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Evaluating rat and canine microbiota models for predicting human colonic prodrug metabolism

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ABSTRACT

The rise of microbiome-aware drug development has placed growing emphasis on the need for reliable preclinical tools to evaluate microbiota-mediated drug metabolism. While human faecal models are used, they suffer from practical limitations such as donor recruitment and regulatory constraints. Larger animals like dogs are often assumed to be more translationally relevant yet are resource-intensive and subject to more complex regulatory and logistical requirements. Rats offer a more accessible, cost-effective and scalable alternative. However, it remains unclear whether their faecal material alone accurately reflects colonic metabolism. Specifically, it is unknown whether faecal samples capture the same metabolic activity as more invasive caecal or colonic contents, or how closely they reflect drug degradation in larger animal models or humans. This study aimed to: (i) compare degradation of three prodrugs across Wistar rat faecal, caecal, and colonic compartments; (ii) determine how rat degradation profiles differ from those observed in Labradors; and (iii) evaluate how closely rat and canine data align with published human in vitro results. Degradation kinetics of sulfasalazine, balsalazide, and olsalazine were first assessed. Bioreactors prepared from 10% faecal, caecal, and colonic contents in rats were used. Faecal material showed equivalent metabolic activity to colonic and caecal material across all drugs (two-way ANOVA, $p = 0.233$), with sulfasalazine degrading most rapidly ($t_{1/2} = 29.1$ min), followed by balsalazide ($t_{1/2} = 47.9$ min), and olsalazine ($t_{1/2} = 84.1$ min). These findings indicate that faecal material can reliably substitute for more invasive gut content sampling, offering practical and procedural advantages. Subsequent interspecies comparisons revealed that rats exhibited significantly higher degradation rates than dogs ($P < 0.05$), reflecting known differences in gut microbial density and composition. When benchmarked against published human in vitro data, rat degradation rates were closely aligned with human values, particularly for sulfasalazine (rat: $K = 0.025$ min^{-1}; human: $K = 0.021$ min^{-1}) and balsalazide (rat: $K = 0.015$ min^{-1}; human: $K = 0.009$ min^{-1}). These findings highlight rat faecal material as a practical and translationally relevant model for microbiota-sensitive prodrug metabolism, offering a low-impact alternative to invasive sampling and larger animal studies.

1. Introduction

The human gut microbiome is now understood to be a major influencer of drug fate, shaping both the efficacy and safety of therapeutics. This complex and densely populated ecosystem comprises trillions of bacteria with distinct enzymatic capabilities (Collins and Patterson, 2020; Guzmán-Rodríguez et al., 2018; Qin et al., 2010; Sousa et al., 2014; Wilson and Basit, 2005). These microorganisms perform a broad spectrum of biochemical transformations, including transformation of dietary compounds, endogenous metabolites, and importantly xenobiotics including drugs (McCoubrey et al., 2022). Microbiota-mediated drug metabolism can alter bioavailability, enhance or reduce therapeutic efficacy and generate toxic by-products (Bakshi et al., 2021; Basit et al., 2002; Sehgal et al., 2018). This makes it a critical consideration in the development of orally administered therapies. As understanding of these interactions grows, so too does the need for reliable preclinical

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models that can accurately replicate microbial drug metabolism in the human gastrointestinal tract.

Safety concerns related to toxicity preclude the use of human in vivo studies during the early stages of drug development (Sousa et al., 2008). These limitations have prompted interest in alternative preclinical models that offer better reproducibility, scalability, and regulatory compliance. One method employed to assess microbiota-mediated drug metabolism involves the use of in vitro fermentation systems (Javdan et al., 2020; Van de Steeg et al., 2018; Sousa et al., 2008). These models simulate key aspects of the colonic environment, enabling the study of drug degradation by intestinal microbiota over defined time periods. In vitro models such as human faecal mixtures have been used with suitable in vivo relevance (Van de Steeg et al., 2018; Isenring et al., 2023; Tannergren et al., 2014; Zhu et al., 2024). However, the practical use of human faecal samples is limited: regulatory constraints, and logistical issues related to sample collection and handling can complicate experimental reproducibility (Isenring et al., 2023; Qi et al., 2023). Samples are often too aqueous for uniform processing and vary day-to-day with diet, hydration, and health status (Watson et al., 2019). These challenges have driven interest in animal-based models that are more logistically feasible and operationally practical.

Animal models are frequently employed in gastrointestinal drug research due to their anatomical and physiological relevance (Hatton et al., 2015; Mukherjee et al., 2022). However, using animal microbiota for drug metabolism modelling to study microbiota-mediated metabolism requires careful consideration due to species-specific differences in gut microbial colonisation. Differences at both the taxonomic and functional levels can limit the translatability of findings to humans. Intestinal colonisation depends on a range of physiological factors which vary between species including pH, motility, secretory activity, and transit time (Hatton et al., 2015; Sousa et al., 2008). Additional variability arises from age, diet, and disease state making the selection of an appropriate animal model critical for ensuring translational relevance. Larger species are often assumed to offer greater translational relevance due to physiological similarities with humans. Dogs have long been used for studying gastrointestinal transit and drug absorption due to their size and ease of handling. However, their use is resource-intensive and carries regulatory complexity. This is particularly problematic for early-stage metabolic screening, where lower-impact models may offer comparable insights (Coelho et al., 2018; Koziolek et al., 2019).

As one of the most widely used species in microbiota research, the rat offers a practical low impact alternative. They are cost-effective, logistically manageable, and can be maintained under tightly controlled dietary and environmental conditions (Čoklo et al., 2020; Shah et al., 2023). This presents the opportunity for a more uniform and reliable source of faecal material. However, the suitability of rat faecal material as a surrogate for microbiota-mediated human colonic metabolism of drugs remains unclear. It remains unclear whether faecal samples from rats perform equivalently to more invasive colonic or caecal material, and how closely their degradation profiles align with those observed in humans. Our previous studies have demonstrated that bacterial metabolism can differ markedly between human and rat matrices depending on the drug substrate, highlighting the importance of compound-specific validation when extrapolating preclinical in vitro data to human outcomes. If faecal material can serve as a valid proxy, it could streamline preclinical workflows, reduce animal use, and support the principles of the 3Rs (Replacement, Reduction, Refinement) (Grimm et al., 2023; Wange et al., 2021). Addressing these questions is critical to evaluating the rat's translational relevance as a model for microbiota-sensitive prodrugs. Importantly, rat faecal samples may provide a viable surrogate because they capture much of the gut microbial community while avoiding the need for invasive sampling. The present study investigated whether rat faecal material can serve as a practical and predictive in vitro model for microbiota-mediated metabolism. This study aimed to: (i) determine whether faecal material performs equivalently to more invasive colonic and caecal contents in rats; (ii) compare rat degradation profiles with those of a larger species in dogs, and (iii) evaluate how well these data align with published in vitro human results.

As a test case, we employed a panel of well-characterised microbiota-sensitive compounds - the azo-linked prodrugs of 5-aminosalicylic acid (5-ASA) including sulfasalazine, balsalazide, and olsalazine. These compounds are used to treat mild-to-moderate ulcerative colitis, a chronic inflammatory disorder of the large intestine (Sehgal et al., 2018; Sousa et al., 2014). To achieve targeted delivery, these compounds remain intact in the upper gastrointestinal tract and are activated in the colon by bacterial azoreductases (Bakshi et al., 2021; Beeck et al., 2021). The resulting liberation of 5-ASA at the site of inflammation is essential for their therapeutic efficacy (Le Berre et al., 2020; Crouwel et al., 2021). Because their activation is entirely dependent on microbial enzymatic activity, these prodrugs serve as robust indicators of microbiota-mediated metabolism. This biotransformation is largely mediated by anaerobic bacteria via flavin-dependent, NADH-driven reductive cleavage of the azo bond as shown in Fig. 1 (Aragaw, 2024; Crouwel et al., 2021; Sousa et al., 2014). However, the efficiency of this activation depends on both drug structure and the enzymatic capacity of the host microbiota which can vary significantly between individuals and species (Zahran et al., 2019; Zhang et al., 2018).

2. Materials and methods

2.1. Materials

Sulfasalazine (MW=398.39 g/mol)) was acquired from Sigma-Aldrich CO., St Louis, USA (BN: 094K0957), with a purity of 98 %. Balsalazide disodium (MW=437.3 g/mol) was acquired from Beijing Mediking Pharmaceutical CO., Beijing, China (BN: 050,791) with a purity of ≥99 %. Olsalazine Sodium (MW=364.2 g/mol) was acquired from Beijing Huameihuli Bio-Chem Trade Centre, Beijing, China (BN: 0509,209) with a purity of 99.5 %. 5-aminosalicylic acid (MW=153.1 g/mol) was acquired from Sigma- Aldrich CO., St Louis, USA (BN 093K1055) with a purity of 99 %.

2.2. Animals and sample collection

2.2.1. Rat faecal and intestinal collection

All procedures were approved by the School of Pharmacy's Ethical Review Committee and were conducted in accordance with the Home Office standards under the Animals (Scientific Procedures) Act 1986.

Five male Wistar rats were housed individually with ad libitum access to food and water. Freshly voided faeces were collected over a two-hour period. Each rat was then sacrificed, the abdominal cavity was opened, and the caecum and colon excised. Luminal contents were carefully expressed from both the caecum and colon using gentle manual pressure to avoid mucosal contamination. All collected material (faecal, caecal, and colonic) was weighed and immediately frozen at −80 °C. Samples were subsequently freeze-dried under low pressure to remove moisture by sublimation. Freeze-drying was used to enable long-term sample storage and to standardise moisture content across replicates, preserving the enzymatic and metabolic activity of the gut microbiota upon rehydration under anaerobic conditions (Bensch et al., 2022; Pourrat et al., 2025). To preserve microbial viability, the time between sample collection and freezing was kept under 30 min, after which samples were later reintroduced into anaerobic conditions during bioreactor preparation (Section 2.3.1).

2.2.2. Dog faecal samples

Faecal samples were collected from four male Labrador dogs (2–7 years old, 25–35 kg) supplied by Terje Gammelsrud and Jonas Falck (Norway). The animals were maintained on a controlled diet to ensure consistent faecal composition. Rats were the primary model of interest and were therefore analysed as independent biological replicates (individual animals) to capture biological variability and assess

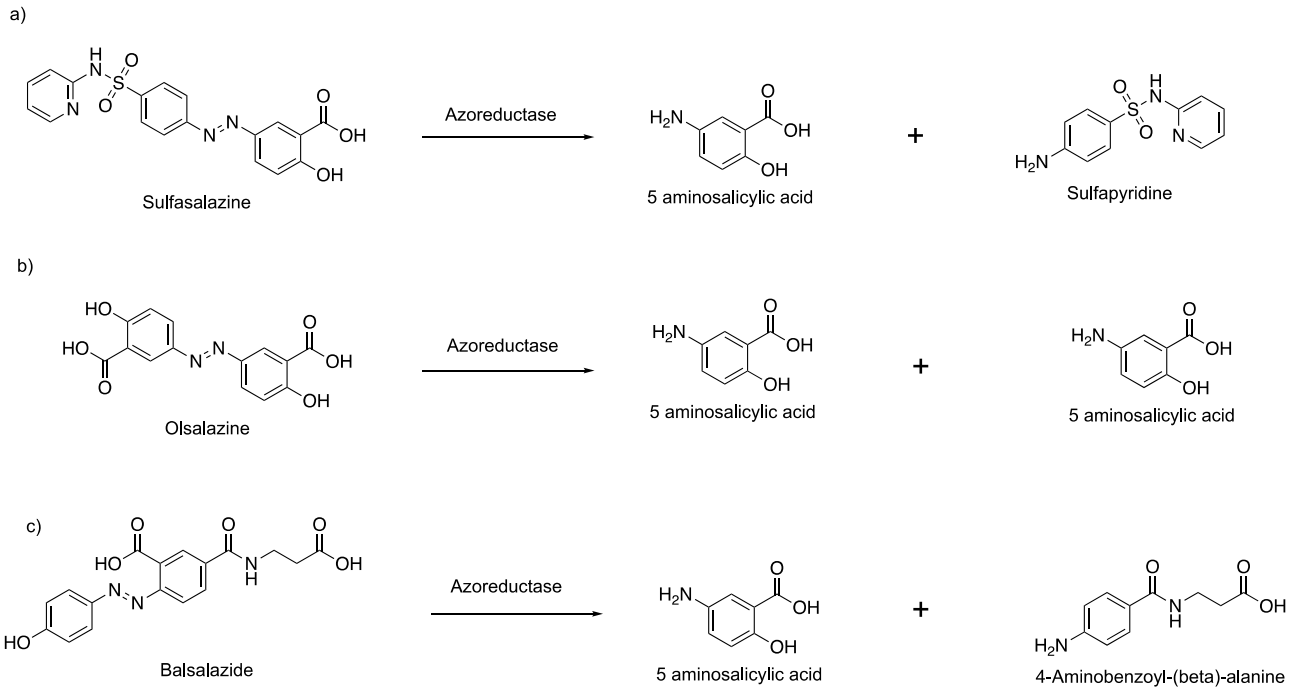

Fig. 1. Azoreduction of 5-ASA prodrugs by colonic bacteria: (a) sulfasalazine, (b) olsalazine, and (c) balsalazide are cleaved at the azo bond to release active 5-aminosalicylic acid (5-ASA) in the colon.

reproducibility. In contrast, canine faecal material was pooled in equal proportions from four donors to minimise inter-individual variation (typically higher in dogs due to dietary and environmental factors) and provide a representative composite microbiota for cross-species comparison. The pooled canine samples were available in sufficient volume from a single collection and were therefore processed immediately under anaerobic conditions, making freeze-drying unnecessary.

2.3. Faecal bioreactor preparation

2.3.1. Rat faecal, caecal, colonic bioreactor preparation

Dried faecal, caecal, and colonic contents were transferred to an anaerobic workstation (Electrotek AW500TG) under strict anaerobic conditions (37 °C, 70 % humidity). Contents from three rats were weighed and suspended in saline phosphate buffer (pH 6.8) to achieve a final mixture concentration of 10 % (w/v). Homogenisation was performed using a disperser for 5–10 min, followed by sieving through a 350 μm mesh. The resulting mixture was used to assess the degradation of sulfasalazine, balsalazide, and olsalazine, each at a final concentration of 500 μM. A total volume of 600 μL was used for each sample, with 50 μL aliquots withdrawn at 0, 15, 30, 60, 120, 180, and 240 min. Samples were centrifuged at 10,000 rpm for 10 min prior to analysis.

2.3.2. Dog faecal bioreactor preparation

Fresh faecal samples were collected from four healthy male Labrador dogs and pooled to minimise inter-individual variability. Equal proportions were used to avoid over-representation of any single donor and to standardise microbial input across conditions. The pooled material was transferred into an anaerobic workstation (Electrotek AW500TG) within 30 min of collection. Samples were then homogenised in saline phosphate buffer (pH 6.8) for 10 min using a disperser, followed by sieving through a 350 μm mesh. The resulting mixture was diluted to a final concentration of 10 % (w/v). Sulfasalazine, balsalazide, and olsalazine were added to the bioreactor at a final concentration of 500 μM (see Section 2.4 for rationale). Each degradation study was conducted in a final volume of 600 μL, from which 50 μL aliquots were collected at 0, 15, 30, 60, 120, 180, and 240 min. All samples were centrifuged at 10,000 rpm for 10 min to separate the supernatant for subsequent analysis.

2.4. Drug incubation studies

Stock solutions of sulfasalazine, balsalazide, and olsalazine were prepared by dissolving the compounds in saline phosphate buffer (pH 6.8) before being added to the mixtures (faecal, caecal, colonic) to achieve a final concentration of 500 μM. A concentration of 500 μM was selected because it falls within physiologically relevant ranges for colonic exposure and enables robust kinetic measurement over the incubation period. This concentration is also consistent with previous in vitro studies of these prodrugs, including the published human data used for benchmarking (D'Incà et al., 2013; Sousa et al., 2014). The drug-incubated mixtures were maintained under anaerobic conditions at 37 °C. At predetermined time points (0, 15, 30, 60, 120, 180, and 240 min), 50 μL aliquots were withdrawn and immediately mixed with ice-cold acetonitrile in a 1:3 (v/v) ratio to quench enzymatic activity and precipitate proteins. Control samples, consisting of drug incubated in saline phosphate buffer without microbial material, were collected at the same time points to confirm chemical stability of the compounds under assay conditions. All samples were prepared in triplicate. Following sample collection, the mixtures were centrifuged at 10,000 rpm for 10 min, and the supernatant was collected for drug quantification via High-Performance Liquid Chromatography (HPLC).

2.5. HPLC/UV analysis

The drug samples were analysed using an HPLC system (1260 Infinity, Agilent Technologies, Stockport, UK) equipped with diode-array detector (DAD). Separation of sulfasalazine, balsalazide, and olsalazine was performed on a Waters Atlantis T3 column (5 μm, 4.6 × 150 mm) with UV detection at 310 nm. 5-aminosalicylic acid was analysed on a Merck Lichrospher 100 RP-18 endcapped column (5 μm, 250 × 4 mm) with UV detection at 228 nm. The instrumental control, data acquisition and analysis were performed via the supporting software Agilent OpenLAB. The standard curves of sulfasalazine, balsalazide,

olsalazine and 5-ASA were constructed using triplicates at each concentration, with the linearity recorded of 0.9925, 0.9866, 0.9913 and 0.9809 respectively. 5-ASA was monitored as a degradation product of all three prodrugs to confirm azo bond cleavage, rather than as an independent control compound.

2.6. Data analysis

All experiments were performed in triplicate, and results are reported as mean ± standard deviation (SD). For rat studies, replicates represent independent biological samples derived from different animals. For dog studies, faecal material was pooled prior to analysis with replicates representing technical repeats from the pooled mixture. Degradation kinetics were determined by fitting the percentage of drug remaining over time to a first-order decay model using non-linear regression (least squares fit) in GraphPad Prism 10. From this model, degradation rate constants (K) and corresponding half-lives ($t_{1/2}$) were derived for each replicate. These constants are derived parameters from the fitted model, not direct measurements of concentrations. Fits were performed with the plateau constrained to zero, ensuring consistency across replicates. Statistical analysis was performed using two-way ANOVA, with drug type and compartment (rat study) or drug type and species (rat vs. dog study) specified as fixed factors, followed by Šídák's multiple comparisons test to identify significant pairwise differences. K values were analysed without transformation, and ANOVA outputs are reported as P values for main effects and interactions. Post hoc Šídák comparisons tested (i) species differences for each drug and (ii) drug differences within each species or compartment. Outputs are presented as mean differences with 95 % confidence intervals and adjusted P values. Assumptions of normality and homogeneity of variance were evaluated by inspection of residuals and found to be acceptable. Non-linear regression fits used to derive K values showed high goodness of fit, with R^2 values exceeding 0.90. Statistical significance was defined as $p < 0.05$.

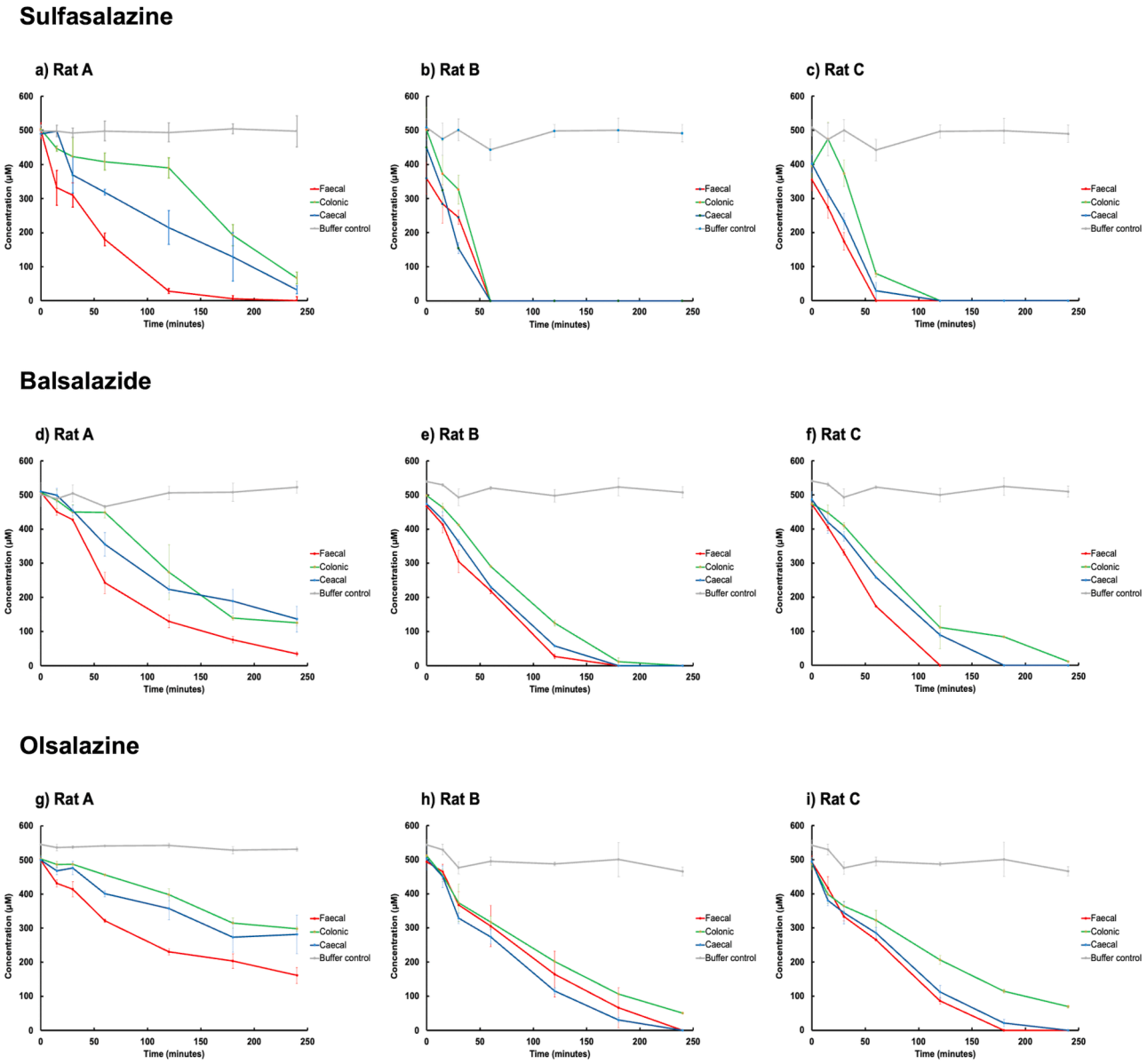

Fig. 2. In vitro degradation kinetics of azo-linked prodrugs in 10 % rat gastrointestinal content mixtures. Drug stability was assessed using faecal, caecal, and colonic contents collected from individual rats (n= 3 per drug). Panels (a–c) show the degradation of sulfasalazine in faecal, caecal, and colonic mixtures from Rat A, B, and C respectively. Panels (d–f) display balsalazide degradation under identical conditions. Panels (g–i) present olsalazine degradation profiles from the same three rats.

3. Results and discussion

The degradation profiles of sulfasalazine, balsalazide, and olsalazine were first assessed across different gastrointestinal compartments in rats shown in Fig. 2. This figure illustrates the degradation kinetics for each compound across faecal, caecal, and colonic mixtures, while Table 1 summarises the corresponding rate constants (K) and half-lives ($t_{1/2}$) derived from non-linear regression modelling. A two-way ANOVA was performed to statistically assess the effects of drug type and mixture type within rats on the metabolic degradation rate of the three prodrugs. The analysis identified significant main effects of drug type ($p = 0.0027$) but no significant effect of mixture type ($p = 0.233$) or interaction between drug type and mixture type ($p = 0.962$). Pairwise comparisons using Šídák's multiple comparisons test revealed that for all three drugs, there were no significant differences in K values between the different compartments (faecal, caecal, and colonic) for any of the drugs. For sulfasalazine, comparisons of faecal vs. caecal ($p = 0.993$), faecal vs. colonic ($p = 0.435$), and caecal vs. colonic ($p = 0.587$) K values were not significant. Similarly, for balsalazide and olsalazine, no significant differences were found between any pairwise comparisons of faecal, caecal, and colonic K values ($p > 0.05$).

The caecum is considered the primary site of microbial fermentation and xenobiotic biotransformation, making it a relevant benchmark for assessing microbiota-mediated drug metabolism (Brown et al., 2018; Čoklo et al., 2020; Hatton et al., 2015). Despite the faecal mixture showing slightly higher mean degradation rates across all three drugs (Fig. 2), the lack of significant difference indicates that the faecal mixture performs equivalently to caecal and colonic content in supporting drug degradation. These results suggest that microbial metabolic capacity is broadly conserved across these colonic regions in rats, at least in the context of azo reduction of sulfasalazine, balsalazide, and olsalazine. Despite higher variability in caecal and colonic half-lives, particularly for sulfasalazine, the larger standard deviations likely reflect greater heterogeneity in microbial density and activity within these compartments. Nonetheless, the consistent lack of statistically significant differences across compartments indicates that this variability does not alter the overall interpretation of equivalent metabolic activity. Visual inspection indicated that Rat A exhibited slower degradation for some conditions (notably olsalazine; Fig. 2), which is reflected in the wider SDs and CIs in Table 1. However, the two-way ANOVA confirmed no significant effect of compartment and no significant pairwise differences among faecal, caecal, and colonic K values. This is consistent with the known distribution of gut microbiota in rats, where bacterial density and enzymatic activity remain high throughout the stomach and gastrointestinal tract. This differs from humans and has been attributed to the rats' higher gastric pH, allowing greater bacterial survival in proximal regions (Hatton et al., 2015; Hurst et al., 2007; Kararli, 1995). These results support the feasibility of using rat faecal material as a surrogate for more invasive sampling methods in screening colonic drug metabolism, especially in the early stages of formulation development. Rat faecal material offers a less invasive approach for ex vivo studies, avoiding the need to sacrifice the animal to obtain colonic or caecal contents.

Table 1
First-order degradation rate constants (K) and half-lives ($t_{1/2}$) for sulfasalazine, balsalazide, and olsalazine across rat gastrointestinal compartments. Values represent mean ± SD from three independent animals (biological replicates), with K values derived from one-phase exponential decay fits.

Drug	Faecal K (min^{-1})	Faecal $t_{1/2}$ (min)	Caecal K (min^{-1})	Caecal $t_{1/2}$ (min)	Colonic K (min^{-1})	Colonic $t_{1/2}$ (min)
Sulfasalazine	0.025 ± 0.006	29.1 ± 7.6	0.024 ± 0.014	43.7 ± 35.1	0.017 ± 0.011	67.9 ± 62.3
Balsalazide	0.015 ± 0.004	47.9 ± 12.8	0.011 ± 0.005	71.5 ± 36.5	0.009 ± 0.003	81.4 ± 32.4
Olsalazine	0.010 ± 0.004	84.1 ± 43.1	0.009 ± 0.006	120.9 ± 112.5	0.006 ± 0.003	157.2 ± 120.8

A species comparison investigating the metabolic degradation rates of sulfasalazine, balsalazide, and olsalazine in rat and canine faecal material was subsequently made. Fig. 3 shows the degradation kinetics of the three prodrugs in canine faecal mixtures, with sulfasalazine in panel (a), balsalazide in panel (b), and olsalazine in panel (c). A two-way ANOVA analysis identified significant main effects of drug type ($p < 0.0001$) and species ($p = 0.0005$), but no significant interaction between these factors ($p = 0.9971$). Pairwise comparisons using Šídák's multiple comparisons test revealed that for all three drugs, rats exhibited significantly higher K values than dogs. The mean difference (95 % CI) was −0.006 (−0.011 to −0.001, $p = 0.021$) for sulfasalazine, −0.006 (−0.012 to −0.001, $p = 0.018$) for balsalazide, and −0.006 (−0.012 to −0.001, $p = 0.018$) for olsalazine.

This interspecies disparity likely reflects differences in gut microbial ecology. Rat microbiota may harbour a greater abundance of azoreductase-expressing taxa than the canine gut, where microbial density and functional redundancy appear lower in faecal samples (Pilla and Suchodolski, 2020). Additional factors such as differences in diet, age, and baseline microbiota composition of the donor dogs may also have contributed to the lower degradation rates observed. The absence of an interaction effect indicates that the drug differences were consistent regardless of species. When comparing degradation rates between drugs within each species, sulfasalazine was found to have significantly higher K values than both balsalazide ($p = 0.010$) and olsalazine ($p = 0.001$) in dogs, while the difference between balsalazide and olsalazine was not significant ($p = 0.615$). A similar trend was observed in rats, where sulfasalazine degradation was significantly higher than that of balsalazide ($p = 0.012$) and olsalazine ($p = 0.002$), again with no significant difference between balsalazide and olsalazine ($p = 0.626$).

Sulfasalazine consistently exhibited the highest degradation rate, suggesting a more rapid cleavage of its azo bond compared to balsalazide and olsalazine. This enhanced degradation could be attributed to sulfasalazines simpler azo linkage and reduced steric hindrance near the cleavage site. This may facilitate more efficient access and processing by FMN-dependent azoreductases. The slower degradation observed for olsalazine, despite consisting of two 5-ASA moieties may reflect conformational or electronic factors that impair enzymatic binding or catalysis (Sousa et al., 2014; Zhang et al., 2018). Interestingly, Ryan et al. investigated the substrate specificity of three azoreductase-encoding genes from Pseudomonas aeruginosa (aAzoR1, paAzoR2, and paAzoR3) and found that, depending on the isoform, higher binding affinity was observed for balsalazide or olsalazine rather than sulfasalazine (Crouwel et al., 2021; Ryan et al., 2010). This trend highlights the complexity of bacterial azo-reduction and underscores the role of molecular structure in determining susceptibility to microbial azo-reduction. This further demonstrates the relevance of the in vitro faecal model for simulating the colonic environment, as it encompasses a diverse community of azoreductase-producing bacterial species, offering a more physiologically representative system than studies limited to individual bacterial strains.

To assess the translational relevance of these findings, the K values for dogs and rats were compared to published human degradation rates from our previous paper shown in Table 2 (Sousa et al., 2014). The human data were derived from Sousa et al. (Sousa et al., 2014), who performed in vitro faecal mixture incubations under comparable conditions (10 % w/v, 500 μM drug concentration, pH 7.0, 37 °C, anaerobic environment, HPLC/UV quantification). Samples in Sousa et al. and in the present canine study were prepared fresh, whereas rat materials were lyophilised and rehydrated under anaerobic conditions. This approach preserves microbial enzymatic activity, thereby supporting the validity of direct cross-species comparison (Bensch et al., 2022; Pourrat et al., 2025). The human degradation rates for sulfasalazine ($K =$

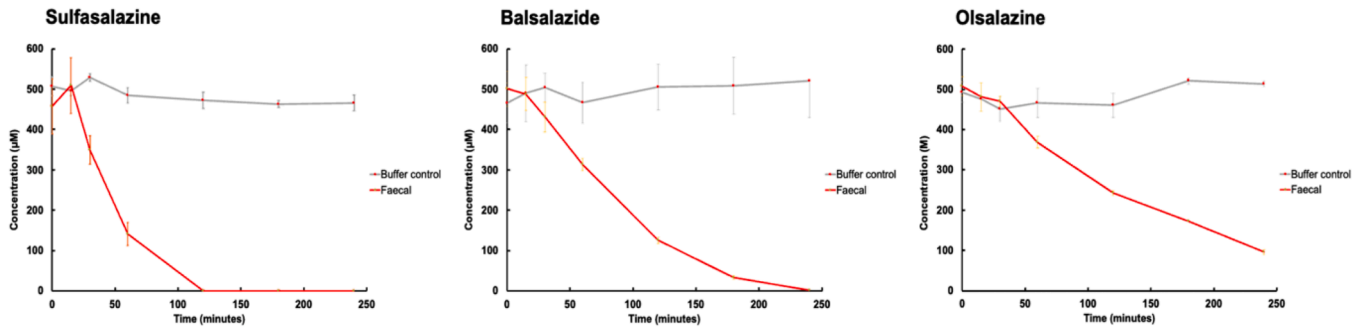

Fig. 3. In vitro degradation of azo-linked prodrugs in pooled canine faecal mixtures. Degradation kinetics of sulfasalazine (a), balsalazide (b), and olsalazine (c) were evaluated in 10 % (w/v) pooled faecal mixtures prepared from four Labrador dogs.

Table 2
First-order degradation rate constants (K) and half-lives ($t_{1/2}$) for sulfasalazine, balsalazide, and olsalazine in rat, dog, and human in vitro systems. Rat data represent mean ± SD from three independent animals. Dog data represent mean ± SD from technical replicates of pooled faecal samples from four animals. Human data are single reference values reported by Sousa et al., 2014.

Compound	K (rat, min^{-1})	$t_{1/2}$ (rat, min)	K (dog, min^{-1})	$t_{1/2}$ (dog, min)	K (human, min^{-1})*	$t_{1/2}$ (human, min) *
Sulfasalazine (Azulfidine)	0.025 ± 0.006	29.1 ± 7.6	0.015 ± 0.005	49.31 ± 17.0	0.021	32.9
Balsalazide	0.015 ± 0.004	47.9 ± 12.8	0.007 ± 0.002	104.3 ± 34.4	0.009	80.9
Olsalazine	0.010 ± 0.004	84.1 ± 43.1	0.004 ± 0.001	181.8 ± 68.5	0.003	203.9

0.021) closely aligned with those observed in rats (K= 0.025) rather than dogs (K= 0.015). For balsalazide, the human value (K= 0.009) was comparably close to both the rats (K= 0.015) and dogs (K= 0.007). For olsalazine, the human rate (K= 0.003) was lower than that of both rats (K= 0.010) and dogs (K= 0.004), though more similar to the dog model. The greater deviation of olsalazine between rats and humans may reflect its unique dimeric structure, making it more susceptible to interspecies differences in microbiota composition, colonic pH, and water content. These results support the relevance of rat faecal material as the most appropriate in vitro model for approximating human colonic degradation of azo-linked prodrugs, especially for sulfasalazine and balsalazide.

Although this study employed Wistar rats, their gastrointestinal physiology is broadly representative of other rat strains (Flemer et al., 2017; Hatton et al., 2015; McConnell et al., 2008). Other common strains such as Sprague–Dawley also exhibit comparable microbiota responses to dietary interventions (e.g., high-fat diet (Marques et al., 2016-2–3)), supporting the broader applicability of rat faecal material as a model system. This is despite notable physiological and microbial differences between species; rodents exhibit a higher gastrointestinal water content, lower colonic pH (as low as 6.6), and routinely practice coprophagy, all of which can influence microbial composition and enzymatic activity. Important compositional differences exist between the human and rat gut microbiota. In humans, Firmicutes dominate with Clostridia clusters IV and XIVa alone comprising nearly half of colonic bacteria. By contrast, rats typically harbour a higher proportion of Bacteroidetes and fewer Clostridia (Čoklo et al., 2020; Hatton et al., 2015; McConnell et al., 2008; Wos-Oxley et al., 2012). These differences underscore the distinct microbial ecology of each species, yet do not preclude functional overlap in drug-metabolising activity. The observed similarities in bacterial degradation rates between rats and humans may reflect the nature of the enzymatic systems involved. Some prodrugs are metabolised by broadly expressed bacterial enzymes, enabling similar degradation profiles across species. In contrast, other compounds may require highly specific enzymatic recognition as per our previous findings, leading to species-dependent differences in metabolic rate (Sousa et al., 2014). Despite these potential differences, the current findings suggest that the metabolic activity relevant to azo-prodrug cleavage is broadly conserved between rats and humans. While microbial community composition was not directly characterised in this study, taxa-level inferences were drawn from established literature on rat, canine, and human microbiota. Future studies incorporating 16S rRNA sequencing or metagenomic profiling could directly link community composition to functional degradation capacity and further strengthen these observations.

4. Conclusions

This study systematically evaluated the in vitro degradation of three azo-linked prodrugs across rat gastrointestinal compartments. Rat faecal material was subsequently compared with canine faecal material, and the findings were benchmarked against published human data. Rat faecal mixtures demonstrated equivalent degradation capacity to colonic and caecal mixtures, highlighting the conserved metabolic potential throughout the large intestine. Its comparable metabolic performance to caecal and colonic compartments suggests that terminal sampling is unnecessary, supporting a refinement in animal sampling protocols. This makes it suitable for repeated or high-throughput studies. Rat faecal mixtures closely replicated human degradation profiles, particularly for sulfasalazine and balsalazide. The logistical challenges and interindividual variability associated with human faecal samples further emphasise the value of the rat as a low impact model. Its controlled diet, reproducible microbiota, and non-terminal sampling make it a robust alternative for early-stage screening. In doing so, this approach advances the principles of the 3Rs by reducing reliance on larger animal models, refining sampling protocols, and supporting replacement of more invasive methods. Collectively, these findings support the potential use of rat faecal material as a practical and translationally relevant model for evaluating microbiota-mediated metabolism of azo-linked prodrugs in preclinical development. Future work may benefit from extending this approach to broader classes of microbiota-sensitive compounds and include in vivo validation to further establish translational relevance.

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CRediT authorship contribution statement

Tiago Sousa: Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Conor Beaupres De Monsales:** Writing – review & editing, Writing – original draft, Formal

analysis, Data curation. **Charlotte Yeung:** Writing – review & editing, Writing – original draft, Formal analysis, Data curation. **Anders Borde:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Bertil Abrahamsson:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Abdul W. Basit:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors report there are no competing interests to declare.

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Data availability

The data that supports the findings in this study are available on the request from the corresponding authors.

References

Aragaw, T.A., 2024. Potential and prospects of reductases in azo dye degradation: a review. Microbe 4, 100162.
Bakshi, H.A., Quinn, G.A., Aljabali, A.A.A., Hakkim, F.L., Farzand, R., Nasef, M.M., Abuglela, N., Ansari, P., Mishra, V., Serrano-Aroca, Á., 2021. Exploiting the metabolism of the gut microbiome as a vehicle for targeted drug delivery to the colon. Pharmaceuticals 14, 1314.
Basit, A.W., Newton, J.M., Lacey, L.F., 2002. Susceptibility of the H2-receptor antagonists cimetidine, famotidine and nizatidine, to metabolism by the gastrointestinal microflora. Int. J. Pharm. 237, 85–91.
Beeck, R., Glöckl, G., Krause, J., Schick, P., Weitschies, W., 2021. Mimicking the dynamic colonic microbiota in vitro to gain a better understanding on the in vivo metabolism of xenobiotics: degradation of sulfasalazine. Int. J. Pharm. 603, 120704.
Bensch, H.M., Tolf, C., Waldenström, J., Lundin, D., Zöttl, M., 2022. Freeze-drying can replace cold-chains for transport and storage of fecal microbiome samples. PeerJ 10, e13095.
Brown, K., Abbott, D.W., Uwiera, R.R.E., Inglis, G.D., 2018. Removal of the cecum affects intestinal fermentation, enteric bacterial community structure, and acute colitis in mice. Gut Microbes 9, 411–425.
Čoklo, M., Maslov, D.R., Kraljević Pavelić, S., 2020. Modulation of gut microbiota in healthy rats after exposure to nutritional supplements. Gut Microbes 12, 1779002.
Collins, S.L., Patterson, A.D., 2020. The gut microbiome: an orchestrator of xenobiotic metabolism. Acta Pharm. Sin. B 10, 19–32.
Coelho, L.P., Kultima, J.R., Costea, P.I., Fournier, C., Pan, Y., Czarnecki-Maulden, G., Hayward, M.R., Forslund, S.K., Schmidt, T.S.B., Descombes, P., Jackson, J.R., Li, Q., Bork, P., 2018. Similarity of the dog and human gut microbiomes in gene content and response to diet. Microbiome 6, 72.
Crouwel, F., Buiter, H.J.C., de Boer, N.K., 2021. Gut microbiota-driven drug metabolism in inflammatory bowel disease. J. Crohns Colitis 15, 307–315.
D'Incà, R., Paccagnella, M., Cardin, R., Pathak, S., Baldo, V., Giron, M.C., Sturniolo, G.C., 2013. 5-ASA colonic mucosal concentrations resulting from different pharmaceutical formulations in ulcerative colitis. World J. Gastroenterol. 19, 5665–5671.
Flemer, B., Gaci, N., Borrel, G., Sanderson, I.R., Chaudhary, P.P., Tottey, W., O'Toole, P. W., Brugère, J.F., 2017. Fecal microbiota variation across the lifespan of the healthy laboratory rat. Gut Microbes 8, 428–439.
Grimm, H., Biller-Andorno, N., Buch, T., Dahlhoff, M., Davies, G., Cederroth, C.R., Maissen, O., Lukas, W., Passini, E., Törnqvist, E., Olsson, I.A.S., Sandström, J., 2023. Advancing the 3Rs: innovation, implementation, ethics and society. Front. Vet. Sci. 10, 1185706.
Guzmán-Rodríguez, M., McDonald, J.A.K., Hyde, R., Allen-Vercoe, E., Claud, E.C., Sheth, P.M., Petrof, E.O., 2018. Using bioreactors to study the effects of drugs on the human microbiota. Methods 149, 31–41.
Hatton, G.B., Yadav, V., Basit, A.W., Merchant, H.A., 2015. Animal farm: considerations in animal gastrointestinal physiology and relevance to drug delivery in humans. J. Pharm. Sci. 104, 2747–2776.
Hurst, S., Loi, C.M., Brodfuehrer, J., El-Kattan, A., 2007. Impact of physiological, physicochemical and biopharmaceutical factors in absorption and metabolism mechanisms on the drug oral bioavailability of rats and humans. Expert Opin. Drug Metab. Toxicol. 3, 469–489.
Isenring, J., Bircher, L., Geirnaert, A., Lacroix, C., 2023. In vitro human gut microbiota fermentation models: opportunities, challenges, and pitfalls. Microbiome Res. Rep. 2, 2.
Javdan, B., Lopez, J.G., Chankhamjon, P., Lee, Y.J., Hull, R., Wu, Q., Wang, X., Chatterjee, S., Donia, M.S., 2020. Personalized mapping of drug metabolism by the human gut microbiome. Cell 181, 1661–1679.e22.
Kararli, T.T., 1995. Comparison of the gastrointestinal anatomy, physiology, and biochemistry of humans and commonly used laboratory animals. Biopharm. Drug Dispos. 16, 351–380.
Koziolek, M., Grimm, M., Bollmann, T., Schäfer, K.J., Blattner, S.M., Lotz, R., Boeck, G., Weitschies, W., 2019. Characterization of the GI transit conditions in Beagle dogs with a telemetric motility capsule. Eur. J. Pharm. Biopharm. 136, 221–230.
Le Berre, C., Roda, G., Nedeljkovic Protic, M., Danese, S., Peyrin-Biroulet, L., 2020. Modern use of 5-aminosalicylic acid compounds for ulcerative colitis. Expert Opin. Biol. Ther. 20, 363–378.
Marques, C., Meireles, M., Norberto, S., Leite, J., Freitas, J., Pestana, D., Faria, A., Calhau, C., 2016. High-fat diet-induced obesity rat model: a comparison between Wistar and Sprague-Dawley rat. Adipocyte 5, 11–21.
McConnell, E.L., Basit, A.W., Murdan, S., 2008. Measurements of rat and mouse gastrointestinal pH, fluid and lymphoid tissue, and implications for in vivo experiments. J. Pharm. Pharmacol. 60, 63–70.
McCoubrey, L.E., Gaisford, S., Orlu, M., Basit, A.W., 2022. Predicting drug-microbiome interactions with machine learning. Biotechnol. Adv. 54, 107797.
Mukherjee, P., Roy, S., Ghosh, D., Nandi, S.K., 2022. Role of animal models in biomedical research: a review. Lab Anim. Res. 38, 18.
Pilla, R., Suchodolski, J.S., 2020. The role of the canine gut microbiome and metabolome in health and gastrointestinal disease. Front. Vet. Sci. 6, 498.
Pourrat, A., Baillieu, V., Ansel, S., Leonardi, M., Poiron, P., Bellais, S., Paul, M., Nebbad, B., 2025. Standardized freeze-dried FMT: is the ideal protectant out there? Front. Microbiol. 16, 1618067.
Qi, Y., Yu, L., Tian, F., Zhao, J., Zhai, Q., 2023. In vitro models to study human gut-microbiota interactions: applications, advances, and limitations. Microbiol. Res. 270, 127336.
Qin, J., Li, R., Raes, J., Arumugam, M., Burgdorf, K.S., Manichanh, C., Nielsen, T., Pons, N., Levenez, F., Yamada, T., Mende, D.R., Li, J., Xu, J., Li, S., Li, D., Cao, J., Wang, B., Liang, H., Zheng, H., Xie, Y., Tap, J., Lepage, P., Bertalan, M., Batto, J.-M., Hansen, T., Le Paslier, D., Linneberg, A., Nielsen, H.B., Pelletier, E., Renault, P., Sicheritz-Ponten, T., Turner, K., Zhu, H., Yu, C., Li, S., Jian, M., Zhou, Y., Li, Y., Zhang, X., Li, S., Qin, N., Yang, H., Wang, J., Brunak, S., Doré, J., Guarner, F., Kristiansen, K., Pedersen, O., Parkhill, J., Weissenbach, J., Bork, P., Ehrlich, S.D., Wang, J., 2010. A human gut microbial gene catalogue established by metagenomic sequencing. Nature 464, 59–65.
Ryan, A., Wang, C.-J., Laurieri, N., Westwood, I., Sim, E., 2010. Reaction mechanism of azoreductases suggests convergent evolution with quinone oxidoreductases. Protein Cell 1, 780–790.
Sehgal, P., Colombel, J.F., Aboubakr, A., Narula, N., 2018. Systematic review: safety of mesalazine in ulcerative colitis. Aliment. Pharmacol. Ther. 47, 1597–1609.
Shah, T., Hou, Y., Jiang, J., Shah, Z., Wang, Y., Li, Q., Xu, X., Wang, Y., Wang, B., Xia, X., 2023. Comparative analysis of the intestinal microbiome in *Rattus norvegicus* from different geographies. Front. Microbiol. 14, 1283453.
Sousa, T., Paterson, R., Moore, V., Carlsson, A., Abrahamsson, B., Basit, A.W., 2008. The gastrointestinal microbiota as a site for the biotransformation of drugs. Int. J. Pharm. 363, 1–25.
Sousa, T., Yadav, V., Zann, V., Borde, A., Abrahamsson, B., Basit, A.W., 2014. On the colonic bacterial metabolism of azo-bonded prodrugs of 5-aminosalicylic acid. J. Pharm. Sci. 103, 3171–3175.
Tannergren, C., Borde, A., Boreström, C., Abrahamsson, B., Lindahl, A., 2014. Evaluation of an in vitro faecal degradation method for early assessment of the impact of colonic degradation on colonic absorption in humans. Eur. J. Pharm. Sci. 57, 200–206.
van de Steeg, E., Schuren, F.H.J., Obach, R.S., van Woudenbergh, C., Walker, G.S., Heerikhuisen, M., Nooijen, I.H.G., Vaes, W.H.J., 2018. An ex vivo fermentation screening platform to study drug metabolism by human gut microbiota. Drug Metab. Dispos. 46, 1596–1607.
Wange, R.L., Brown, P.C., Davis-Bruno, K.L., 2021. Implementation of the principles of the 3Rs of animal testing at CDER: past, present and future. Regul. Toxicol. Pharmacol. 123, 104932.
Watson, E.-J., Giles, J., Scherer, B.L., Blatchford, P., 2019. Human faecal collection methods demonstrate a bias in microbiome composition by cell wall structure. Sci. Rep. 9, 16888.
Wilson, P.J., Basit, A.W., 2005. Exploiting gastrointestinal bacteria to target drugs to the colon: an in vitro study using amylose coated tablets. Int. J. Pharm. 300, 89–94.
Wos-Oxley, M.L., Bleich, A., Oxley, A.P., Kahl, S., Janus, L.M., Smoczek, A., Nahrstedt, H., Pils, M.C., Taudien, S., Platzer, M., Hedrich, H.-J., Medina, E., Pieper, D.H., 2012. Comparative evaluation of establishing a human gut microbial community within rodent models. Gut Microbes 3, 234–249.
Zahran, S.A., Ali-Tammam, M., Hashem, A.M., Aziz, R.K., Ali, A.E., 2019. Azoreductase activity of dye-decolorizing bacteria isolated from the human gut microbiota. Sci. Rep. 9, 5508.
Zhang, J., Zhang, J., Wang, R., 2018. Gut microbiota modulates drug pharmacokinetics. Drug Metab. Rev. 50, 357–368.
Zhu, W., Zhang, X., Wang, D., Yao, Q., Ma, G.L., Fan, X., 2024. Simulator of the human intestinal microbial ecosystem (SHIME®): current developments, applications, and future prospects. Pharmaceuticals 17, 1639.