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Investigating the bidirectional interactions between senotherapeutic agents and human gut microbiota

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ABSTRACT

Biological ageing is a time-dependent process that has implications for health and disease. Cellular senescence is a key driver in ageing and age-related diseases. Senotherapeutic agents have been shown to slow biological ageing by eliminating senescent mammalian cells. Given the increasing awareness of the gut microbiome in regulating human health, this study aimed to investigate the effects of senotherapeutic agents as pharmacological interventions on the human gut microbiota. In this study, the bidirectional effects of four senotherapeutic agents, quercetin, fisetin, dasatinib, and sirolimus, with the gut microbiota sourced from healthy human donors were investigated. The results revealed that quercetin was completely biotransformed by the gut microbiota within six hours, while dasatinib was the most stable of the four compounds. Additionally, metagenomic analysis confirmed that all four compounds increased the abundance of bacterial species associated with healthy ageing (e.g., *Bacteroides fragilis, Bifidobacterium longum*, and *Veillonella parvula*), and decreased the abundance of pathogenic bacteria primarily associated with age-related diseases (e.g., *Enterococcus faecalis* and *Streptococcus* spp.). The findings from this study provide a comprehensive understanding of the pharmacobiomics of senotherapeutic interventions, highlighting the potential of microbiome-targeted senolytics in promoting healthy ageing.

1. Introduction

Cellular senescence, first described in 1961, describes a state in which cell division is permanently arrested (Herranz and Gil, 2018). Any cell in the human body has the potential to enter senescence, often triggered by DNA damage, telomere shortening, and mitochondrial dysfunction (Di Micco et al., 2021). Senescent cells have a distinct senescence-associated secretory phenotype (SASP) that can affect healthy cells and tissues (Huang et al., 2022). SASP components, which include cytokines, growth factors, and chemokines, can have both beneficial and pathological effects. For example, cellular senescence is biologically useful for embryonic development, cancer suppression, and wound healing by halting proliferation of dysfunctional cells (Burton and Krizhanovsky, 2014). However, persistence and accumulation of senescent cells can increase inflammation, impair tissue regeneration, and increase susceptibility to infection, among other detrimental impacts (Naylor et al., 2013; Palmer et al., 2019; Soto-Gamez et al., 2019). Accumulation of senescent cells naturally occurs during biological ageing and is thought to play a major role in the aetiology of age-related diseases (Tchkonia and Kirkland, 2018; Schmauck-Medina et al., 2022). Cellular senescence has been mechanistically associated with a plethora of age-related conditions, including osteoarthritis, Alzheimer's disease, atherosclerosis, chronic kidney disease, and cancer (Schroth et al., 2020; Mylonas and O'Loghlen, 2022).

Due to its role in unhealthy ageing, cellular senescence is increasingly being investigated as a therapeutic target. Here, four pharmacological strategies exist: prevention of senescence, elimination of senescent cells (senolysis), restoration to a healthy cell type (senoreversion), and inhibition of the SASP (senomorphism) (Di Micco et al., 2021; Huang et al., 2022; Zhang et al., 2022). Of these, senolysis is the most researched approach and is typically achieved via initiation of apoptosis in senescent cells (Di Micco et al., 2021). Key investigatory senolytics include the flavonoids, quercetin (Q) and fisetin (F), and the pan-tyrosine kinase inhibitor, dasatinib (D) (Novais et al., 2021). These molecules target senescent cell anti-apoptotic pathways (SCAPs) leading to apoptosis of senescent, but not healthy, cells (Zhang et al., 2021).

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Amongst activity in other SCAPs, quercetin and fisetin inhibit intracellular phosphoinositide 3-kinase (PI3K) expression leading to decreased synthesis of mammalian target of sirolimus (S) (mTOR), a regulator of apoptosis (Di Micco et al., 2021; Castedo et al., 2002). Conversely, dasatinib inhibits multiple tyrosine kinases that reduce expression of ephrin ligand B proteins, which triggers cell death via inactivation of Eph receptors (Zhu et al., 2015; Furne et al., 2009). Due to their activities on different SCAPs, dasatinib and quercetin ($D + Q$) are commonly co-administered for their synergistic effects as documented in preclinical and clinical studies (Novais et al., 2021; Saccon et al., 2021; Justice et al., 2019; Hickson et al., 2019). The antibiotic sirolimus is also under investigation for its senotherapeutic effects (Huang et al., 2022; Harrison et al., 2009). As an inhibitor of mTOR, sirolimus has a multi-modal mechanism: promotion of autophagy, delay of cell entry into senescence, and suppression of SASP (Di Micco et al., 2021; Wang et al., 2017). As such, quercetin, fisetin, dasatinib, and sirolimus represent promising candidates for the promotion of healthy ageing by targeting multiple SCAPs. Following oral administration, the majority of senolytic compounds are predominantly absorbed via the proximal gastrointestinal (GI) tract (Crespy et al., 1999; Christopher et al., 2008). Alongside cellular senescence, disturbances in gut microbiome composition and functioning is considered a hallmark of ageing (Schmauck-Medina et al., 2022). Multiple high-impact studies have revealed that gut microbiome composition is associated with both lifespan and health span (Wilmanski et al., 2021; Gregory et al., 2020; DeJong et al., 2020; Chaudhari et al., 2020; Ghosh et al., 2022). There is also evidence for a connection between cellular senescence and gut microbiome disturbance in the aetiology of age-related conditions. For example, both hallmarks are associated with a decline in immune function and inflammation in later life (Bosco and Noti, 2021). Further, a bidirectional relationship may exist whereby metabolites produced by gut microbiota affect the development and accumulation of senescent cells; and the SASP of senescent cells may affect the gut microbiome (Sharma, 2022). There is also evidence that oral administration of senolytic substances such as quercetin, fisetin, dasatinib and sirolimus improves gut microbiome composition and function in preclinical ageing models, including senescent-driven disease models (Saccon et al., 2021; Shi et al., 2020; Lin et al., 2020; Schinaman et al., 2019). Unlike traditional strategies using probiotics and prebiotics to support the growth of beneficial bacteria and promote healthy ageing, we hypothesise that senolytic compounds may offer superior pharmacological benefits by selectively targeting senescent cells. However, their impact on gut microbiota and ability to promote beneficial bacteria remain unexplored, while at the same time, the gut microbiome likely plays a significant role in the efficacy of senotherapeutics. Additionally, there is limited evidence that delves into interactions between human gut microbiota and senolytic compounds. Therefore, it is important to characterise senotherapeutics' interactions with gut microbiota to better understand their roles in the pharmacological promotion of healthy ageing.

In this study, the bidirectional interaction of four senolytic compounds and healthy human gut microbiota was fully investigated, aiming to establish the concept of senotherapeutics targeted at the gut microbiome for healthy ageing. The effect of human gut microbiota on stability of senolytic compounds was assessed with high-performance liquid chromatography (HPLC) while the effect of senolytic compounds on modulating human gut microbiota composition was assessed with metagenomic analysis. So far, over 150 drugs are known to be significantly metabolised or accumulated by gut microbiota, so it is important to understand how the gut microbiota may impact drug bioavailability in the GI tract and whether stability is variable across individuals (Shi et al., 2020; Lin et al., 2020; Schinaman et al., 2019; McCoubrey et al., 2022). Concurrently, many drugs are also known to impact the growth and functioning of gut microbiota (McCoubrey et al., 2022; Zimmermann et al., 2019). The insights from this study contribute towards knowledge on the senotherapeutics' behaviour in the GI tract that could be leveraged to develop optimal dosage forms for promoting healthy ageing.

2. Materials and methods

2.1. Materials

Quercetin dihydrate was purchased from MP Biomedicals, LLC (California, USA). Fisetin and dasatinib monohydrate were purchased from Cambridge Biosciences (Cambridge, UK). Sirolimus was purchased from ApexBio (Taxas, USA). Methanol and acetonitrile were purchased from Thermofisher Scientific (Massachusetts, US) and all were of high-performance liquid chromatography (HPLC) grade. Orthophosphoric acid, Bryant and Burkey medium (BBM), sodium phosphate dibasic heptahydrate, and sodium phosphate monobasic monohydrate were purchased from Sigma Aldrich (Dorset, UK). All chemicals were of HPLC reagent grade with no further purification before use. HPLC-grade water was obtained from an ELGA purification system (ELGA LabWater, High Wycombe, UK). Penicillin-Streptomycin, Trypsin-EDTA (0.25 %), etoposide (Etoposide), Dulbecco's Modified Eagle Medium (DMEM) and Dimethysulfoxide (DMSO) were purchased from Thermofisher Scientific (Massachusetts, US). Senescence-beta-Galactosidase Staining kit was purchased from Cell Signaling Technology (Massachusetts, US).

2.2. Preparation of human faecal slurry and microbial aliquots

Five gut microbiota samples were prepared from faecal samples provided by three males and two female healthy volunteers aged 27 - 35. The donors had not taken antibiotics within the 6 months preceding donation. Ethical approval to collect human faeces was obtained by Intract Pharma Ltd. from UCL Biobank Ethical Review Committee at Royal Free London NHS Foundation Trust (reference no NC2017.010). Fresh faecal samples from each donor were deposited into sterile plastic containers and immediately sealed with an AnaeroGen sachet (Thermo Fisher Scientific, Loughborough, UK) to maintain the quality and viability of the microbiota. Faecal samples were stored overnight at - 20 °C and subsequently moved to an anaerobic chamber the next morning (Electrotek 500TG workstation, Electrotek, West Yorkshire, UK; containing 5 % CO_2, 5 % H_2, 90 % N_2). Conditions within the anaerobic chamber were set to simulate the intra-intestinal environment at a temperature of 37 °C and 70 % relative humidity.

Human faecal slurry was produced by homogenising each donor's sample and combining it with basal medium at a ratio of 1:3 (w/v), thus producing 25 % w/v faecal slurry. The composition and preparation of the basal medium are detailed by Coombes et al. (Coombes et al., 2020). Then, to culture the faecal microbiota, 0.5 mL of faecal slurry was combined with 100 mL sterile BBM and incubated for 24 h in the anaerobic chamber. This primary culture was then re-established by inoculating 1.0 mL into a secondary 100 mL sterile BBM and incubated for a further 24 h under anaerobic conditions. Afterwards, the final culture of each donor was mixed with 30 % glycerol in ¼ Ringer's solution to obtain a final concentration of 15 % v/v microbial glycerol stock suspension. These stocks were subsequently frozen as aliquots at −80 °C. Each aliquot was thawed and used only once to protect the viability of the gut microbiota (Karatza et al., 2016).

2.3. Preparation of drug stock solutions and culture medium

All drug stock solutions were freshly prepared on the same day of experiment, using HPLC-grade water and methanol as a co-solvent. The concentrations of stock solutions of each compound were at 2000 μM. The clarity and appearance of solutions were inspected under light to ensure total drug dissolution.

BBM was prepared by dissolving 16.5 g of BBM powder with 500 mL distilled water. The medium was autoclaved prior to use at 126 °C and 1.8 bar for 30 min. Then, 50 mL of sterile BBM was transferred into sterile tubes and equilibrated in an anaerobic chamber (A25 Sleeved

Anaerobic Workstation, Don Whitley Scientific, Bingley, UK; containing 5 % CO_2, 5 % H_2, 90 % N_2) for at least eight hours to eliminate oxygen and warm to 37 °C.

2.4. Isothermal microcalorimetry

Isothermal microcalorimetry was performed using a 2277 Thermometric Activity Monitor (TAM, TA Instruments Ltd., UK) to observe the influence of methanol concentration (as a co-solvent) on gut microbiota growth and viability. The temperature of the microcalorimeter was set to 37 °C. All samples were prepared in 3.0 mL sterile glass ampules. 2.8 μL of thawed faecal microbiota sample from a donor was inoculated into 2.8 mL sterile BBM containing methanol at concentrations of 2, 3, 4, 5, 6 and 7 % v/v. Samples were prepared in triplicate inside an anaerobic workstation. All ampoules were hermetically sealed within the workstation before loading into the microcalorimeter's channels for 30 min to allow thermal equilibration. After 30 min, recording was started with power readings taken every 10 s with an amplifier range of 1000 μW using the Digitam 4.1 software package.

2.5. Culture of human faecal microbiota

An aliquot of each faecal microbiota sample ($n = 5$ healthy human donors) was thawed within the anaerobic workstation (A25 Sleeved Anaerobic Workstation, Don Whitley Scientific, Bingley, UK; containing 5 % CO_2, 5 % H_2, 90 % N_2). The thawed suspension was vortexed for 15 s, and 1.0 mL was combined with 50 mL sterile BBM in a sterile tube. Microbiota was allowed to proliferate for 16 h under mild agitation at 100 rpm (Vibrax VXR basic, IKA, Germany). BBM was chosen as the culture medium as it has been shown to support the balanced growth of human faecal microbiota in a previous study (Javdan et al., 2020).

2.6. Chromatographic condition

Drug concentrations in microbiota samples and controls were quantified by High Performance Liquid Chromatography (HPLC) using an Agilent 1260 Infinity II (Agilent, USA) system. The standard curve of each compound was constructed with triplicate measurements of each standard concentration (linearity (R^2) $\geq$ 0.995). The instrumental control, data acquisition and analysis were performed via the supporting software (Agilent openLAB, version 2.5). Table 1 shows the chromatographic separation methods used for each drug.

2.7. Experimental samples and controls

The stability of the senotherapeutic drugs in the presence of human gut microbiota was carried out under anaerobic conditions within a Whitney A20 anaerobic workstation (Don Whitney Scientific, Germany). Each senolytic drug stock solution was combined with 5.0 mL of microbiota cultures to achieve a final drug concentration of 30 μM. All drug-microbiota incubations were performed in triplicate for each human donor sample ($n = 5$) and a single drug concentration. The optical density was observed before and after 24 h of incubation. Controls consisted of drug stock solutions combined with sterile BBM to assess the stability of the drugs in the absence of microbiota. Incubations were shaken at 100 rpm on a shaker plate (Vibrax VXR basic, IKA, Germany) within the anaerobic chamber for 24 h. At 0.5, 2, 4, 6 and 24 h, 600 μL of each incubation sample was transferred into a microtube, and 600 μL of methanol was added to suspend microbial activity. These timepoint samples were centrifuged at 14,000 rpm for 5 min at 4 °C (Fresco 21, Thermo Scientific, Germany) to separate the incubation medium from microbial cells. The supernatant was collected and filtered through Millex 0.22 μm filters and stored at 4 °C. Once all samples had been collected, HPLC was employed to analyse drug stability (Table 1).

2.8. Metagenomics analysis

The impact of the four senolytic compounds on human gut microbiota was analysed using 16S rRNA gene sequencing (16S Illumina sequencing). In brief, a gut microbiota sample ($n = 3$ per donor) was taken from the bioreactor after 24 h of incubation with or without the presence of senolytic compounds. The DNA from samples was extracted by ultrasonication (Covaris LE220 ultrasonicator, US), purified (AMPure XP magnetic beads) before being amplified as follows in a protocol from a previous study (He et al., 2013). A standard operating procedure was used to filter and assemble reads via the readfq (version 1.0) package. Operational taxonomic units (OTUs) clustering was achieved via two methods depending on the type of sequences. The USERACH (version 7.0.1090) tool was applied to annotate clustering sequences into OTUs with the similarity threshold set to 97 %. For pair-end sequencing, the FLASH (version 1.2.11) tool was applied to generate OTUs. The amplicon sequence variants (ASVs) were generated via Divisive Amplicon Denoising Algorithm (DADA2) in QIIME2 package (version 1.80). The OTU annotation was achieved by using Ribosome Database Project (RDP) classifier (version 2.2). The functional profiling was achieved through the Phylogenetic investigation of communities by reconstruction of unobserved states (PICRUST2 version 2.3.0-b). In brief, the generated OTU was then imported into PICRUST2 and The Kyoto encyclopedia of genes and genomes (KEGG) orthology (KO) to predict the functional of genetic content of the microbial community which was represented in the Greengenes database.

2.9. Data analysis

The stability of senotherapeutics in the presence of human faecal microbiota was measured in triplicate. The average drug concentrations at each timepoint with their corresponding standard deviation were used to calculate the percentage of drug remaining in the sample. The concentration found at the initial time point of each drug was considered to be 100 %. The stability of the compounds in the human gut microbiota cultures over 24 h was compared with the experimental control. A two-way ANOVA analysis was performed to assess the statistical

Table 1
The parameters used to detect validation drugs with HPLC. Stationary phase A: Luna C18, 110 Å, 5 μM, 150 × 3.00 mm (Phenomenax, California, US). Stationary phase B: Roc C8, 100 Å, 5 μM, 150 × 4.60 mm (Shimudzu, Wolverton, UK).

Drug	Stationary phase (see Table legend for further details)	Mobile phase A identity (ratio)	Mobile phase B identity (ratio)	Flow rate (mL/min)	Injection volume (μL)	Detection wavelength (nm)	Drug elution time (min)
Dasatinib	A	5 mM phosphate buffer (40)	Methanol, ratio (60)	1.0	10	260	2.55
Quercetin	A	1 % orthophosphoric acid in water (40)	Methanol, ratio (60)	1.0	10	370	6.10
Fisetin	A	1 % orthophosphoric acid in water (56)	Methanol, ratio (44)	1.0	10	355	3.19
Sirolimus	B	Water (20)	Methanol, ratio (80)	1.0	10	277	4.40

significance of the activity of gut microbiota to decay senolytic drugs across different donors and time points. The significance level was justified using a p-value threshold of <0.05.

For microbial community analysis, the impact of senotherapeutics was evaluated. The alpha diversity analysis was performed using the Mothur package (Version 1.31.2) to calculate the Chao, Ace, Shannon, and Simpson indices. Then, a one-way ANOVA was performed to assess significant differences. The beta-diversity analysis was performed using the QIIME package (Version 1.80). The phylogenetic tree was generated for beta diversity metrics and core diversity matrices using weighted UniFrac, unweighted UniFrac, and Bray-Curtis dissimilarity. Then, principal component analysis (PCA) was used to visualise the beta diversity. The Permutational Multivariate Analysis of Variance (PERMANOVA) was applied to assess significant differences which were used to generate a heatmap. All experiments were conducted in triplicate, and significance was determined as a p-value of <0.05. All ANOVA analyses were performed via GraphPad Prism (Version 10.2.3; GraphPad Software 10.2.3, San Diego, CA, US)

3. Results and discussion

3.1. The stability of senotherapeutics in the presence of healthy human gut microbiota

Fig. 1 shows the stability profiles of quercetin, fisetin, dasatinib, and sirolimus in the presence of the faecal microbiota sourced from 5 healthy humans. The drugs were solubilised in the incubation medium with <1 % methanol; this cosolvent concentration did not significantly alter microbial growth as measured by microcalorimetry (Figs. S1 and S2). Of the four drugs, quercetin was the most rapidly and significantly ($p < 0.05$) depleted. The microbiota from Female 2 depleted all quercetin within 2 h, whereas the microbiotas from Male 2 and Female 1 required 6 h to completely deplete the drug. This *ex vivo* variability suggests that quercetin's microbial depletion could be significantly different within patients. The drug with the next most prominent, and variable microbiome depletion was sirolimus. All microbiota samples except for Male 1′s significantly depleted the drug after 24 h compared with the control ($p < 0.05$). The average percentage of sirolimus remaining after 24 h ranged from 62.77 - 93.65 %. Fisetin showed a similar stability profile to sirolimus, with all the donors' microbiota except for Male 1′s significantly depleting the drug after 24 h ($p < 0.05$). Here, the average percentage of fisetin remaining after 24 h spanned 71.43 - 98.35 %. As with quercetin, the variability of sirolimus and fisetin's stabilities could suggest variable microbial depletion in vivo. Conversely, dasatinib showed good stability in the presence of the microbiota. Microbiotas from Male 1 and Female 1 significantly depleted dasatinib compared with the control ($p < 0.05$), however, the vast majority of dasatinib remained intact after 24 h (89.19 - 99.97 %). None of the senotherapeutics showed an antimicrobial effect, observed from the increase from initial $OD_{600} = 0.7$ to $OD_{600} \geq 1$ in all donors after 24 h of incubation, corresponding to the number of colonies over 10^9 CFU/mL.

These results demonstrate that quercetin is highly unstable, sirolimus and fisetin are moderately stable, and dasatinib is stable in the presence of human faecal microbiota. The instability of quercetin in the gut is well documented; It is known that the human enzyme lactate phlorizin hydrolase significantly metabolises quercetin in the small intestine prior to absorption (Bischoff, 2008; Arts et al., 2004). Furthermore, gut bacterial enzymes can transform quercetin to various phenolic

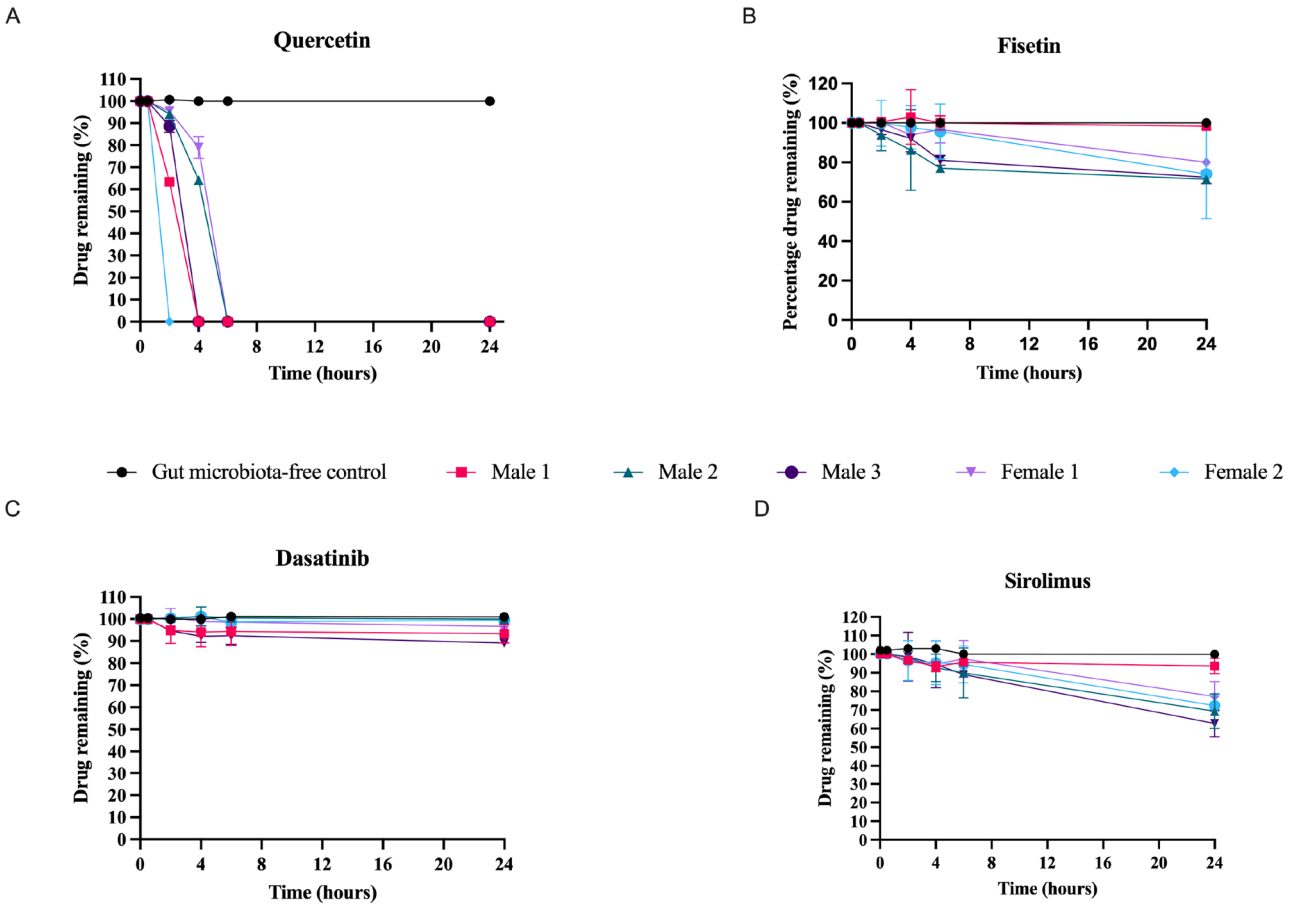

Fig. 1. Stability of quercetin (A), fisetin (B), dasatinib (C), and sirolimus (D) in the presence of faecal microbiota sourced from 5 healthy humans. Incubations were conducted for 24 h in triplicate. Datapoints represent means and standard deviations.

compounds (Zhang et al., 2014; Shabbir et al., 2021; Santangelo et al., 2019). One of quercetin's microbial metabolites, 3,4-dihydroxyphenylacetic acid, has substantial anti-inflammatory properties (Gao et al., 2006; Lofft et al., 2022). This could imply that the microbiome metabolism of quercetin plays a role in its therapeutic action. Compared with quercetin, much less is known about the microbiome stability of fisetin. Fisetin includes several functional groups that could promote susceptibility to gut bacterial metabolism, including phenol groups and a flavone moiety. The moderate depletion of fisetin in this study could have been a result of reactions at these chemical sites. Work by Kawabata et al. found that co-incubation of fisetin with *Bifidobacterium adolescentis* enhanced bacterial anti-inflammatory activity by suppressing nitric oxide production (Gonçalves et al., 2016). As such, the bacterial metabolism of fisetin could ameliorate the microbiome's anti-inflammatory potential.

3.2. The effect of senotherapeutics on a healthy gut microbial community

In this study, 16S rRNA amplicon sequencing was performed to assess the effect of senolytic compounds on the bacterial community to the controls. The alpha diversity was observed through Shannon index and Sobs index. The Sobs index is commonly used to assess the diversity within the community while the Shannon index is used to assess both diversity and evenness within communities (Walters and Martiny, 2020). The Shannon index (Fig. 2A) and Sobs index (Fig. 2B) confirmed a significant ($p < 0.05$) change in alpha diversity after the gut microbiota samples were treated with the senolytic compounds compared with the control. The quercetin and sirolimus groups show significantly lower values of the index in both metrics, while fisetin and dasatinib only significantly show lower values in the index in the Sobs index. This implies that senolytic compounds modify the diversity of certain gut microbiota communities compared with the control, making the species in the community less diverse while improving the evenness of the species.

To observe the effect of senolytic compounds in changing the proportions of OTUs, the Chao index was used to estimate richness addition to diversity. Fig. 3 shows the Chao index of each interventional group compared with the control. Of the four compounds, dasatinib and sirolimus significantly reduced the abundance of present OTU in the gut microbiota samples, denoted by the significant reduction in the Chao

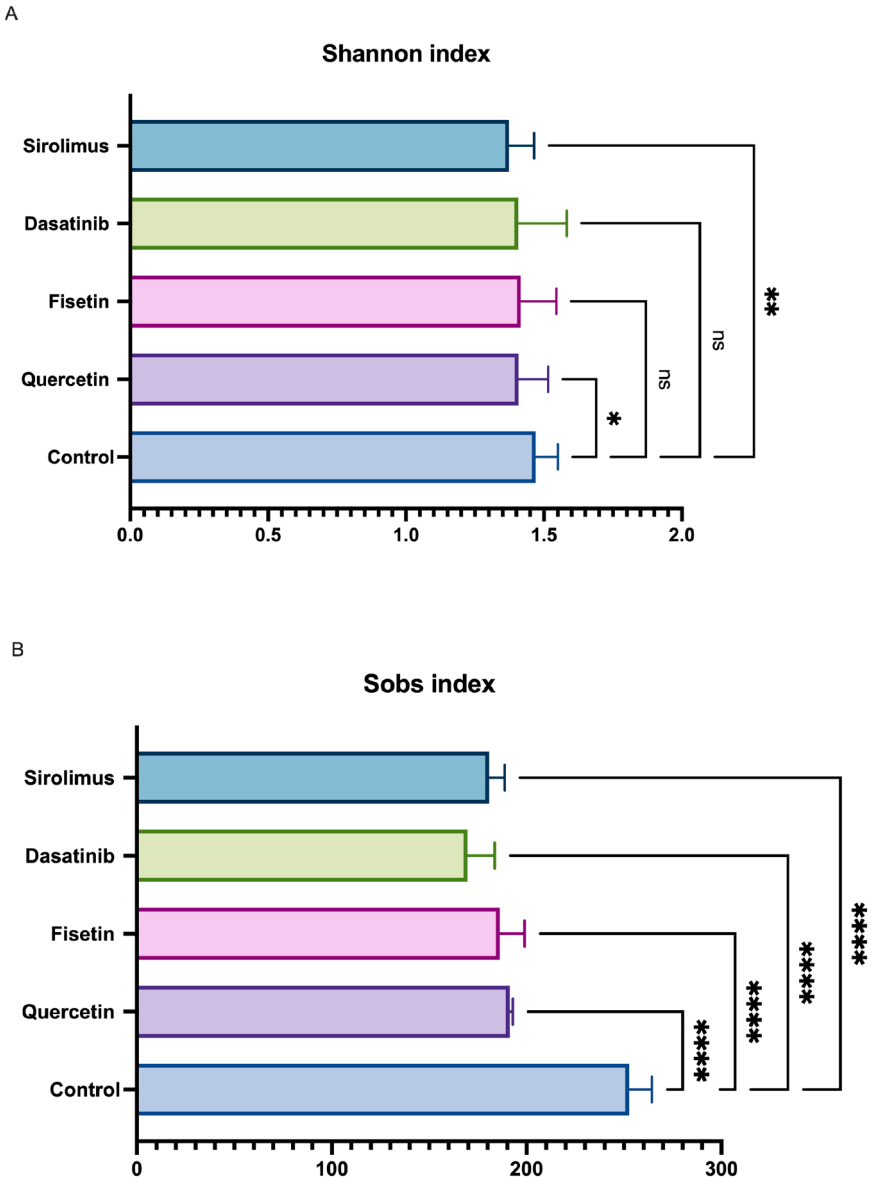

Fig. 2. Shannon index (A) and Sobs (B) index of healthy gut microbiota classified by different senolytic drugs compared to control where the ****, ** and * refer to p-value <0.0001, 0.001, and 0.01, respectively.

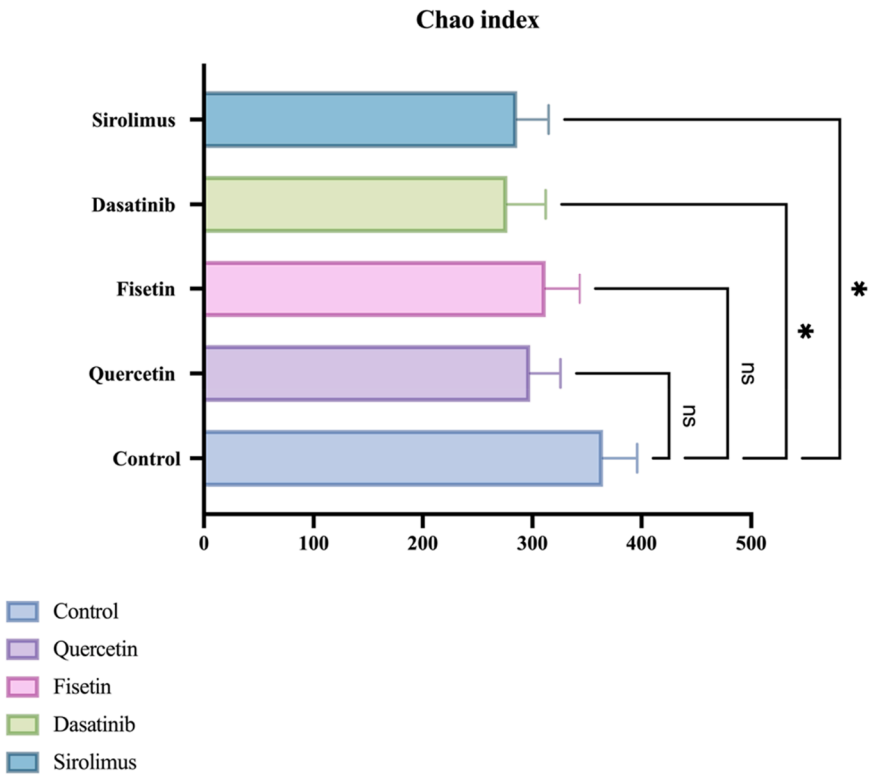

Fig. 3. The Chao index of the intervention group compared to the control where * refers to p-value < 0.01.

index.

The beta-diversity is applied to observe the effect of four senolytic compounds on the variability in gut microbiome community composition compared with the control. The unweighted (Fig. 4A) and weighted (Fig. 4B) UniFrac distances were applied to construct the phylogenetic tree. The unweighted UniFrac only assesses the absence or presence of taxa, while the weighted UniFrac takes the relative abundance into account in the calculation (Tang et al., 2016).

These results confirm that beta diversity in gut microbiome composition was not significantly different in the treated group (with different compounds) compared to the control group. However, the subtle variations within gut microbiota structure after exposure to treatments were further investigated. At the genus level, weighted UniFrac was used to confirm the impact of senolytic compounds on community diversity in both common and rare genera which may constitute a less significant portion of the community (Lozupone et al., 2011). All senolytic compounds showed the same trend of increasing the variety of certain microbial species such as *Bacilli, Bacteroidia*, and *Actinobacteria*, while decreasing the variety of *Clostridia* in the majority of donors (Fig. 5). Moreover, all senolytic compounds increased the abundance of *Veillonella* in donor 4 and decreased the abundance of *Pediococcus* in donor 3. *Veillonellae* use lactate in a lactate-to-propionate catabolic pathway and work to lower lactate levels in the body (Zhang and Huang, 2023). The increase in *Veillonellae* may contribute a positive effect on propionate and acetate production, playing a role in maintaining colonic immune homeostasis and other essential regulatory pathways (Zhang et al., 2024). *Pediococcus spp*. are lactic-producing bacteria that naturally occur in human gut microbiota alongside well-known lactic acid bacteria such as *Lactobacilli* (Holland et al., 2011). In a healthy colon, *Pediococci* mainly produce a racemic mixture of lactate forms (L-lactate and D-lactate), inhibiting pathogen proliferation, and consequently helping to regulate human gut microbiota composition (Shan et al., 2021; Kandler, 1983). However, in the elderly who have health complications, the lactate produced from lactic-producing bacteria, especially D-lactate, can accumulate to high levels that risk progression into lactic acidosis (Wang et al., 2020; Remund et al., 2023). Moreover, *Pediococcus* has been documented as a case report as an opportunistic pathogen in an immunocompromised 70-year-old patient (Mantzios et al., 2024).

The gut microbiota composition was identified with 16S rRNA sequencing targeted Illumina sequencing. Fig. 6A shows the ranking of relative abundance of gut microbiota species before and after exposure to the four senolytic compounds. The core species in all donors are consistent across the donors, composed of *Bacteroides fragilis* (average = 50.5 %), *Clostridiumsensustricto spp* (average = 30.9 %), *Bidifidobacterium longum* (average = 10.9 %) and *Veillonella atypica* (average = 3.5 %) with less abundance of *Eggerthella lenta* (average = 1.7 %) and *Streptococcus anginosus* (average = 0.1 %). The individual sequencing data of each donor after being treated with senolytic drugs is shown in Fig. S3. In Fig. 6B, all senolytic compounds show a potential anti-senescent effect through gut microbiome modulation as they increase the abundance of *Bacteroides fragilis, Bidifidobacterium longum* and *Veillonella parvula* and decrease *Clostridiumsensustricto* genera. A recent study of centenarians found that increasing the abundance of *Bacteroides fragilis* potentially offers a positive effect in anti-ageing through the upregulation of anti-inflammatory factor IL-10 gene expression (Wang et al., 2022). *Bidifidobacterium longum* shows promising anti-senescent effects through potent anti-oxidant activity and anti-microbial action against certain pathogenic bacteria species (Xia et al., 2020). Therefore, the enrichment in this specific species offers a positive effect on preventing senescent accumulation inside the colon.

Veillonella atypica is mainly responsible for enzymatically metabolising lactate inside the colon into propionic acid (Scheiman et al., 2019). Balancing lactate levels is important in ageing because excess lactate is absorbed through colonic epithelial cells via the proton-dependent monocarboxylate transporter 1 (MCT-1) and accumulates in other organs, resulting in extracellular lactic acidosis (Remund et al., 2023; Andreucci et al., 2023). The high level of lactate could disrupt the interaction of Akt and regulation of the downstream Akt/p21/p27/cyclin D and Akt/Nrf2/HO-1 pathways, consequently inducing cellular senescence and oxidative stress production (Zhang

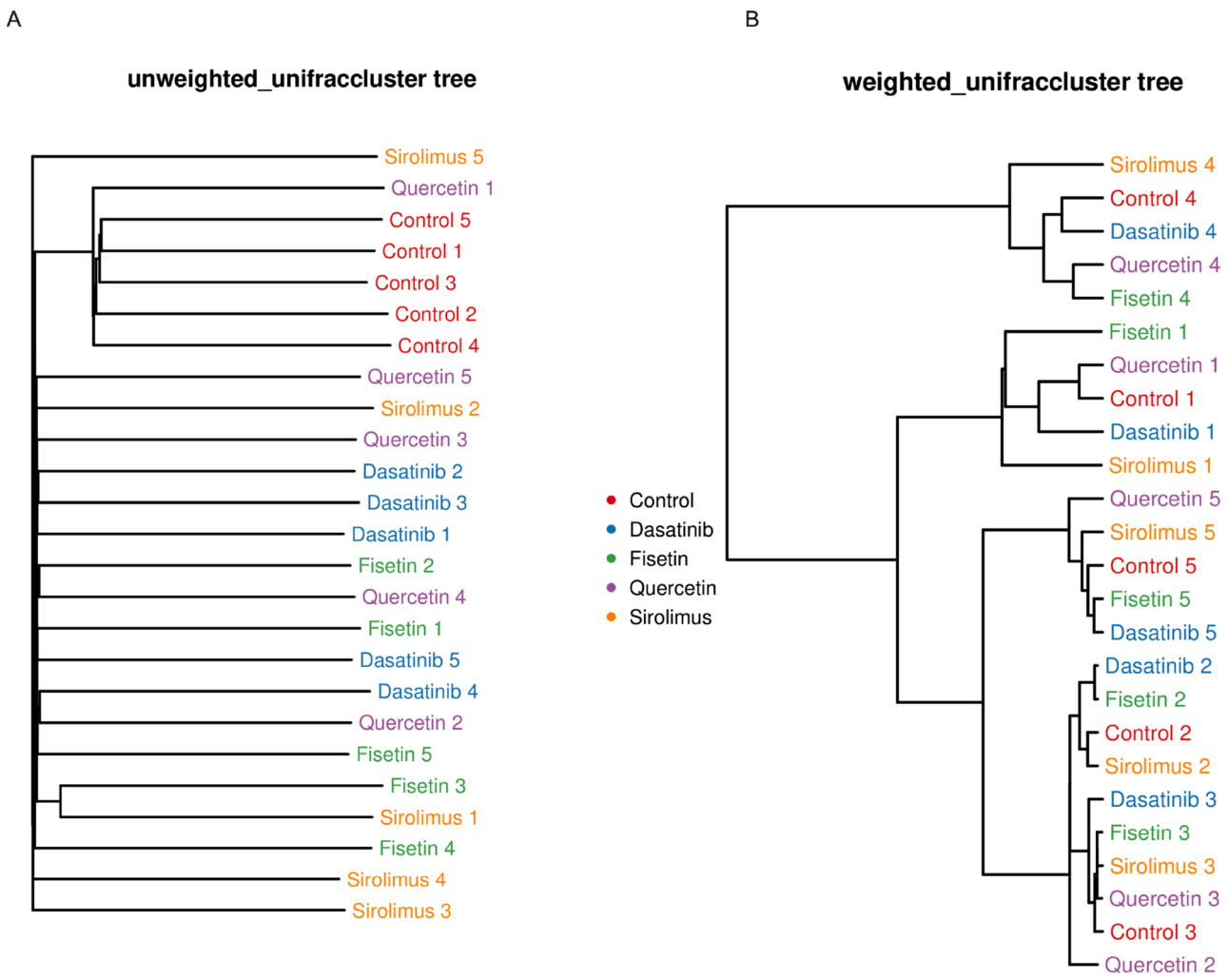

Fig. 4. The phylogenetic tree constructed from the unweighted UniFrac (A) and weighted UniFrac (B) of gut microbiota before and after treated with four senolytic compounds.

et al., 2024). Both cellular senescence and oxidative stress are important hallmarks of cellular damage and telomere erosion, leading to ageing and morbidity in later life (Erusalimsky, 2020; Zhou et al., 2021; Höhn et al., 2017). Therefore, the enrichment of *Veillonella atypica* after exposure to senolytic compounds may offer a beneficial environment. A recent in vivo study found that the abundance of *Clostridium sensu stricto* increases with age in an aged animal model and is highly abundant in enterocolitis subjects, suggesting that this genus could act as a biomarker of ageing (Ke et al., 2021). Thereby, the reduction in abundance of this genus may yield anti-ageing effects from senolytic compounds.

In addition, the data reveals the majority of senolytic compounds, except fisetin, enrich *Eggerthella lenta,* which can invade the blood and lead to bacteraemia (Maffei et al., 2017; Gardiner et al., 2015; Viehof et al., 2024). Another significant aspect of fisetin is its ability to decrease the abundance of several bacteria significantly, such as *Enterococcus faecalis* and *Streptococcus* spp., while dasatinib and sirolimus significantly reduce only *Streptococcus* spp. These species are opportunistic pathogens and are closely associated with ageing (Gonçalves et al., 2016; Dahl et al., 2019). Meanwhile, fisetin also significantly decreases the abundance of *Bidifidobacterium breve, Limosulactobacillus reuteri*, and *Veillonella parvula* while dasatinib and sirolimus significantly reduce the abundance of latter two species. These species play a role in regulating immune function while potentially eliciting anti-senescence effects.

The effect of senotherapeutic agents on the microbiota, with respect to the heterogeneity of donors, was also investigated. A heatmap illustrates changes in species abundance under the influence of senolytic compounds compared with the control (Fig. 7). The relative abundance is indicated by log10-transformed relative abundance of the most common taxa (rows) across intervention groups in each donor (columns). The red zone indicates an increase in relative abundance, while the blue zone indicates a decrease. The findings reveal that senolytic compounds do not significantly modulate gut microbiota between individuals, likely due to the high heterogeneity of gut microbiota among donors. This confirms that human host factors shaped gut microbiota is an important factor that cannot be overlooked. The diet, host environment, maternal effect and other factors contribute to the unique gut microbiota fingerprint, leading to heterogeneity in therapeutic outcomes due to diverse interaction between individual gut microbiota and xenobiotics (Yip and Chan, 2015; Spor et al., 2011; Rothschild et al., 2018).

3.3. *The effect of senotherapeutics on metabolic pathways*

The effect of senolytic compounds on the functional diversity of healthy gut microbiota is revealed in this study. The microbial communities play vital roles in nearly every biological process, mediating the host functions (Bier et al., 2015). However, uncovering changes in the composition of the gut microbiota alone does not link the functional information of gut microbiota with host interactions (Lozupone et al.,

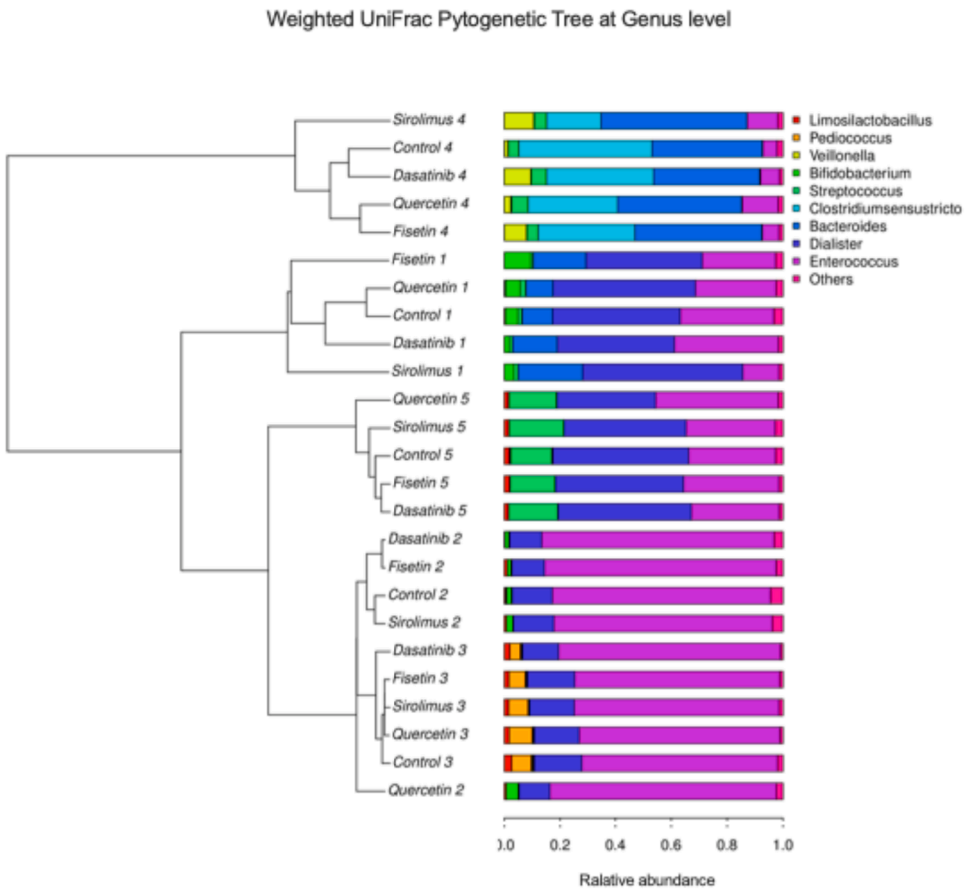

Fig. 5. The beta diversity of five different donors across four different interventions at the genus level.

2012). Therefore, functional profiling is the best way to assess the bioactivities of senotherapeutics. The Kyoto Encyclopedia of Genes and Genomes (KEGG) and Kyoto Orthology (KO) are databases used in this study to identify functional features. KO reference hierarchy was divided into three tiers. While KO level 1 offers the broadest functional categories, KO level 2 is a sub-division of KO level 1 that specifically refines biological processes or pathways. In addition, KO level 3 offers the finest data on specific biological pathways among other hierarchies (Kanehisa et al., 2017).

KEGG offers a more expansive vantage due to its coverage of genetic information and diseases, including drug interactions (Altman et al., 2013). At KO level 2 KEGG pathways, all senolytic compounds modulate the expression of gene or protein abundance in metabolic pathways (supplementary table S1). All senolytic compounds decrease the expression of the gene which is associated with xenobiotic biodegradation, environmental adaptation, signal transduction, cell motility, and metabolism of terpenoids and polyketides. From Fig. 8, quercetin reduces gene expression in several pathways such as the immune system, environmental adaptation, signal transduction, cell motility, cell growth and death, biosynthesis of other secondary metabolites, and metabolism, translation, glycan biosynthesis and metabolism, amino acid metabolism, and metabolism of cofactor and vitamins, assessed from the darker purple colour zone compared to control. Dasatinib reduces the expression of genes associated with amino acid metabolism, while sirolimus and fisetin increase the expression of genes associated with the aforementioned pathway.

Delving into the specific signalling pathways offered by the KO level 3, all senolytic compounds modulate the expression of genes involved in several metabolic pathways known for ageing and longevity (supplementary table S2). Surprisingly, all senolytic compounds reduce the expression of genes relevant to nucleotide excision repair (0.4–0.9 %) while increasing gene expression in the citric acid cycle (TCA cycle) (Fig. 9). Both the nucleotide excision repair and TCA cycle play vital roles in regulating ageing. Nucleotide excision repair (NER) functions in maintaining genome stability through the removal of DNA lesions (Kuper and Kisker, 2023; Cohen and Adar, 2023). There is mounting evidence indicating that the NER capacity decline in ageing is associated with the progression of age related-diseases relevant to DNA instability such as Alzheimer's disease. However, newer evidence found that defective NER repairment is a more important factor in preventing the formation of senescent cells (Suzuki et al., 2024). The TCA cycle generates cellular energy, free radicals, and critical metabolites required for cellular function (Kurhaluk, 2024). Increasing the flux within the TCA cycle has been connected to longevity in vitro and in vivo models (Perron et al., 2000; Borkum, 2023). The findings found a reduction in gene abundance in NER, otherwise, the decrease in gene expression is very small and not significant enough to justify for lack of anti-ageing activity. Moreover, the anti-senescent effect may be compensated by the increase of other pathways such as TCA. The potential finding is supported by findings in a previous study accomplished in the NER dysfunction *C. elegans* model (Fang Evandro et al., 2016).

Moreover, quercetin greatly reduces the expression of genes involved in apoptosis and NOD-like receptor signalling pathways while slightly decreasing the expression of genes involved in insulin signalling pathway, RNA degradation, base excision repair, homologous recombination, folate metabolism, and mismatch repair. Quercetin also increases gene expression in several pathways such as the proteasome, lysosome, TCA cycle, glycolysis, and pentose phosphate pathway. Fisetin tends to increase gene expression in several pathways involved with ageing and longevity. Similar to quercetin, fisetin increases the gene expression in the proteasome, and lysosome while decreasing gene expression in fatty acid degradation, RNA degradation, base excision

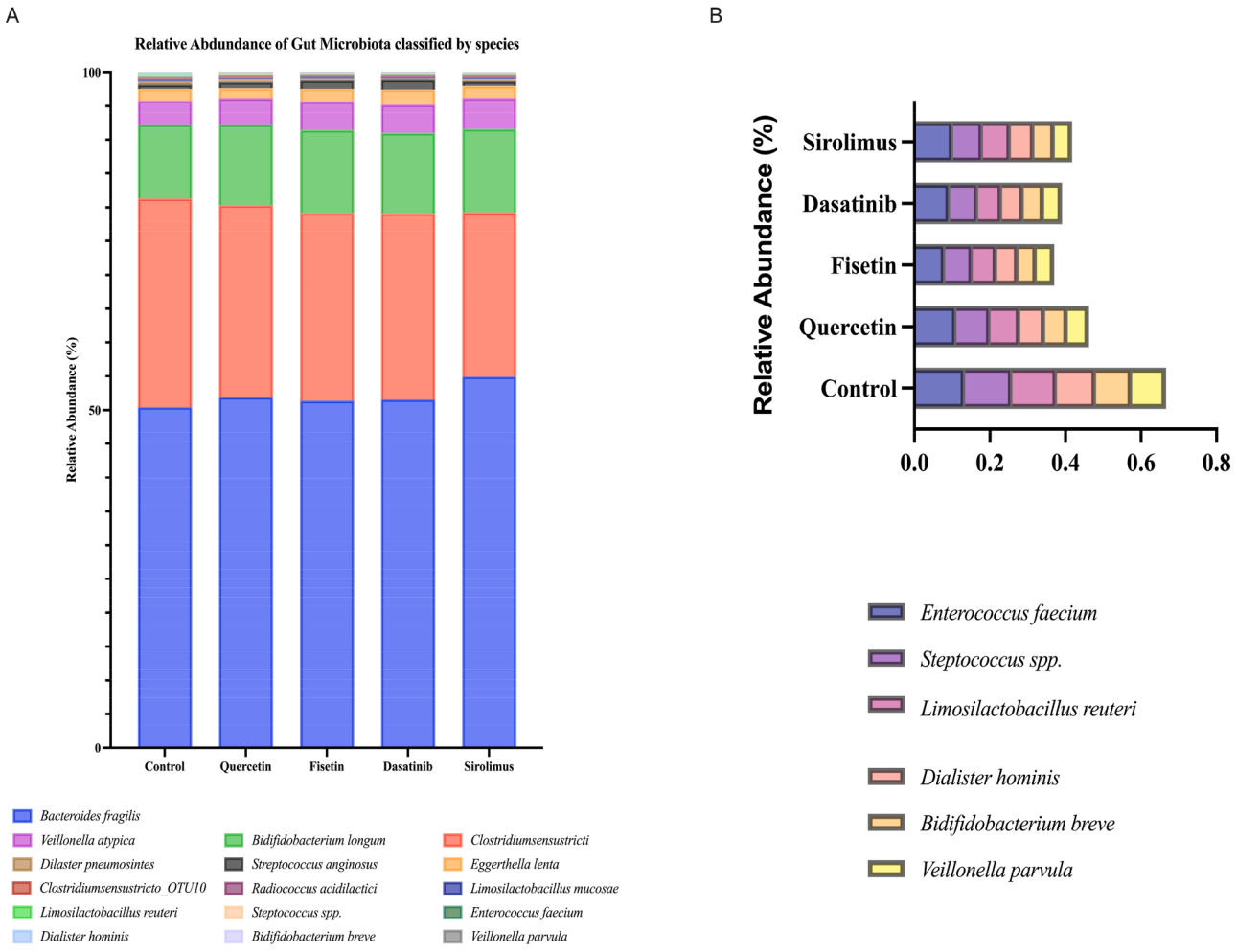

Fig. 6. Gut microbiota abundance in response to senolytic compounds. The relative abundance of gut microbiota species before (control) and after exposure to senolytic compounds (A). The relative abundance of specific gut microbiota species that are significantly modulated after the presence of senolytic compounds (B).

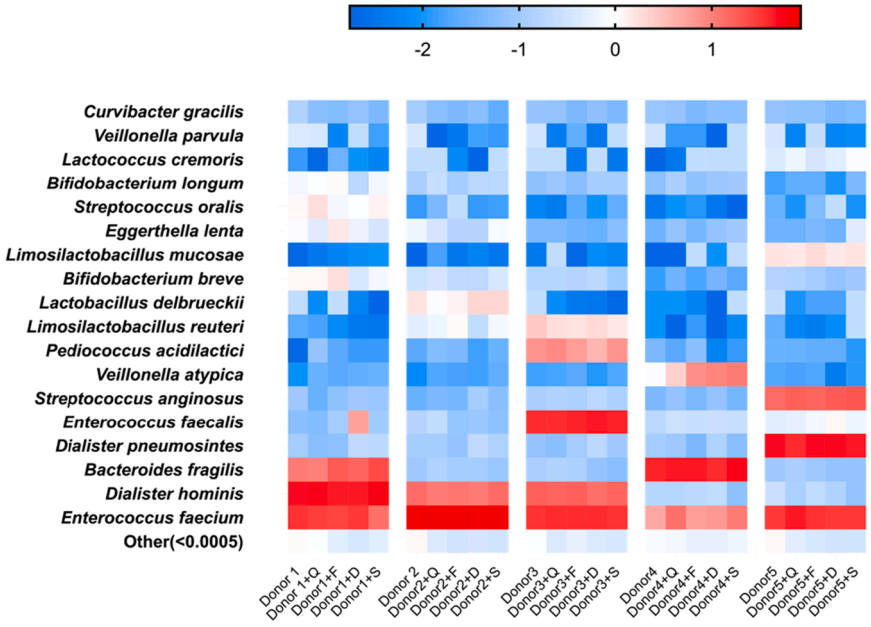

Fig. 7. A heatmap of gut microbiota composition displays the log10-transformed relative abundance of the most common taxa among donors before and after being treated with senolytic compounds.

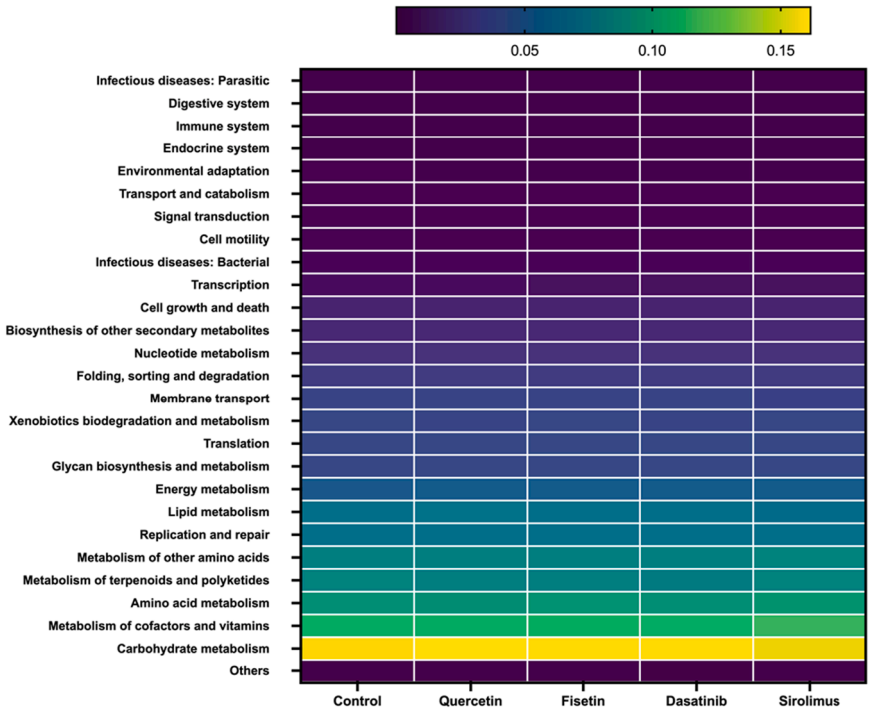

Fig. 8. A heatmap representing gene expression associated with metabolic pathways (rows) across four different senolytic compounds compared to the control (column). The darker zone denotes higher gene expression compared to the lighter colour zone.

repair and pentose phosphate pathway. Fisetin, dasatinb, and sirolimus follow a similar trend in increasing the expression of genes in several pathway such as apoptosis and NOD-like receptor signalling pathway, oxidative phosphorylation, homologous recombination, and folate metabolism. Unlike quercetin, most senolytic compounds reduce the expression of genes in glycolysis and pentose phosphate pathways.

The proteasome pathway maintains the homeostasis of proteins, degrading normal proteins for physiological regulation and eliminating the abnormal proteins for cellular protection (Hegde et al., 2023). The newer studies found a close association between the reduction of proteasome activity and ageing, amplifying the accumulation of damaged and misfolded proteins in many organs and causing aged-related diseases such as neurodegenerative diseases (Davidson and Pickering, 2023; Mishra and Thakur, 2023). The increase in gene expression involved in the proteasome pathway due to several senolytic compounds through the modulation of gut microbiota is a promising mechanism to elicit an anti-ageing effect. Dysregulation within the apoptosis pathway is considered the hallmark of ageing, leading to cellular senescence (Lucas et al., 2023). Therefore, the increase in expression of the gene involved in the apoptosis pathway contributes to the promising anti-senescent effect. Unlike apoptosis, lysosome breaks down and recycles cellular waste (Settembre and Perera, 2024). This function declines during ageing, leading to the accumulation of cellular waste and eventually contributing to cellular senescence (Tan and Finkel, 2023). New evidence reveals that increasing the lysosome processing and apoptosis pathway potentially promotes healthy ageing while alleviating age-related pathology due to less macroautophagy (Zhang et al., 2023; Roh et al., 2023). The increase in gene expression in preventive-like pathways such as oxidative phosphorylation, mismatch repair, homologous recombination, and folate metabolism increases the stability of DNA from damage due to oxidative stress (Dominguez-Valentin et al., 2023; Salmón et al., 2023; Talibova et al., 2023; Choi and Friso, 2023). The increase in gene expressions from those pathways potentially offers anti-ageing and improves longevity through the delaying of cell damage which is the key mechanism associated with cellular senescent. Glycolysis and the pentose phosphate pathway run in parallel to produce ribose-5-phosphate and nicotinamide adenine dinucleotide phosphate (NADPH) (TeSlaa et al., 2023). Both products play a vital role in regulating redox homeostasis which is a key for longevity and healthy ageing (Horecker, 2002). NADPH is a reducing agent that removes free radicals, improving oxidative stress tolerance and eventually extending lifespan (Shen et al., 2023). There is still ambiguity in the role glycolysis plays in ageing. Some studies indicate that the upregulation of the glycolysis pathway activates mTOR and S6K activities which enhance the expression of HIF-1 α, a key mediator in expanding the longevity in the nutrient-dependent manner of animal models (Snyder et al., 2022; Bjedov and Rallis, 2020). However, another study reveals that shifting energy production towards glycolysis in the cytoplasm elevates pyruvate concentration (Borkum, 2023). Pyruvate mediates the production of reactive oxygen species in the mitochondria (Kiesel et al., 2021). Moreover, glycolysis is aligned with the insulin signalling pathway, and upregulating glycolysis results in insulin resistance, escalating the progression of cellular senescence and age-related diseases (Rabbani et al., 2022).

4. Conclusion

In this study, the interactions between human gut microbiota and four senolytic drugs, quercetin, fisetin, dasatinib and sirolimus, have been explored. Human gut microbiota degraded the senolytic drugs to various extents, with dasatinib being the most stable and quercetin the least stable after 24 h of incubation. Fisetin and sirolimus demonstrated moderate stability in the presence of gut microbiota. The senolytic drugs modified the human gut microbiota by reducing microbial diversity (alpha diversity), while increasing the abundance of bacteria associated with anti-ageing and reducing the abundance of opportunistic pathogens responsible for age-related inflammation (16S rRNA sequencing). Moreover, the drugs modified gene expression in pathways involved in anti-ageing strategies and longevity. This study provides an understanding of the pharmacobiomics of senotherapeutics and their bidirectional interactions with gut microbiota. This research bridges the gap towards the clinical translation of gut microbiome-targeted strategies

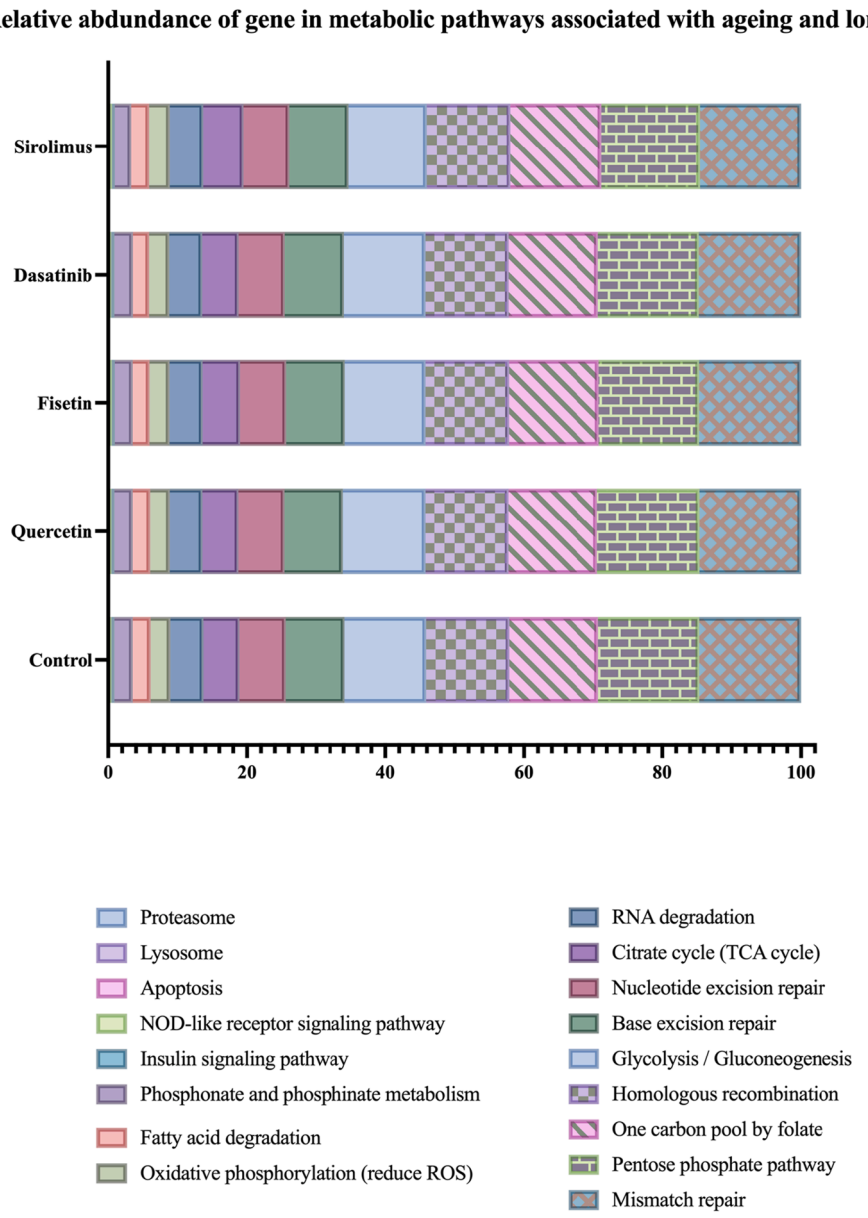

Fig. 9. Abundance of genes associated with metabolic pathways involved in ageing and longevity across four different senolytic compounds compared to a control.

for healthy ageing, including highlighting the importance of optimizing senotherapeutic delivery to enhance their direct effects on gut microbiota. While the findings offer promising evidence of the modulatory effects of senolytic compounds on gut microbiota as potential agents for healthy ageing, further research is needed. Future studies should replicate these findings using larger and more diverse microbiome samples while also exploring the long-term effects, effects of multiplebdoses and possible toxicity of senolytic compounds. Additionally, a more comprehensive study design is essential to validate the therapeutic effects of senotherapeutics and gain deeper insights into their interplay with gut microbiota.

CRediT authorship contribution statement

Nannapat Sangfuang: Writing – review & editing, Writing – original draft, Visualization, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Yuan Xie:** Investigation, Formal analysis, Data curation. **Laura E. McCoubrey:** Project administration, Funding acquisition, Conceptualization. **Marissa Taub:** Writing – review & editing, Investigation. **Alessia Favaron:** Investigation, Formal analysis, Data curation. **Yang Mai:** Investigation, Funding acquisition, Formal analysis, Data curation. **Simon Gaisford:** Writing – review & editing, Supervision, Resources. **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. The content of this paper does not reflect the views of GSK.

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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.2025.107098.

Data availability

Data will be made available on request.

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