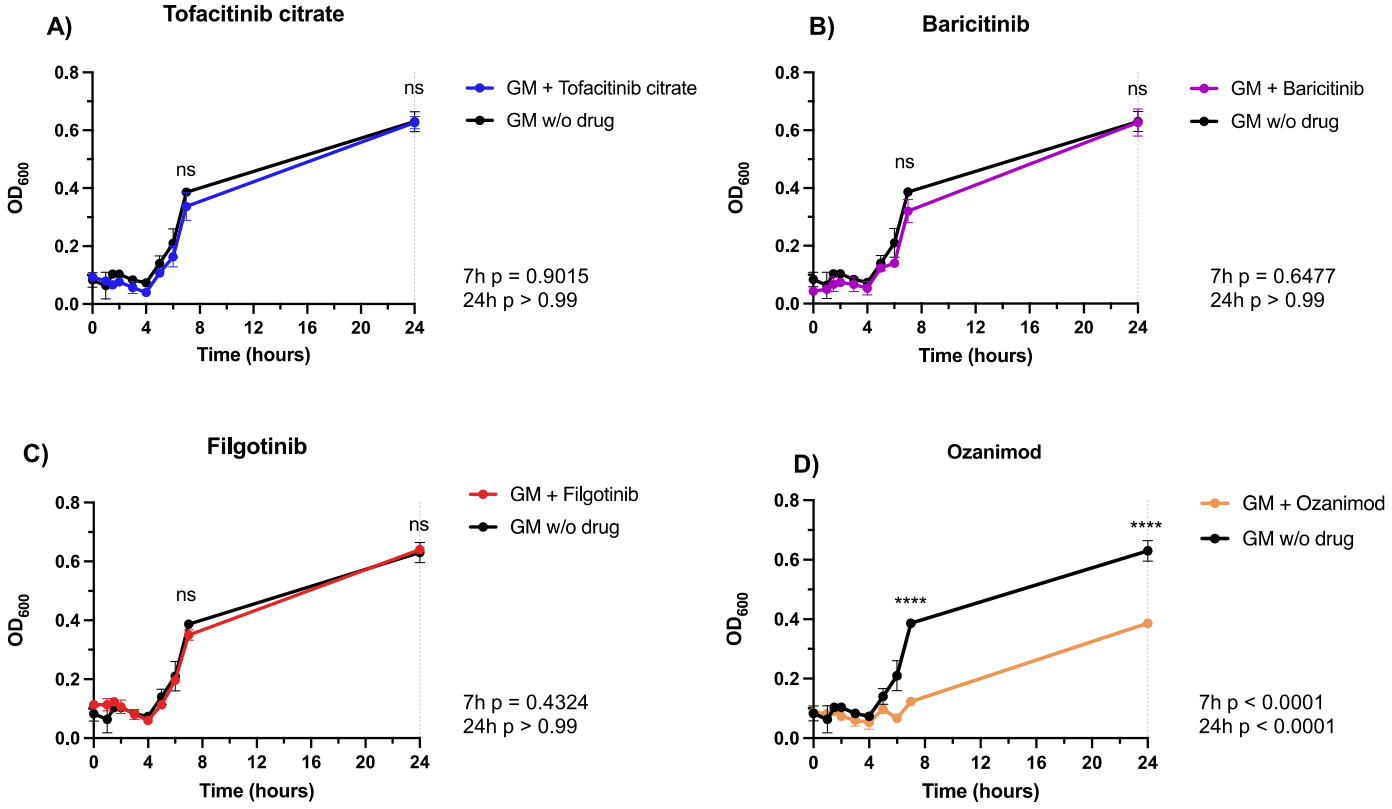

Fig. 3. OD graphs of the growth of the gut microbiota sample, with and without the drug, in 24 h experiment; with respective statistical *t*-test of the OD values at 7 h and 24 h. A) Tofacitinib citrate: the blue line represents the growth of the bacteria in the presence of the drug ($n = 3$), vs the control sample without the drug ($n = 3$). B) Baricitinib: the pink line represents the growth of the bacteria in the presence of the drug ($n = 3$), vs the control sample without the drug ($n = 3$). C) Filgotinib: the red line represents the growth of the bacteria in the presence of the drug ($n = 3$), vs the control sample without the drug ($n = 3$). D) Ozanimod: the yellow line represents the growth of the bacteria in the presence of the drug ($n = 3$), vs the control sample without the drug ($n = 3$).

et al., 2015), were used for taxonomic profiling. The bacterial community constituted > 99.9 % of the microbes in each sample, with their classification based on genera in Fig. 4A, and species in Fig. 4B, in percentage (left) and absolute raw reads (right).

The control GM sample was predominantly composed of the phylum Firmicutes, with *Ruminococcus faecis* representing 45 % of the entire bacterial community, and *Enterococcus* species, specifically *faecium, durans, hirae*, and *faecalis*, comprising 29.44 % in descending concentrations. *Peptoniphilus harei* and *Dorea longicatena* together constituted another 30 % of the GM community. *Lactobacillus delbrueckii* and *Ruminococcus torques* each accounted for 3 % of the bacterial composition. Other species were present in smaller proportions. When analysing the taxonomic profiling data derived from metagenomic sequencing, the introduction of tofacitinib citrate to the GM sample led to an increase of two times the number of absolute raw reads of genera compared to the GM control sample (3.012 vs 5.961 × 10^6). The species *Dorea Longicatena,* increased from approximately 5 % to 30 %, at the expense of *Enterococcus faecium* and *Ruminococcus faecis.* The *Dorea* genus, a member of the Firmicutes phylum, encompasses various species, with *D. longicatena* and *D. formicigenerans* being the most prevalent. The effects of *Dorea spp*. depend on the context of the overall gut microbiota composition and the specific health conditions of the individual. For instance, *Dorea* spp. was found to be increased in the subjects with colorectal adenoma, compared to the normal healthy colon composition (Shen et al., 2010), and its abundance diminished in subjects affected by hypertriglyceridemia-associated acute pancreatitis (Hu et al., 2021). In a

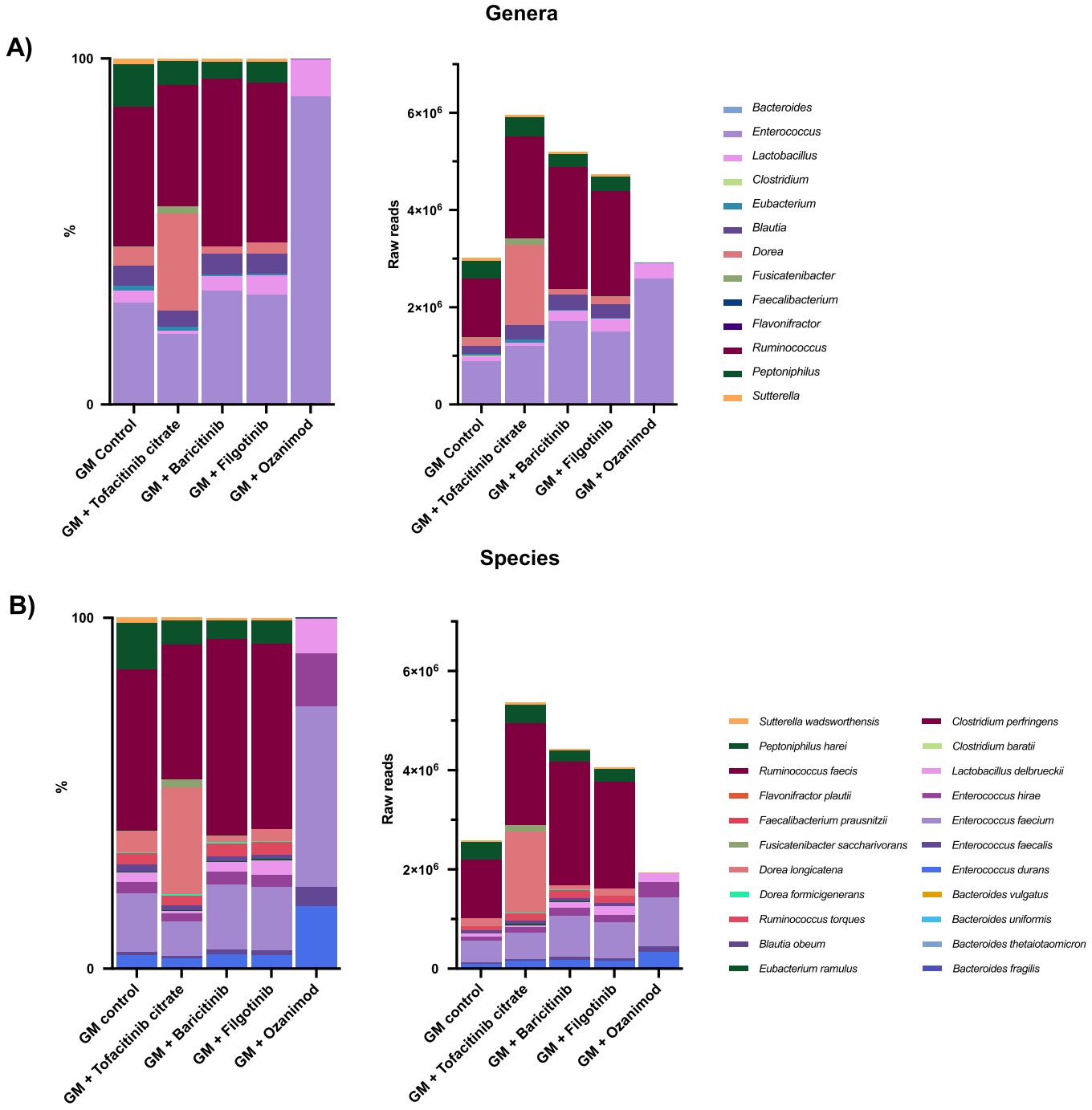

Fig. 4. Taxonomic profiles of GM samples, treated with tofacitinib citrate, baricitinib, filgotinib and ozanimod and compared to control GM sample. A) taxonomic profiling at a genera level, B) species level. Bar plots represent relative bacterial genera and species in percentage (left), and absolute raw reads (right).

study involving Crohn's disease patients, a significant enrichment of *D. longicatena* was observed in those patients experiencing remission after ileocolonic resection. This enrichment co-occurred with heightened levels of bacteria from *Bacteroides, Ruminococcus* and *Dialister* genera (Mondot et al., 2016), highlighting a potential negative correlation between *Dorea* genus abundance and IBD. This correlation was also reinforced by the discovery that the abundance of *D.longicatena* was significantly reduced by 39.7-fold in an IBD group of 11 patients when compared to a healthy group (Dahal et al., 2023). Given this context of diminished richness of the *Dorea* genus in IBD patients, the findings of our study suggest that the stimulation of *Dorea* growth by tofacitinib citrate could potentially serve as an additional beneficial mechanism of action, supplementing its anti-inflammatory activity. As an increasing body of research is confirming an intricate connection between IBD and dysbiotic gut microbiota, it is important to explore therapeutic approaches that not only alleviate inflammation but also contribute to the preservation and potential restoration of a balanced and healthy gut microbiota in these patients. Such dual-action therapies may hold promise in addressing the multifactorial nature of IBD, offering a new strategy towards improved clinical outcomes and long-term gut health for affected individuals.

Baricitinib and filgotinib did not display disruptive effects on the composition of the control GM sample; rather, they appeared to stimulate bacterial growth, as evidenced by increased raw read counts of 5.189×10^6 and 4.734×10^6 for baricitinib and filgotinib, respectively, compared to 3.012×10^6 in the control. However, the most noteworthy finding from the metagenomic results was the impact of ozanimod on the gut microbial composition. Ozanimod led to the reduction of both Proteobacteria and Bacteroidetes. Within the Firmicutes phylum, it diminished all genera to <0.2 %, increasing instead the *Enterococcus* genus (88.9 % up from 29.4 % in the control) and *Lactobacillus* genus (L. *delbrueckii*) (10.7 %, up from 3.4 %). Among the *Enterococcus* spp. present, including *E. faecium, E. durans, E. hirae*, and *E. faecalis*, all were identified in the GM sample treated with ozanimod. They exhibited concentrations constituting 51.6 %, 17.6 %, 15.0 %, and 5.6 %, respectively, of the total. Quite consistently, the abundance of *Enterococcus* has been identified as positively associated with IBD, alongside *Fusobacterium, Ruminococcus gnavus, Streptococcus anginosus, Megasphaera, Campylobacter,* sulfate-reducing *Gammaproteobacteria,* and *Deltaproteobacteria* (Pascal et al., 2017; Metwaly et al., 2020; Gevers et al., 2014). Particularly, in murine models, *E. faecalis* has been implicated in chronic intestinal inflammation by compromising epithelial barrier integrity. Hence, the stimulated growth of Enterococcus may raise further investigation into the intricate interplay between ozanimod, gut microbiota, and the inflammatory landscape in IBD patients.

The functional group responsible for the antimicrobial activity of ozanimod is sphingosine, a sphingoid base and key structural component of sphingolipids, whose protonated amino group of sphingosine binds with the negatively charged membrane protein cardiolipin, causing rapid permeabilization of the bacterial membrane. There is no published evidence on the antimicrobial activity of ozanimod drug on the gut microbiota, however, both etrasimod and fingolimod, belonging to the same S1P receptor modulator family, were both discovered to exert powerful activity against *Staphylococcus aureus* (Zore et al., 2022; Zore et al., 2021). Moreover, etrasimod displayed bactericidal activity and reduced viable bacteria within 1 hour of exposure, with the strongest activity against Gram-positive bacteria, while fingolimod showed the strongest antimicrobial activity against the Gram-negative bacteria *Acinetobacter baumannii* and *Pseudomonas aeruginosa* (Zore et al., 2022; Zore et al., 2021). The antibacterial activity of the compounds was evaluated at a concentration ranging from 2.5 to 50 μM (etrasimod) and 25 to 200 μM (fingolimod) (Zore et al., 2022; Zore et al., 2021).

4.1.2.2. Functional profiling. Complementing the taxonomic profiling, the Shannon index was utilised to assess microbial diversity in terms of genera, species and Gene Ontology (GO) (Fig. 5A) (Kim et al., 2017). Tofacitinib citrate caused a slight increase in genus diversity (Shannon index: 1.60) compared with the control (1.57), due to the enrichment of *Dorea*. A Shannon index value of 0.37 for genera confirmed that the strongest alteration in microbial diversity was observed with ozanimod, correlating well with the taxonomic data. However, when examining the functional diversity through GO terms, the Shannon index across all the samples remained similar to the control, suggesting that the functional profile of the microbiota is resilient to the compositional changes induced by the drugs. Moreover, the slight increase in functional diversity with ozanimod may suggest that the surviving gut community induce a compensatory mechanism to maintain metabolic function despite a decrease in taxonomic diversity.

In the Bray-Curtis dissimilarity analysis (Fig. 5B), ozanimod consistently appeared as the most disrupting treatment on the microbial community structure and function, differing from the control and other drug samples across all genera, species, and GO categories. Baricitinib and filgotinib consistently clustered together across all analyses, indicating a similar and less disruptive impact on microbial composition and function.

Delving into the specific biological processes (BP) affected by drug treatments, the UpSet plot illustrated in Fig. 6 selectively examines the intersection of BP terms across untreated and treated samples. It reveals that the second most common shared set of pathways is the one containing all samples aside from those exposed to ozanimod, demonstrating that ozanimod has a different effect on microbiota compared to JAK inhibitors. Notably, the 22 BPs unique to the drug-treated samples are predominantly metabolic pathways, highlighting the substantial impact these drugs have on microbial metabolism. These pathways include crucial processes such as polysaccharide, sphingolipid, and inositol metabolism, as well as other functions like protein catabolism and arginine deiminase pathways. This convergence of metabolic BPs under drug influence led to the decision to refine the functional profiling through a heatmap specifically tailored to categories pertinent to metabolism: carbohydrates, lipids, peptides, nucleotides, secondary metabolites, cofactors, SCFAs, and tryptophan metabolism (Fig. 7). By isolating these categories, we aim to obtain a clearer understanding of each drug's nuanced effects on the microbiome's metabolic functions, as expressed in gene count per million.

One of the main modes by which the gut microbiota interacts with the host is through the synthesis of metabolites. IBD is characterised by alterations in the composition, function, and metabolite profile of the microbiota. Several classes of microbial-derived metabolites play significant roles in IBD pathogenesis, including altered metabolism of bile acids, SCFAs, and tryptophan derivatives (Lavelle and Sokol, 2020). In the current dataset, no genes involved in bile acid metabolism were identified, which may be due to the specific composition of the gut microbiota samples, mainly dominated by Firmicutes. Instead, metabolic pathways involving SCFAs and tryptophan were found. The biosynthesis of poly-hydroxybutyrate, a component of the SCFA profile, was detected when the gut microbiota sample was treated with all the IBD drugs, and not in the control, indicating a potential alteration in the production of this metabolite in response to drugs, while the butyrate metabolic process gene, a key SCFA in colonic health, was absent across the samples except for a minimal detection in the tofacitinib-treated sample. Interestingly, lactate oxidation and the catabolic process to d-lactate via S-lactoylglutathione were increased in the drug-treated samples compared with the control, particularly so with tofacitinib citrate. Indeed, *Dorea spp*. are associated with a $>$ 6-fold increase in lactate oxidation gene expressions. This suggests that tofacitinib citrate, through the modulation of microbial composition, could be encouraging the gut microbiome to support processes related to SCFAs, which could be positive in an inflammatory context. Tryptophan is a precursor of many significant bioactive molecules, including serotonin, melatonin, nicotinamide and vitamin B3. Its metabolism occurs predominantly in

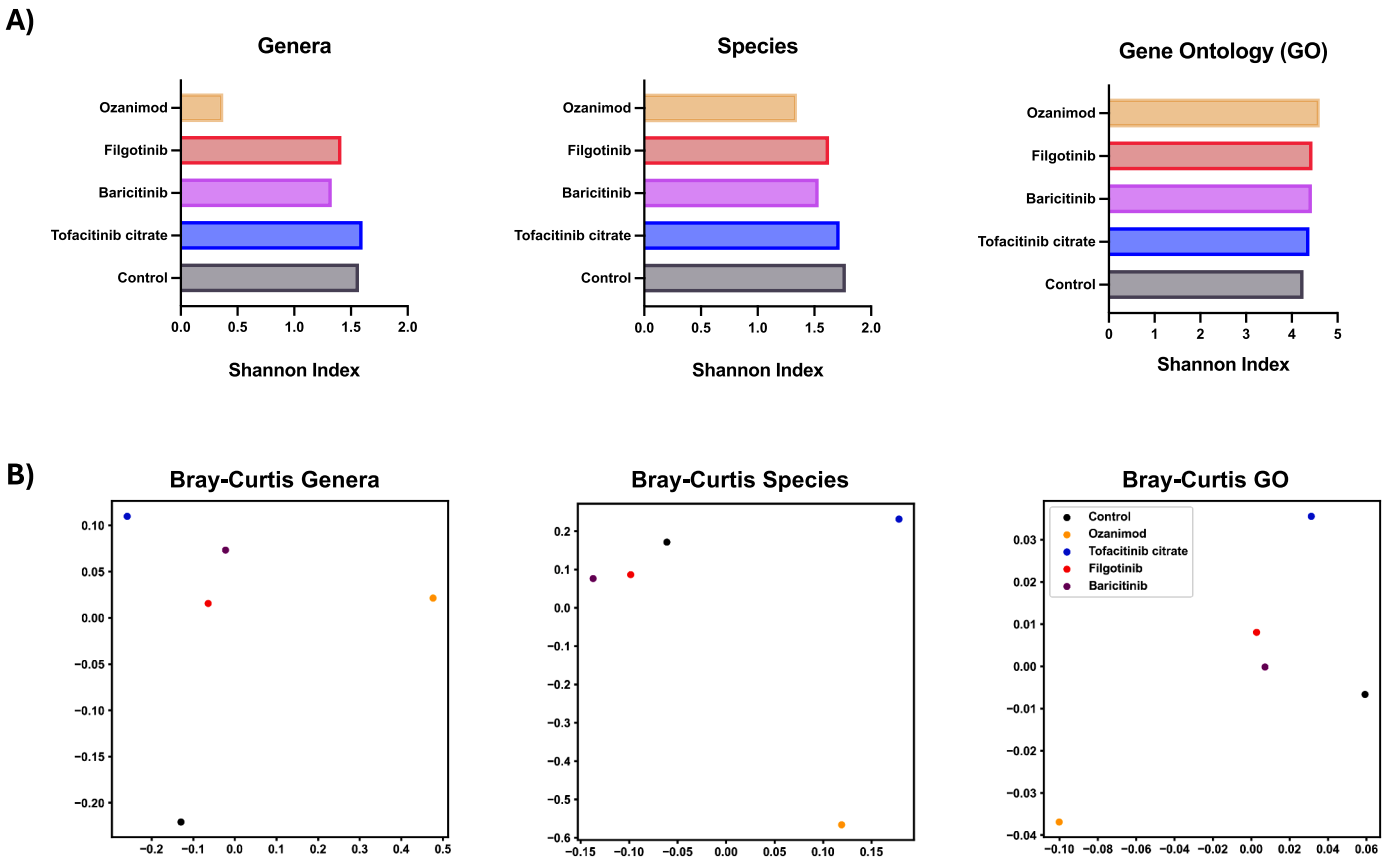

Fig. 5. A) Shannon Diversity Index based on genera, species and gene ontology (GO) terms. B) Bray-Curtis dissimilarity based on genera, species and gene ontology (GO) Terms.

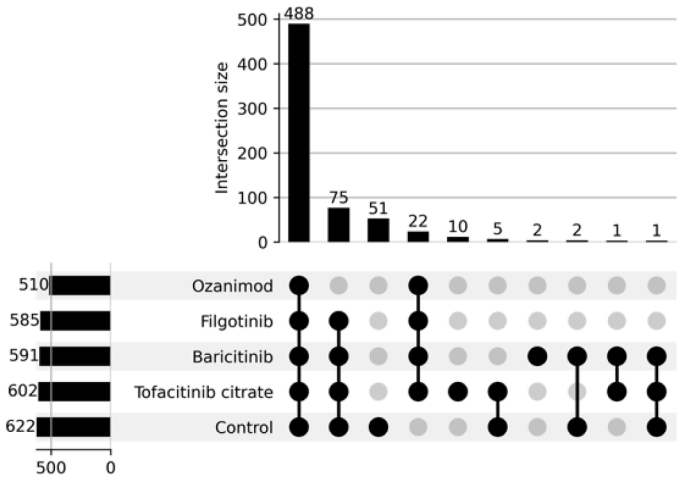

Fig. 6. Comparative analysis of biological processes (BP) affected by the gut microbiota in response to the drugs. The intersection size represents the number of shared and unique biological processes across the control and treated groups. The bar graphs on the left denote the total number of biological processes identified in each treatment, while the connected dots on the right illustrate the overlap between groups.

the gastrointestinal tract, where the gut microbiota is responsible for the production of metabolites with diverse effects on mucosal immunity and homeostasis. Moreover, decreased tryptophan serum levels were associated with increased IBD disease activity, and its deficiency could contribute to IBD development. In our findings, an increase in tryptophan biosynthesis was shown in the presence of JAK inhibitors, through the increased expression of the genes tryptophanyl-tRNA aminoacylation and tryptophan biosynthetic process, when compared with the control samples, therefore indicating a possible beneficial action of these medications in an inflamed gut.

Overall, while the taxonomic diversity of the microbiota was impacted by the treatment with IBD drugs, there is a slight functional resilience of the gut microbiome sample treated with the drugs when looking at specific metabolic processes linked to SCFAs and tryptophan metabolism. These findings, despite being limited in terms of sample size and microbiota variety of the samples, highlight the intricate relationship between the gut microbiota and pharmacological interventions. Further research is needed to understand the full spectrum of microbiome changes in response to these drugs and their implications for IBD treatment strategies.

4.1.2.3. Resistance profiling. Finally, a resistance profiling was conducted on the control and treated samples, to identify whether drugs induced the expression of antimicrobial-resistant genes (ARGs). Compared with the control that showed resistance genes to the treatment of tetracycline and trimethoprim, baricitinib was the only drug to develop the resistance of the GM to aminoglycoside in addition to tetracycline and trimethoprim (Supplementary Data, Fig. 4). This expansion of drug resistance could restrict treatment choices for infections and may worsen the already unbalanced gut microbiota in IBD patients, who often depend on antibiotics for their care.

The present study provides an exploratory valuable insight into the potential impacts of IBD drugs on the gut microbiome. TAM and OD represented appropriateness in investigating the capacity of medications to impact the growth of the gut microbiota. Indeed, both approaches could recognise a significant antimicrobial effect of ozanimod, whose effect was confirmed by the metagenomics sequencing technique.

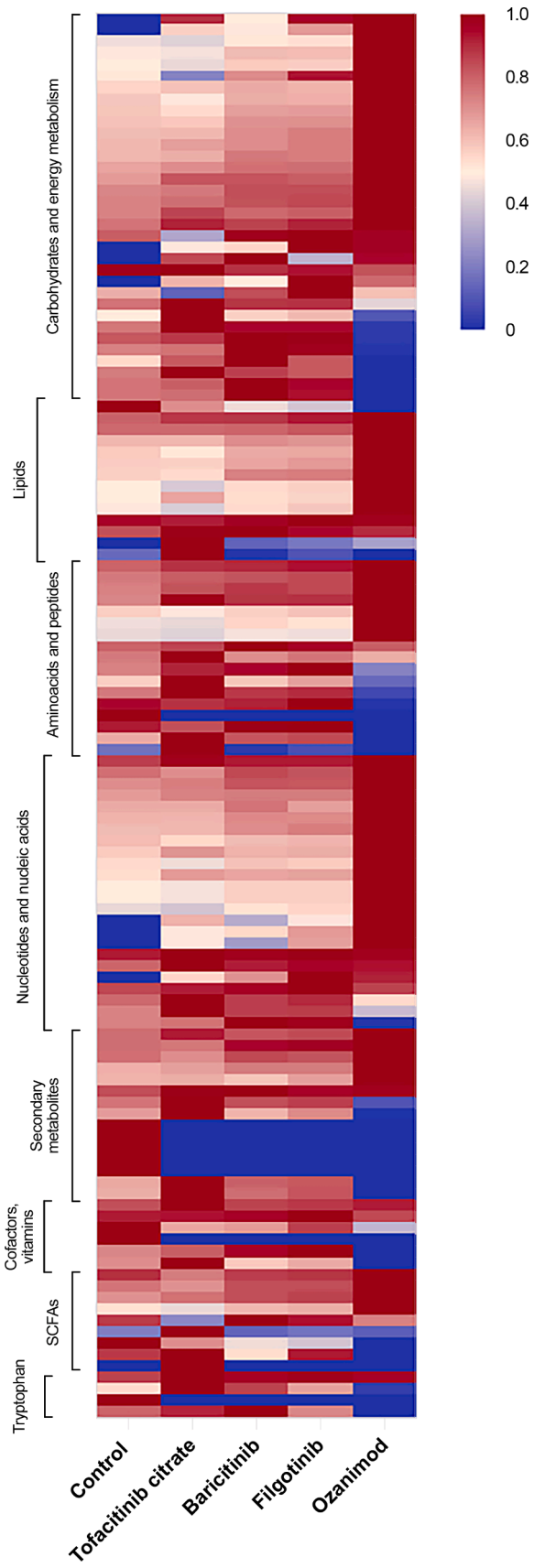

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Fig. 7. Heatmap illustrating the relative activity levels of various metabolic pathways across the four drug treatments. The data is presented as row-normalised counts per million (CPM) for each biological process (BP). Each row corresponds to a specific BP, with values normalised on a scale from 0 to 1 to ensure direct comparability. This normalisation method allows for uniform interpretation of pathway activity irrespective of absolute expression levels, highlighting the differential impacts of each drug on the metabolic functions within the gut microbiome.

However, the small sample size and the absence of replicates limit the ability to generalise these findings. Additionally, the microbiome profiles were predominantly characterised by Firmicutes, reflecting the bacterial composition of the healthy donor samples rather than the more diverse microbiota often associated with IBD. Finally, the short observation period of 24 h might not capture the potential long-term effects of JAK inhibitors and ozanimod on the gut microbiome. While important changes were observed within this short timeframe, as seen with ozanimod, it is plausible that chronic administration of these drugs could lead to even more pronounced and potentially detrimental alterations in gut microbial communities. Over extended periods, the gut microbiome may experience compensatory effects, potentially leading to dysbiosis or other adverse health outcomes. Furthermore, while >71 % of orally administered drugs reach the colon at concentrations of at least 20 μM, as indicated by Maier et al. (Maier et al., 2018), more sophisticated techniques are required to accurately determine the actual colonic concentrations of these drugs. In our previous work (Favaron et al., 2024), we demonstrated the use of the Physiologically based pharmacokinetic (PBPK) modelling to detect the concentration of upadacitinib, a model drug, in the luminal region of the colon in healthy patients, underscoring the importance of such models in future research. This suggests that while the concentrations used in this study provide a valid approximate value, pharmacokinetic modelling could offer more precise data in future studies. Overall, the study's findings lay the groundwork for future research, highlighting the need for more comprehensive studies with increased sample sizes and the inclusion of clinical IBD samples to fully elucidate the microbiome's response to therapeutic interventions.

4.2. Ozanimod, but not JAK inhibitors, is susceptible to bacterial enzymatic activity

>150 orally administered drugs have been found to be metabolised by gut microorganisms (Zimmermann et al., 2019). This mechanism is particularly significant in the context of IBD, where it is crucial for the drug to remain active and not be prematurely metabolised by the gut microbiota, ensuring its efficacy at the local site of inflammation in the colon.

To test the susceptibility of tofacitinib citrate, baricitinib, filgotinib, and ozanimod to microbial biotransformation, we first evaluated the enzymatic activity of the six GM samples for their capability of metabolising metronidazole, a previously tested model drug substrate of the enzyme nitroreductase (Karatza et al., 2017). As a positive control, we demonstrated that the GM samples' efficiency in degrading metronidazole is comparable to that observed in the original faecal slurries from which the GM samples were derived (Supplementary Data, Fig. 3).

Tofacitinib citrate, baricitinib, filgotinib, and ozanimod have low solubility in water (< 0.4 mg/mL), similar to the solubility in BB broth which is an aqueous culture medium. Therefore, the cosolvent DMSO was used at the concentration of 1 % v/v on the final bacterial culture volume, to allow the complete solubilisation of the drugs to evaluate whether they are susceptible to microbial biotransformation. In the context of understanding the complicated relationship between the specific drug and the gut microbiota samples, DMSO should not affect the gut microbiota growth and metabolic function. In addition to the literature which is already published on the safety of 1 %v/v DMSO as a drug cosolvent for bacteria (Maier et al., 2018; Zore et al., 2022), a TAM

experiment was conducted to show that DMSO did not decrease the growth of the gut microbiota sample that was used in this study (Supplementary data, Fig. 2).

The use of static batch culture to evaluate the stability of drugs in the gut microbiota is a well-established technique (Yadav et al., 2013; Vertzoni et al., 2018; Sousa et al., 2014). A drug concentration of 100 μM of the four drugs was incubated with 8.3 % v/v of six healthy donor microbiota samples, in anaerobic conditions for 24 h, and an OD assay was performed at 0 and 24 h to ensure the growth of the bacteria in the static batch culture and to ensure no bacteria grew in the control sample. Tofacitinib citrate, baricitinib and filgotinib were not depleted by the bacteria, with an average of respectively 85 %, 83 % and 85 % of free drug at 24 h (Fig. 8). Even considering interindividual variability, the enzymatic activity of the different gut microbiota samples did not pass the 80 % depletion cut-off over 24 h. Previous literature demonstrated tofacitinib citrate to be stable over 6 h in rat caecal slurry (Yadav et al., 2022), and our experiment confirmed the findings in human-derived samples over 24 h. Filgotinib is reported to have an active major metabolite which represents 92 % of the biotransformed parent drug. The enzyme involved in the metabolism of filgotinib is carboxylesterase (CES), in particular CES isoform 2, which is localised in the epithelia of the metabolic organs including the liver, intestine and kidney (Wang et al., 2018). Additionally to CES 1 and 2, CES was also found to be a bacterial enzyme responsible for xenobiotic metabolism (Yu et al., 2019), but no research showed whether filgotinib is a substrate of this microbial enzyme isoform. In our experimental work, filgotinib was stable when incubated with the donors' GM samples, either meaning that CES, if present, is not specific for the metabolism of the filgotinib ester group, or that the metabolic activity of these specific bacterial species was not strong enough to greatly reduce free drug concentration, which remained above 80 % for all donors. In contrast, in line with our findings, ozanimod has already been shown to undergo extensive metabolism by the liver and the gut microbiota, with 14 metabolites identified (Surapaneni et al., 2021). The gut microbiota is responsible for the oxadiazole ring-opening of ozanimod: the relative metabolites RP112533, RP112374 and RP112480 are found in the feces at the concentrations of 7.7 %, 12.2 % and 5.3 % respectively (Surapaneni et al., 2021). Our in vitro work aligns with the microbial depletion of free ozanimod observed in vivo, whose concentration was below 40 % in three out of the six gut microbiota donors over 24 h.

5. Conclusion

This work represents the first study to explore the bidirectional interaction between JAK inhibitors and ozanimod and the human gut microbiota. Specifically, the complimentary use of isothermal microcalorimetry and optical density was demonstrated as an effective *in vitro* approach to assess the potential impacts of drugs on the growth of the gut microbiota. These techniques offer a rapid, cost-effective means to pre-screen drugs for antimicrobial activity in complex microbial communities, setting the stage for efficient pre-clinical evaluations for new medications. Moreover, based on the preliminary findings of this study, it appears that JAK inhibitors, which do not exhibit a significant impact on bacterial growth and function and show good stability in the gut microbiota environment, could be suited for a colon targeted formulation. In contrast, the suitability of ozanimod for such formulations is questionable. Its pronounced antimicrobial activity significantly hampers the growth of various microorganisms, and its vulnerability to

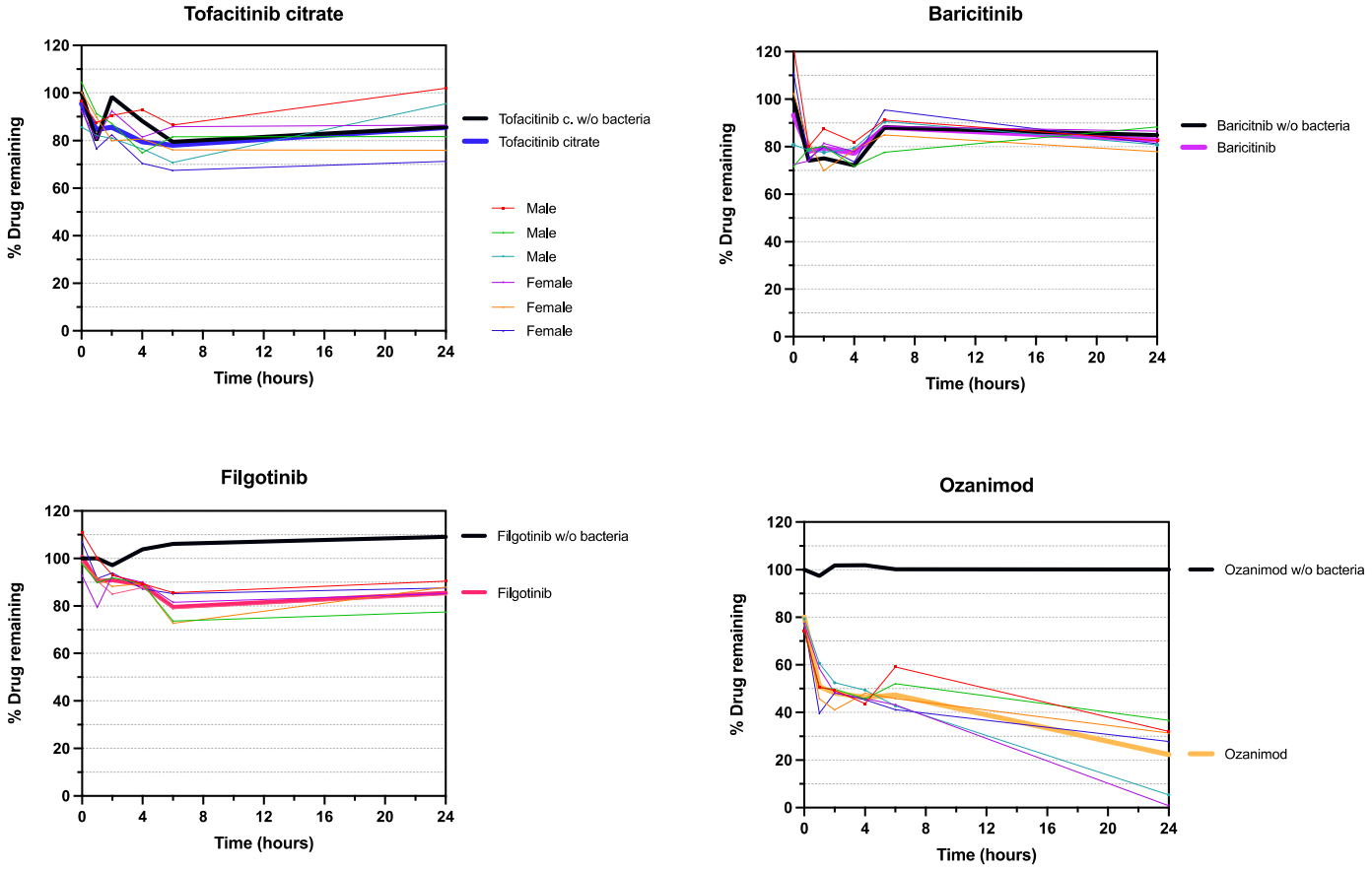

Fig. 8. Incubation of the drugs with the gut microbiota sourced from six healthy donors to evaluate drugs' depletion. The control sample is represented by the drug dissolved in BB broth medium, without the presence of any bacteria. Drugs' concentration is detected over 24 h. 80 % degradation was taken as cut-off value for drugs' depletion. Each coloured bold line represents the average values of the six individual donors (three males and three females).

microbial enzymatic activity could raise concerns regarding the stability of the drug in the ileocolonic region. In conclusion, this study emphasises that interactions between medications and the human microbiome must be considered as a pivotal aspect of drug development.

CRediT authorship contribution statement

Alessia Favaron: Writing – original draft, Investigation, Formal analysis, Data curation, Conceptualization. **Youssef Abdalla:** Writing – review & editing, Formal analysis, Data curation. **Laura E. McCoubrey:** Writing – original draft, Formal analysis, Data curation. **Laxmi Prasanna Nandiraju:** Methodology, Data curation. **David Shorthouse:** Writing – review & editing, Validation, Methodology, Formal analysis. **Simon Gaisford:** Writing – review & editing, Methodology, Conceptualization. **Abdul W. Basit:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Mine Orlu:** Writing – review & editing, Supervision, Resources, Methodology, Funding acquisition, Conceptualization.

Data availability

The data that has been used is confidential.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ejps.2024.106845.

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