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Interplay between senolytic drugs and microbiome preserve cognitive abilities during aging

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Abstract

Aging is characterized by the accumulation of senescent cells, which significantly contributes to the development of age-related diseases, including cognitive impairment and compromised integrity of the gut–brain axis. Senolytic intervention aimed at eliminating senescent cells, particularly the combination of Dasatinib and Quercetin (D+Q), represent a promising strategy to mitigate these dysfunctions. The aim of this dissertation was to examine the multidirectional effects of senolytic treatment (D+Q) on cognitive function, gut microbiota composition, bacterial metabolites, and the integrity of the intestinal and brain barriers in aged Wistar rats.

Using cognitive behavioural tests, especially the Active Allothetic Place Avoidance task (AAPAT), it was shown that D+Q treatment significantly improves spatial memory and cognitive abilities in aged rats, with beneficial effects persisting even after the end of treatment. Analysis of the gut microbiota of these animals demonstrated that D+Q administration led to significant changes in its composition, including an increased abundance of *Lactobacillus acidophilus*, a bacterial species previously associated with beneficial effects on cognitive function and metabolic processes. Metabolomic analysis revealed a marked reduction in faecal levels of short-chain fatty acids (SCFAs) and bile acids, consistent with altered microbial production and/or altered host handling of these metabolites, including changes in utilization, degradation, reabsorption, and absorption, while systemic profiles remained largely stable apart from a selective reduction in serum formic acid.

Histological and molecular analyses revealed region-specific changes in intestinal mucosal architecture, expression of tight junction proteins, mucin production, and inflammatory markers in response to D+Q treatment. In the small intestine, mucosal thickening and increased mucin production were observed, whereas in the large intestine a reduced mucosal thickness and decreased mucin synthesis were noted. These changes were accompanied by a decrease in pro-inflammatory markers (TNF-α, IFN-γ) alongside an increase in anti-inflammatory cytokines (IL-10), indicating a protective and anti-inflammatory effect of D+Q at the intestinal level.

Importantly, D+Q administration contributed to improved blood–brain barrier integrity, confirmed by increased expression of tight junction proteins (zonula occludens-1, Occludin, claudin-1) in brain tissue. Moreover, reduced inflammatory markers and increased IL-10 expression indicate a shift in the brain milieu toward an anti-inflammatory state, which may explain the observed improvement in cognitive function.

These results indicate systemic benefits of senolytic of intermittent D+Q intervention through modulation of the gut microbiota, reduction of inflammation, region-specific remodelling of intestinal barrier-associated features, and improvement of blood–brain barrier integrity. Together, these findings suggest that intermittent administration of D+Q may represent a promising strategy to counteract cognitive impairment, while underscoring the key role of the gut–brain axis as a key mechanistic axis and intervention target in age-related dysfunction.

Streszczenie

Starzenie się organizmu charakteryzuje się gromadzeniem komórek starzejących się (senescentnych), co istotnie przyczynia się do rozwoju chorób związanych z wiekiem, w tym zaburzeń poznawczych oraz upośledzenia integralności osi jelitowo-mózgowej. Interwencje senolityczne, ukierunkowane na eliminację starzenie komórkowe, zwłaszcza kombinacja dasatynibu i kwercetyny (D+Q), stanowią obiecującą strategię łagodzenia tych dysfunkcji. Celem niniejszej rozprawy było zbadanie wielokierunkowego wpływu podawania senolityków (D+Q) na funkcje poznawcze, skład mikrobioty jelitowej, metabolity bakteryjne oraz integralność barier jelitowej i mózgowej u starych szczurów szczepu Wistar.

Przy użyciu behawioralnych testów poznawczych, a zwłaszcza zadania aktywnego unikania miejsca (AAPAT – active allothetic place avoidance task), wykazano, że podawanie D+Q znacząco poprawia pamięć przestrzenną oraz umiejętności poznawcze u starych szczurów, przy czym pozytywne efekty utrzymują się nawet po zakończeniu leczenia. Analiza mikrobioty jelitowej tych zwierząt wykazała, że podawanie D+Q doprowadziło do istotnych zmian w jej składzie, w tym do zwiększenia liczebności *Lactobacillus acidophilus*, gatunku bakterii, którego obecność wiązana była wcześniej z korzystnym wpływem na funkcje poznawcze oraz procesy metaboliczne. Analiza metabolomiczna wykazała znaczące obniżenie poziomu krótkołańcuchowych kwasów tłuszczowych (SCFA) oraz kwasów żółciowych w kale, co może wskazywać na zmniejszoną produkcję mikrobiologiczną, nasiloną degradację lub zwiększone wchłanianie systemowe tych metabolitów.

Analizy histologiczne oraz molekularne ujawniły regionowo-specyficzne zmiany w architekturze śluzówki jelit, ekspresji białek połączeń ścisłych, produkcji mucyn oraz markerów zapalnych w odpowiedzi na leczenie D+Q. W jelicie cienkim zaobserwowano pogrubienie błony śluzowej oraz podwyższoną produkcję mucyn, natomiast w jelicie grubym odnotowano zmniejszenie jej grubości i obniżenie syntezy mucyn. Zmianom tym towarzyszył spadek poziomu markerów prozapalnych (TNF-α, IFN-γ) przy jednoczesnym wzroście cytokin przeciwzapalnych (IL-10), co wskazuje na ochronne i przeciwzapalne działanie interwencji D+Q na poziomie jelitowym.

Co ważne, podawanie D+Q przyczyniło się do poprawienia integralności bariery krew–mózg, potwierdzonego podwyższoną ekspresją białek połączeń ścisłych (Zonulina-1, Okludyna, Klaudyna-1) w tkance mózgowej. Ponadto redukcja markerów stanu zapalnego oraz wzrost ekspresji IL-10 świadczą o przesunięciu równowagi w obrębie środowiska mózgowego w kierunku przeciwzapalnym, co może tłumaczyć zaobserwowaną poprawę funkcji poznawczych.

Wyniki te wskazują na ogólnoustrojowe korzyści interwencji senolitycznej poprzez modulację mikrobioty jelitowej, redukcję stanu zapalnego oraz przywrócenie integralności bariery jelitowej i mózgowej. Niniejsza praca wskazuje, że zastosowanie interwencja D+Q stanowi obiecujące podejście przeciwdziałania zaburzeniom poznawczym, podkreślając istotną rolę osi jelitowo-mózgowej jako ważnej osi interwencji w zaburzeniach związanych z wiekiem.

Abbreviations

AAPAT	Active Allothetic Place Avoidance Task
ANOVA	Analysis of Variance
ANS	Autonomic Nervous System
ASV / ASVs	Amplicon Sequence Variant(s)
ATAC-seq	Assay for Transposase-Accessible Chromatin sequencing
BAs	Bile Acids
BCA	Bicinchoninic Acid (protein assay)
BBB	Blood–Brain Barrier
BDNF	Brain-Derived Neurotrophic Factor
CNS	Central Nervous System
CRP	C-reactive Protein
DDR	DNA Damage Response
D+Q	Dasatinib + Quercetin (senolytic cocktail)
DNA	Deoxyribonucleic Acid
DNA-SCARS	DNA Segments with Chromatin Alterations Reinforcing Senescence
DSS	Dextran-Sodium-Sulfate
DTR	Diphtheria Toxin Receptor (in p16-Ink4a-DTR model)
eGFR	estimated Glomerular Filtration Rate
ENA	European Nucleotide Archive
ENS	Enteric Nervous System
FDR	False Discovery Rate (statistical correction)
FFAR2 / FFAR3	Free Fatty Acid Receptor 2/3 (aliases of GPR43/41)
FITC	Fluorescein Isothiocyanate (FITC-dextran assay)
FMT	Faecal Microbiota Transplant
FOXO4-DRI	FOXO4 D-retro-inverso peptide (senolytic)
GABA	Gamma-Aminobutyric Acid
GBA	Gut–Brain Axis
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GC-MS	Gas Chromatography–Mass Spectrometry
GPC	Glycerophosphocholine
GPR41 / GPR43	G-protein-coupled Receptor 41 / 43 (SCFA receptors)
H&E	Hematoxylin and Eosin
H2AX (γH2AX)	Histone H2A.X
H3K9me3	Trimethyl-lysine 9 marks on histone H3
H3K27me3	Trimethyl-lysine 27 marks on histone H3

HPA	Hypothalamic–Pituitary–Adrenal axis
IFN-γ	Interferon γ
IL-6	Interleukin 6
IL-10	Interleukin 10
IND	Investigational New Drug
LPS	Lipopolysaccharide
MCI	Mild Cognitive Impairment
MS	Mass spectrophotometry
MTBE	Methyl tert-Butyl Ether
NF-κB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
OCT	Optimal Cutting Temperature compound
OIS	Oncogene-Induced Senescence
PBS	Phosphate-Buffered Saline
PCA	Principal Component Analysis
PCoA	Principal Coordinates Analysis
PEG (PEG-400)	Polyethylene Glycol (solvent vehicle)
Ph+	Philadelphia chromosome-positive
QC	Quality Control
qPCR	quantitative Polymerase Chain Reaction
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
SA-β-gal	Senescence-Associated β-Galactosidase
SAHF	Senescence-Associated Heterochromatin Foci
SASP	Senescence-Associated Secretory Phenotype
SCFAs	Short-Chain Fatty Acids
SDS	Sodium Dodecyl Sulfate
TGR5	Takeda G-protein-coupled receptor 5 or GPBAR1
TMA / TMAO	Trimethylamine / Trimethylamine N-oxide
TNF-α	Tumor Necrosis Factor α
UHPLC-HRMS	Ultra-High-Performance Liquid Chromatography–High-Resolution MS
USA	United States of America
UV	Ultraviolet (radiation)
ZO-1	Zonula Occludens-1 (tight-junction protein)

I. Introduction

1.1 Background on Aging and Age-related Diseases

- ### Definitions and Demographic Relevance

Aging is a complex, universal biological process marked by a progressive decline in physiological function and a rising susceptibility to diseases and death (López-Otín et al., 2013). Although the concept of aging is broadly understood, its precise definition remains challenging due to its multifactorial nature, encompassing molecular, cellular, and systemic changes (Kirkwood, 2005). The accumulation of these changes over time results in diminished resilience and functional capacity, ultimately leading to senescence and organismal deterioration (Childs et al., 2015).

Cellular senescence, a central hallmark of aging, describes an irreversible state of cell cycle arrest induced by various stressors, including DNA damage, oxidative stress, and oncogene activation (Hayflick & Moorhead, 1961; Campisi, 2013). Senescent cells accumulate with age and secrete numerous pro-inflammatory cytokines, chemokines, and growth factors collectively known as the senescence-associated secretory phenotype (SASP), thereby contributing significantly to chronic inflammation and tissue dysfunction, collectively termed "inflammaging" (Franceschi & Campisi, 2014; Sikora et al., 2021).

Age-related diseases are defined as conditions for which aging itself is a major risk factor. These diseases include neurodegenerative disorders (e.g., Alzheimer's disease and Parkinson's disease), cardiovascular diseases, type 2 diabetes, cancer, osteoporosis, arthritis, and chronic respiratory diseases (Kennedy et al., 2014; Niccoli & Partridge, 2012; Ferrucci et al., 2018). Alzheimer’s disease and other dementias exemplify aging-associated neurodegeneration, characterized by progressive cognitive impairment, loss of neuronal function, and distinctive neuropathological features such as amyloid-beta plaques and tau neurofibrillary tangles (Wyss-Coray, 2016). Similarly, Parkinson’s disease, marked by dopaminergic neuron loss and Lewy body pathology, progressively impairs motor and cognitive functions (Poewe et al., 2017).

The global demographic shift towards an aging population underscores the increasing relevance of studying age-related conditions. According to the United Nations, the proportion of the global population aged 65 and over is projected to rise from 10% in 2022 to 16% by 2050, nearly doubling from approximately 761 million to over 1.6 billion individuals (Figure 1; United Nations, 2022). This demographic transition is particularly pronounced in developed countries, where the elderly population is rapidly expanding, posing significant public health and socioeconomic challenges (Harper, 2014; Bloom et al., 2015). In Poland, demographic forecasts indicate a substantial increase in the elderly population, expected to reach 14 million people (about 40% of the population) by 2050 (Krawczyk, 2021).

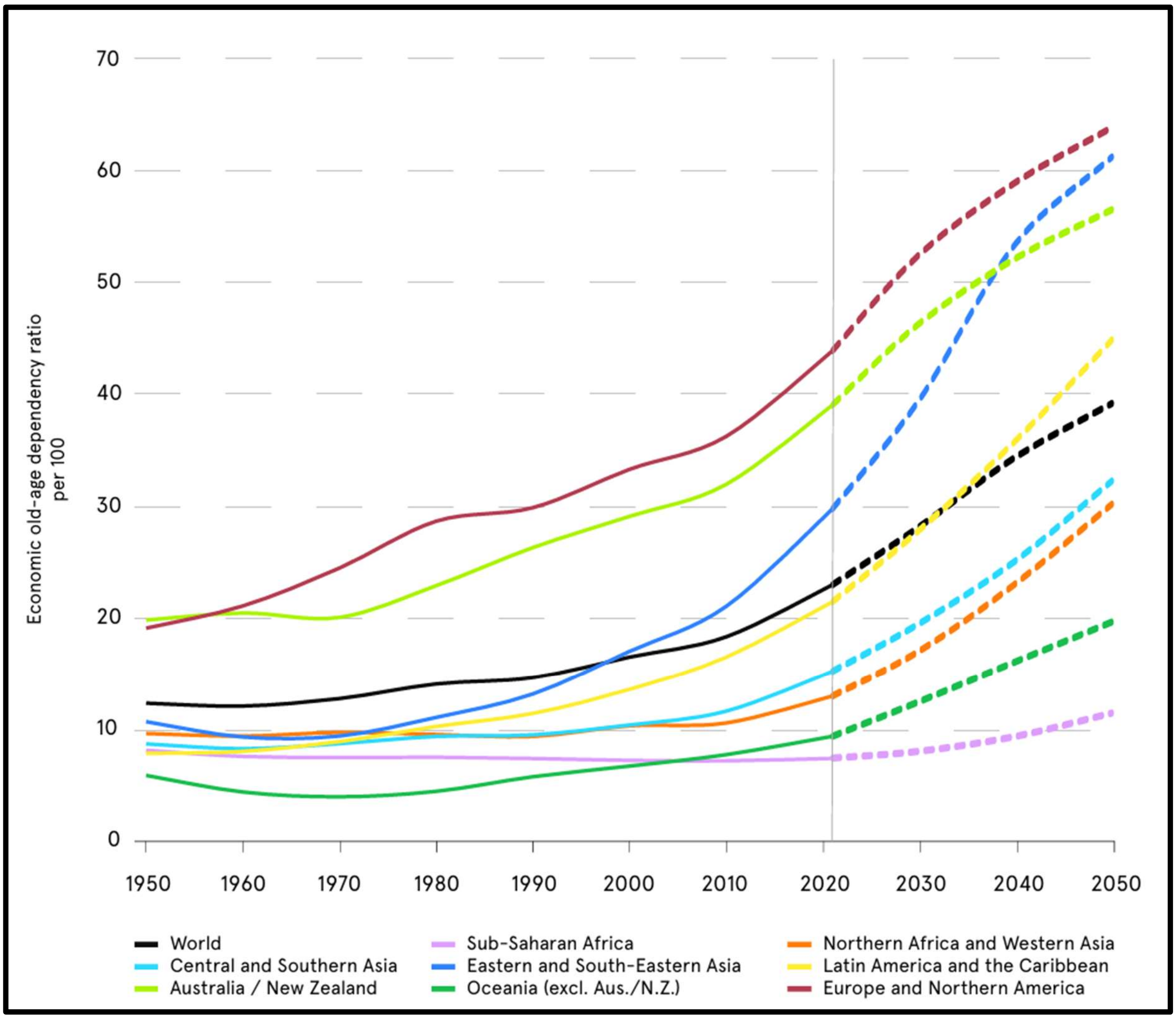

Figure 1. Percentage of people aged 65 years or over, world and regions. Estimates for 1950–2021 and projections for 2022–2050. In 2021, 1 in 10 people worldwide was aged 65 or over compared to 1 in 20 in 1950. In 2050, this age group may account for 1 in 6 people worldwide.

(Source: United Nations, 2017)

The economic impact of aging-related diseases is profound. Chronic age-associated conditions account for substantial healthcare expenditures, with increasing demands on medical resources, long-term care facilities, and social support systems (Prince et al., 2015; Olesen et al., 2012). Dementia alone, including Alzheimer's disease, represents a significant economic burden, estimated at approximately USD 1 trillion annually worldwide, with costs expected to double by 2030 (World Health Organization, 2021). Consequently, understanding aging processes and developing effective interventions to delay or prevent age-related diseases have become top priorities for biomedical research and public health policy (Kennedy et al., 2014; Partridge et al., 2020).

Given this context, aging research has increasingly focused on identifying interventions targeting fundamental biological mechanisms of aging, such as senescence, genomic instability, loss of proteostasis, deregulated nutrient sensing, mitochondrial dysfunction, and altered intercellular communication (López-Otín et al., 2023). Intervention strategies that aim to modulate these aging-associated mechanisms, such as caloric restriction, pharmacological interventions (e.g., rapamycin, senolytics), and modulation of the microbiome, represent promising avenues to extend health span and mitigate the burden of age-associated diseases (Longo et al., 2015; Zhu et al., 2015; Petrazzo et al., 2026).

Aging is characterized by complex, multifactorial changes at cellular, molecular, and systemic levels, contributing significantly to increased vulnerability to numerous chronic diseases. Given the unprecedented demographic shift towards an aging global population, addressing age-related diseases through targeted biomedical interventions has become imperative. **Comprehensive understanding of the biological processes underpinning aging is critical for developing novel approaches aimed at supporting healthy aging and reducing the associated healthcare and societal burdens**.

- Hallmarks of Aging

Aging is increasingly understood as a multifaceted biological phenomenon, characterized by distinct molecular and cellular hallmarks that collectively contribute to organismal decline and susceptibility to age-associated diseases. Among these hallmarks, cellular senescence, chronic inflammation ("inflammaging"), and compromised barrier integrity have emerged as particularly critical in the pathogenesis of various age-related conditions (López-Otín et al., 2013; Kennedy et al., 2014; López-Otín et al., 2023).

Cellular Senescence is an irreversible state of permanent cell cycle arrest initially described by Leonard Hayflick in human fibroblasts (Hayflick & Moorhead, 1961). Senescence occurs as a response to diverse cellular stressors including DNA damage, oxidative stress, telomere attrition, oncogene activation, and epigenetic alterations (Figure 2; Campisi, 2013; Childs et al., 2015; Muñoz-Espín & Serrano, 2014). Although initially considered beneficial as an anti-tumorigenic mechanism, the prolonged accumulation of senescent cells has deleterious consequences. These cells exhibit distinctive morphological and metabolic changes, along with an enhanced resistance to apoptotic signals (Campisi & d'Adda di Fagagna, 2007). Critically, senescent cells adopt a characteristic secretory phenotype known as SASP, secreting inflammatory cytokines, chemokines, growth factors, and extracellular matrix remodelling enzymes. The SASP not only reinforces senescence through paracrine signalling but also promotes chronic tissue inflammation, cellular dysfunction, and structural deterioration (Coppé et al., 2010; Rodier & Campisi, 2011; Sikora et al., 2021). Consequently, senescence contributes to the pathogenesis of cardiovascular diseases, metabolic disorders, osteoarthritis, cancer, and neurodegenerative diseases such as Alzheimer's and Parkinson's diseases (Tchkonia et al., 2013; Baker et al., 2016).

Chronic Inflammation (Inflammaging) represents another critical hallmark of aging, characterized by a persistent, low-grade inflammatory state arising from dysregulated immune responses and the accumulation of senescent cells (Franceschi et al., 2000; Franceschi & Campisi, 2014). This age-related inflammatory response is associated with elevated levels of pro-inflammatory cytokines, including IL-1β, IL-6, TNF-α, and CRP, which collectively drive tissue degeneration and impaired regeneration (Ferrucci & Fabbri, 2018; Fulop et al., 2018; Franceschi et al., 2018). Inflammaging contributes significantly to the progression of various age-associated conditions, such as diabetes, atherosclerosis, osteoporosis, Alzheimer's disease, and cancers, by exacerbating cellular stress and perpetuating tissue dysfunction (Ferrucci & Fabbri, 2018; Rea et al., 2018). Emerging evidence indicates that inflammaging not only results from the accumulation of senescent cells but also from chronic activation of immune cells, dysbiosis of the microbiome, metabolic dysregulation, and increased gut permeability (Nagpal et al., 2018).

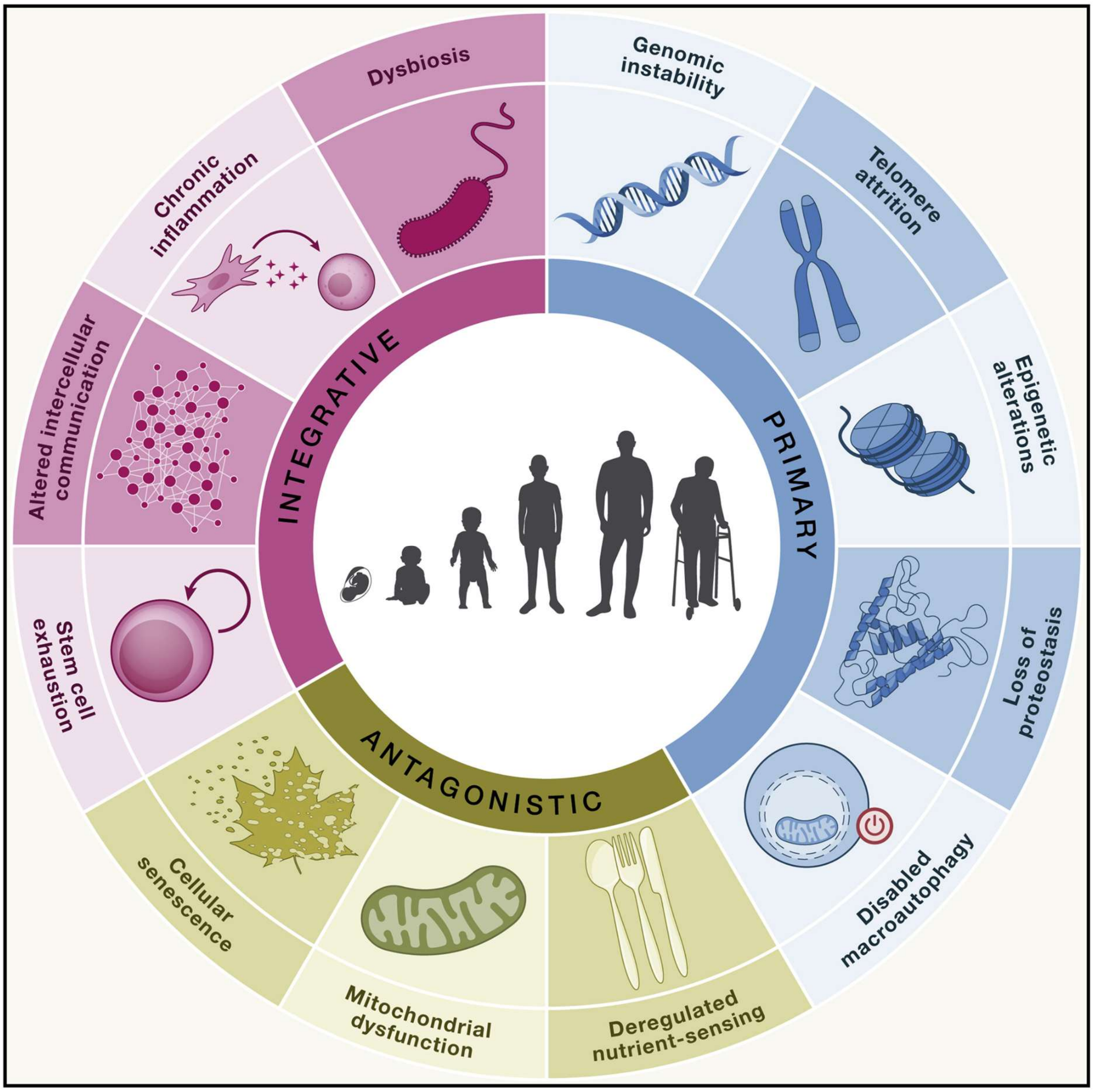

Figure 2. Hallmarks of aging.

The scheme compiles the 12 hallmarks of aging proposed in this work: genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, disabled macroautophagy, deregulated nutrient-sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, altered intercellular communication, chronic inflammation, and dysbiosis. These hallmarks are grouped into three categories: primary, antagonistic, and integrative.

(Source: López-Otín, Carlos et al. 2023)

Barrier Dysfunction has recently been recognized as a critical component of aging pathology, encompassing compromised integrity of physiological barriers such as the intestinal mucosal barrier, blood-brain barrier (BBB), and skin barrier (Tran & Greenwood-Van Meerveld, 2013; Camilleri et al., 2012; Sweeney et al., 2018). Biological barriers serve essential functions, protecting tissues from environmental insults and maintaining selective permeability and homeostasis. Aging profoundly impairs these barriers through a variety of mechanisms, including decreased epithelial and endothelial cell renewal, altered expression of tight junction proteins (such as claudins, Occludin, and zonula occludens proteins), and disrupted mucin production (Nagpal et al., 2018). The gut barrier is particularly sensitive to age-related changes, leading to increased intestinal permeability ("leaky gut"), allowing bacterial products such as lipopolysaccharides (LPS) and microbial metabolites to translocate into systemic circulation. This translocation exacerbates systemic inflammation, metabolic disturbances, and cognitive dysfunction (Camilleri et al., 2012; Tran & Greenwood-Van Meerveld, 2013; Nagpal et al., 2018). Similarly, blood-brain barrier dysfunction, characterized by endothelial cell senescence, decreased expression of tight junction proteins, and altered neurovascular coupling, significantly contributes to neuroinflammation and neurodegeneration observed in Alzheimer's disease, Parkinson’s disease, and vascular dementia (Montagne et al., 2015; Sweeney et al., 2018; Nation et al., 2019).

Collectively, the interactions between these hallmarks, cellular senescence, inflammaging, and compromised barrier integrity, create a self-reinforcing cycle that accelerates aging and increases susceptibility to age-related diseases. **Interventions targeting these mechanisms, such as senolytics (e.g., Dasatinib and Quercetin), anti-inflammatory agents, dietary interventions, microbiome modulation, and barrier-strengthening approaches,** represent promising strategies to extend health span and reduce age-associated morbidity (Zhu et al., 2015; Xu et al., 2018; Petrazzo et al., 2026; López-Otín et al., 2023). Given the projected global demographic aging, understanding these fundamental biological mechanisms remains imperative for the development of effective, targeted interventions aimed at improving the quality of life in aging populations.

1.2 Targeting Cellular Senescence in Age-Related Pathologies

- ### Mechanisms of Cellular Senescence

Cellular senescence represents a critical biological state, originally described as a permanent cell cycle arrest of normal human fibroblasts after repeated population doublings (Hayflick & Moorhead, 1961). Since this seminal discovery, cellular senescence has been extensively studied as a major contributor to the aging process and associated diseases, establishing it as an important intervention target for mitigating age-related pathologies (Campisi, 2013; Muñoz-Espín & Serrano, 2014; Childs et al., 2015).

The induction of cellular senescence occurs in response to multiple intrinsic and extrinsic cellular stresses. These include DNA damage, oxidative stress, telomere attrition, oncogene activation, epigenetic changes, mitochondrial dysfunction, metabolic dysregulation, and mechanical stresses (Campisi & d'Adda di Fagagna, 2007; López-Otín et al., 2013; Gorgoulis et al., 2019). Cellular senescence acts initially as an essential tumour-suppressive mechanism, halting proliferation of cells at risk of malignant transformation. However, chronic accumulation of senescent cells exerts detrimental effects due to their altered secretory profile and persistent presence in tissues (Kuilman et al., 2010; Childs et al., 2015).

Morphological changes. Senescent cells exhibit distinctive features and biomarkers that define their state (Figure 3). Morphologically, these cells typically display enlarged and flattened cytoplasm, enhanced granularity, increased lysosomal mass, and prominent nucleoli (Dimri et al., 1995; Hernandez-Segura et al., 2018).

Lysosomal compartment. One of the most widely recognized molecular markers of senescence is increased activity of lysosomal β-galactosidase at acidic pH, termed senescence-associated β-galactosidase (SA-β-gal) activity (Debacq-Chainiaux et al., 2009). While SA-β-gal is an important marker, cellular senescence identification typically involves assessing multiple complementary biomarkers, including elevated expression of cyclin-dependent kinase inhibitors (CDKIs) such as p16^INK4a (CDKN2A), p21^CIP1 (CDKN1A), and p53, which regulate cell cycle arrest at the G1/S transition (Alcorta et al., 1996; Stein et al., 1999; Baker et al., 2011).

DNA damage. At a molecular level, the senescence program is triggered and maintained through complex and interconnected pathways. Key among these pathways is the DNA damage response (DDR), activated by genotoxic stressors such as ionizing radiation, UV exposure, and oxidative DNA damage (Rodier et al., 2009; d'Adda di Fagagna, 2008). Persistent DDR signalling involves phosphorylation of histone variant H2AX (γH2AX), ATM kinase activation, and accumulation of DDR proteins such as 53BP1, leading to sustained activation of tumour suppressors p53 and p21 (Herbig et al., 2004). Additionally, telomere shortening or dysfunction activates DDR and triggers replicative senescence through the p53/p21 signalling pathway (Harley et al., 1990).

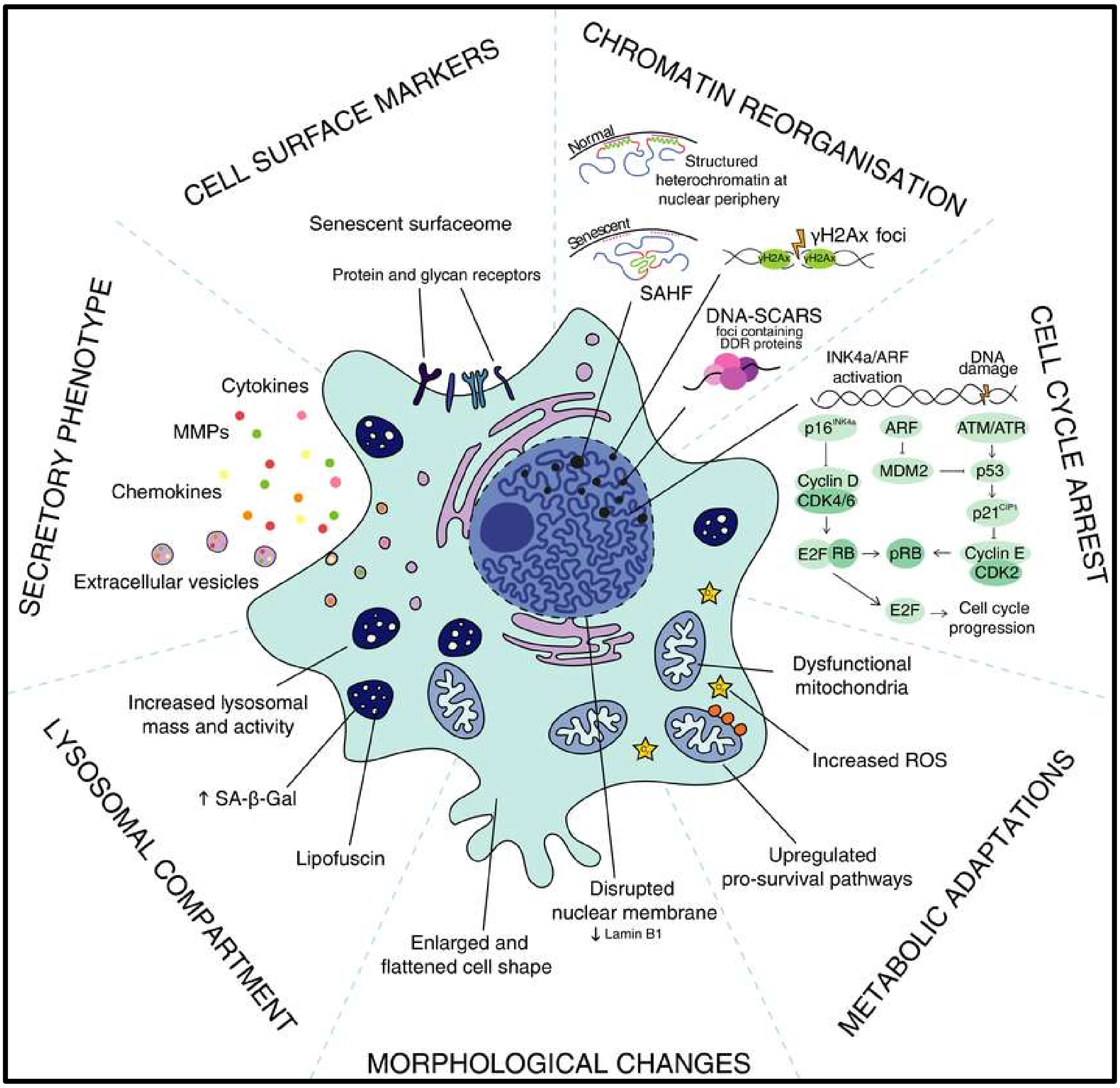

Figure 3. General hallmarks of cellular senescence.

Several cellular mechanisms and alterations constitute the so-called hallmarks of cellular senescence. These include a stable cell cycle arrest, driven by the action and cooperation of several proteins implicated in the p16INK4a/Rb and p21CIP1/p53 axes, depending on the senescence driver; chromatin alteration and reorganization, which includes SAHFs and DNA-SCARS marks and also results in the disruption of the nuclear envelope; macromolecular damage and metabolic changes, including increased ROS levels and dysfunctional mitochondria; resistance to apoptosis, resulting from the upregulation of pro-survival pathways; increased lysosomal compartment, which is characterized by the well-known overexpression of SA-β-gal, and the implementation of a strong paracrine secretion termed SASP.

(Source: González-Gualda E. et al., 2021)

Oncogene-induced senescence. Another significant mechanism driving cellular senescence is oncogene-induced senescence (OIS). Activated oncogenes, such as Ras, Raf, or BRAF, can stimulate senescence through excessive proliferative signalling, hyper-replication stress, and reactive oxygen species (ROS) accumulation, culminating in DDR and activation of tumour suppressor pathways, prominently p53 and p16^INK4a/Rb (Serrano et al., 1997; Kuilman et al., 2010). These pathways are critical for halting cell proliferation and preventing malignant transformation in early-stage tumours, further highlighting the dual role of cellular senescence in cancer prevention and promotion of aging-related pathologies (Collado et al., 2007; Braig et al., 2005).

Chromatin reorganisation. Epigenetic modifications also profoundly influence cellular senescence. Senescent cells display altered DNA methylation patterns, histone modifications, and chromatin remodelling, resulting in transcriptional changes that sustain the senescent phenotype (Cruickshanks et al., 2013; Sen et al., 2016). Notably, global hypomethylation, site-specific hypermethylation at CpG islands, loss of heterochromatin structure, and altered histone marks such as increased H3K9me3 and decreased H3K27me3 play fundamental roles in gene silencing or activation of senescence-related genes (Narita et al., 2003; Sedivy et al., 2008; Rai & Adams, 2012). This epigenetic remodelling stabilizes the irreversible nature of senescence and contributes to its persistence in tissues.

Secretory phenotype. Central to the detrimental impact of senescent cells is the senescence-associated secretory phenotype (SASP). SASP involves secretion of pro-inflammatory cytokines (IL-6, IL-1α, IL-1β), chemokines (CXCL8, CCL2), growth factors (VEGF, IGF-1), proteases (matrix metalloproteinases), and bioactive lipids (Coppé et al., 2008; Rodier & Campisi, 2011; Acosta et al., 2013). SASP factors reinforce senescence via autocrine and paracrine signalling, alter tissue microenvironments, promote chronic inflammation, and disrupt normal cellular and tissue functions. SASP also promotes immunological dysregulation, contributing significantly to aging and age-related diseases (Franceschi et al., 2018; Sikora et al., 2021).

Metabolic adaptations. More recently, mitochondrial dysfunction and metabolic shifts have been identified as crucial determinants in senescence induction and maintenance. Dysfunctional mitochondria result in impaired energy metabolism, increased ROS production, and activation of AMPK and mTOR pathways, which further sustain senescence (Wiley et al., 2016; Correia-Melo et al., 2016). Senescent cells exhibit enhanced glycolysis, altered lipid metabolism, and compromised autophagy, collectively promoting senescent cell survival and persistence in tissues (Narita et al., 2011; Correia-Melo & Passos, 2015).

Given these comprehensive mechanistic insights, selective targeting of senescent cells has emerged as a promising strategy to attenuate senescence-associated dysfunction and delay age-related decline. Pharmacological elimination of senescent cells using senolytic drugs, agents that selectively induce apoptosis in senescent cells, has gained substantial attention. Prominent examples include the combination of Dasatinib and Quercetin, Navitoclax, Fisetin, and others, each targeting specific pro-survival pathways in senescent cells (Zhu et al., 2015; Xu et al., 2018; Kirkland & Tchkonia, 2020). Alternatively, senomorphics, compounds that modulate SASP without killing senescent cells, represent another strategy to manage age-related conditions (Short et al., 2019).

Cellular senescence is a multifaceted cellular state driven by complex interlinked mechanisms, including DDR, oncogene activation, telomere dysfunction, epigenetic alterations, mitochondrial dysfunction, metabolic dysregulation, and the potent inflammatory SASP. Deepening our understanding of these mechanisms continues to highlight cellular senescence as an attractive and a promising mechanistic target in aging research, offering opportunities to improve health span and quality of life during aging.

Role of Senescent Cells in Age-Related Pathologies

Cellular senescence, initially identified as a stable, irreversible cell cycle arrest mechanism, is increasingly recognized as a pivotal factor driving numerous age-related pathologies through both direct cellular effects and paracrine mechanisms mediated by the senescence-associated secretory phenotype (SASP) (Figure 4) (Campisi & d'Adda di Fagagna, 2007; Childs et al., 2015; Sikora et al., 2021). Senescent cells accumulate with advancing age across diverse tissues, significantly influencing their function and contributing to the clinical manifestation of age-associated diseases, including cardiovascular disorders, neurodegeneration, metabolic syndrome, osteoporosis, osteoarthritis, chronic kidney disease, and cancer (Tchkonia et al., 2013; Baker et al., 2016; Kirkland & Tchkonia, 2020).

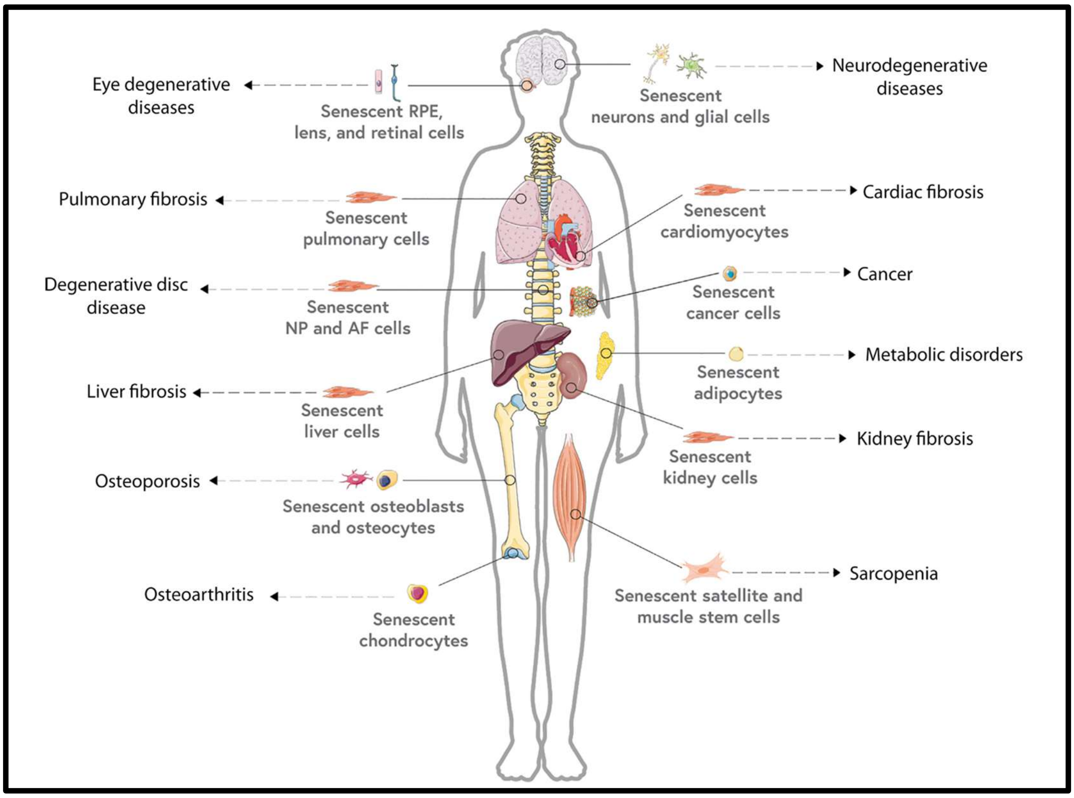

Figure 4. Senescent cells play a role in age-associated diseases.
Senescence-promotion through SASP together with a decline in the immune system activity, converge to induce organismal accumulation of senescent cells. In aged individuals, chronic accumulation of senescent cells contributes to tissue dysfunction and increased risk of age-associated diseases development.

(Source: Borghesan et al., 2020)

Cardiovascular Diseases represent a leading cause of morbidity and mortality in aging populations worldwide, significantly influenced by cellular senescence. Senescent cells accumulate in the cardiovascular system, including endothelial cells, vascular smooth muscle cells (VSMCs), and cardiac fibroblasts, exacerbating pathological conditions such as atherosclerosis, hypertension, and heart failure (Childs et al., 2016; Roos et al., 2016). Senescent endothelial cells compromise vascular function, reducing nitric oxide (NO) bioavailability and increasing reactive oxygen species (ROS) production, thus promoting endothelial dysfunction, arterial stiffness, and vascular inflammation (Ungvari et al., 2018). Moreover, senescent VSMCs enhance SASP factor secretion, facilitating the recruitment of inflammatory cells and perpetuating chronic vascular inflammation, thereby accelerating atherosclerotic plaque formation and instability (Gardner et al., 2015).

Neurodegenerative Diseases, notably Alzheimer's and Parkinson's diseases, are increasingly linked to senescent cell accumulation within brain tissues. Senescent astrocytes, microglia, oligodendrocyte precursor cells (OPCs), and endothelial cells of the blood-brain barrier (BBB) have been identified in aged brains and animal models of neurodegeneration (Bussian et al., 2018; Baker & Petersen, 2018; Zhang et al., 2019). Senescent glial cells exacerbate neuroinflammation through SASP secretion, particularly pro-inflammatory cytokines IL-1β, IL-6, TNF-α, and chemokines, driving neuronal dysfunction, synapse loss, and cognitive decline (Zhang et al., 2019; Sikora et al., 2021). Further, senescence-induced BBB disruption permits entry of neurotoxic substances and inflammatory cells, accelerating neurodegenerative processes and promoting cognitive impairment (Montagne et al., 2015; Sweeney et al., 2018).

Metabolic Syndrome and Diabetes have been significantly correlated with increased senescence burden in adipose tissue, pancreatic β-cells, and hepatic tissue. Senescent adipocytes accumulate in obese and aging individuals, contributing to adipose tissue dysfunction, insulin resistance, and chronic systemic inflammation via secretion of pro-inflammatory SASP factors (Palmer et al., 2019; Tchkonia et al., 2010). Similarly, pancreatic β-cell senescence reduces insulin secretion capacity and promotes β-cell dysfunction through inflammatory SASP mediators, thus exacerbating type 2 diabetes mellitus (Aguayo-Mazzucato et al., 2017; Thompson et al., 2019). In the liver, senescent hepatocytes and hepatic stellate cells impair liver function and regeneration, playing a role in non-alcoholic fatty liver disease (NAFLD) progression and fibrosis (Aravinthan et al., 2013; Ogrodnik et al., 2017).

Musculoskeletal Disorders, such as osteoporosis and osteoarthritis, demonstrate substantial links to cellular senescence. Senescent osteoblasts and osteocytes secrete SASP factors, impairing osteoblast function, enhancing osteoclast activity, and driving bone resorption, consequently contributing to osteoporosis and increasing fracture risk (Farr et al., 2017; Khosla et al., 2020). In joints, senescent chondrocytes contribute significantly to osteoarthritis by disrupting cartilage matrix homeostasis through elevated SASP secretion, including matrix-degrading metalloproteinases (MMPs), aggrecanases, and inflammatory cytokines (Jeon et al., 2017; Loeser et al., 2016).

Chronic Kidney Disease (CKD) progression and severity are markedly influenced by renal cell senescence, which impacts podocytes, tubular epithelial cells, and mesangial cells. Senescent renal cells exacerbate renal inflammation, fibrosis, and impaired regenerative capacity via secretion of SASP factors, substantially accelerating CKD development and advancing kidney failure (Sturmlechner et al., 2017; Valentijn et al., 2018).

Cancer and Aging exhibit a complex, bidirectional relationship with cellular senescence. On the one hand, cellular senescence functions as a potent tumour-suppressive mechanism by halting the proliferation of damaged or potentially malignant cells (Campisi, 2013; Serrano et al., 1997). On the other hand, persistent senescent cells in tissues create a pro-inflammatory, pro-oncogenic microenvironment via secretion of SASP factors, promoting cancer initiation and progression, angiogenesis, metastasis, and resistance to chemotherapy (Coppé et al., 2008; Rodier & Campisi, 2011; Demaria et al., 2017).

Immunosenescence also links senescent cells with impaired immune function in aging. Accumulation of senescent immune cells, including T cells and macrophages, reduces immune surveillance capacity and contributes to chronic inflammation, autoimmunity, and increased vulnerability to infections and malignancies (Fulop et al., 2018; Salminen, 2021).

In addition to individual organ dysfunction, systemic accumulation of senescent cells contributes broadly to frailty, decreased resilience, reduced physical performance, impaired wound healing, and overall decline in physiological reserve associated with aging (Xu et al., 2018; Justice et al., 2019).

Given the detrimental impact of senescent cells in various age-related diseases, therapies specifically targeting these cells (senolytics and senomorphics) have emerged as promising strategies. Preclinical studies demonstrate that the elimination or modulation of senescent cells significantly ameliorates pathology and extends health span and lifespan in animal models of aging, cardiovascular diseases, neurodegeneration, metabolic syndrome, osteoporosis, and frailty (Zhu et al., 2015; Baker et al., 2016; Xu et al., 2018; Wissler Gerdes et al., 2020). Clinical trials are underway to translate these findings to human therapies, underscoring the substantial potential of targeting cellular senescence as a novel, broadly applicable intervention strategy in geroscience and chronic diseases (Kirkland & Tchkonia, 2020).

Extensive research clearly identifies cellular senescence and its associated SASP as central mechanisms contributing substantially to multiple aging-related diseases. Deepened understanding of these intricate relationships highlights senescent cells as prime targets for intervention aimed at attenuating senescence-associated phenotypes, improving health span, and reducing the burden of age-associated conditions.

1.3 Senolytic Interventions

- Overview and Rationale for Senolytic Drugs

Cellular senescence, characterized by permanent growth arrest and a pro-inflammatory secretory profile termed the senescence-associated secretory phenotype (SASP), is increasingly recognized as a critical driver of aging and various age-related pathologies (Campisi et al., 2019; López-Otín et al., 2023). While senescence initially functions as an essential tumour-suppressive mechanism to remove damaged or potentially malignant cells, chronic accumulation of these cells in aged tissues paradoxically promotes disease pathology and accelerates organismal aging (Childs et al., 2015; Kirkland & Tchkonia, 2020). Therapeutically targeting and eliminating senescent cells represents a promising strategy to extend health span, delay aging phenotypes, and alleviate age-associated chronic diseases (Baker et al., 2016; Justice et al., 2019; Wissler Gerdes et al., 2021).

Senolytic drugs are a class of compounds designed explicitly to induce apoptosis selectively in senescent cells, thereby alleviating the adverse consequences associated with senescent cell accumulation (Zhu et al., 2015; Kirkland & Tchkonia, 2020). The term "senolytic" originates from "senescence" and "lytic," indicating the specific elimination of senescent cells. The fundamental rationale underpinning senolytic intervention lies in the removal of detrimental senescent cells, consequently reducing inflammation, restoring tissue homeostasis, improving organ function, and attenuating various disease states associated with aging (Xu et al., 2018; Short et al., 2019).

The identification of senolytics emerged from pioneering research elucidating the molecular vulnerabilities of senescent cells (Figure 5). Unlike normal proliferating or quiescent cells, senescent cells exhibit unique resistance to apoptosis, sustained by anti-apoptotic signalling networks (Yosef et al., 2016; Kirkland & Tchkonia, 2020). Senescent cells upregulate anti-apoptotic proteins of the BCL-2 family (e.g., BCL-xL, BCL-W), the p53–p21–serpine signalling axis, PI3K/AKT/mTOR pathways, heat shock proteins, and other pro-survival mediators, granting them resilience to stress-induced apoptosis despite severe genomic or metabolic insults (Chang et al., 2016; Zhu et al., 2016; Baar et al., 2017; Robbins et al., 2021). Leveraging these vulnerabilities, researchers conducted hypothesis-driven screens employing RNA interference and pharmacological strategies, successfully identifying the first generation of senolytic agents (Zhu et al., 2015; Kirkland et al., 2017).

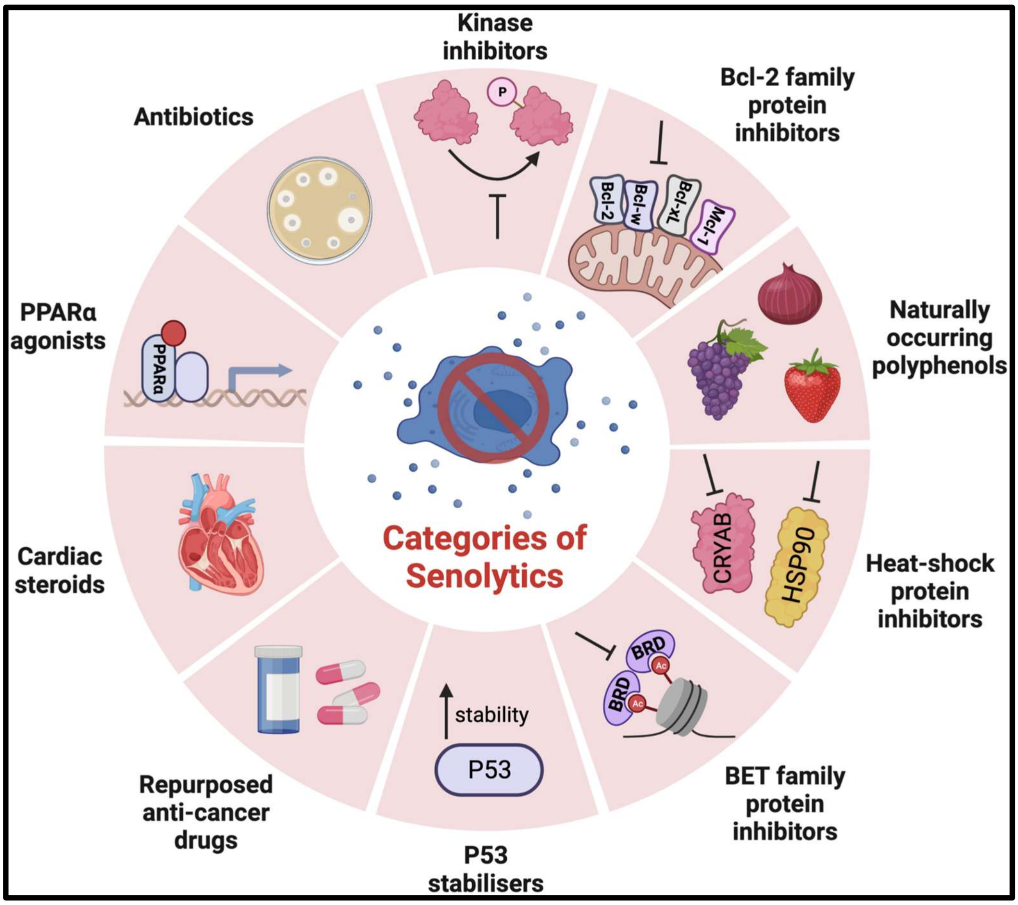

Figure 5. Current categories of senolytics.

Current senolytics include Bcl-2 family inhibitors; natural polyphenols; heat-shock-protein inhibitors; BET inhibitors; p53 stabilisers; repurposed anti-cancer drugs; cardiac steroids; PPAR-α agonists; and selected antibiotics. Each class eliminates senescent cells through distinct molecular targets

(Source: Power H. et al., 2023)

Dasatinib and Quercetin. One of the earliest and most widely studied senolytic interventions is the combination of Dasatinib and Quercetin (D+Q). Dasatinib, a tyrosine kinase inhibitor originally approved for treating chronic myeloid leukaemia, selectively induces apoptosis in senescent preadipocytes and human endothelial cells, while Quercetin, a naturally occurring flavonoid, targets senescent endothelial cells and bone marrow-derived mesenchymal stem cells (Zhu et al., 2015; Xu et al., 2018). D+Q acts synergistically by selectively disrupting survival networks specifically upregulated in senescent cells, notably targeting the ephrin-dependent survival pathways and the anti-apoptotic protein BCL-xL (Zhu et al., 2015; Hickson et al., 2019). Animal studies have demonstrated the potent efficacy of D+Q in alleviating age-related dysfunction across multiple tissues and organ systems, including improvements in cardiovascular function, cognitive capacity, pulmonary function, adipose tissue metabolism, and lifespan extension in animal models (Xu et al., 2018; Roos et al., 2016; Krzystyniak et al., 2022).

Navitoclax. Another prominent senolytic drug, Navitoclax (ABT-263), originally developed as an anticancer drug targeting BCL-2 family proteins, induces apoptosis in senescent cells by specifically inhibiting BCL-2, BCL-xL, and BCL-W (Chang et al., 2016; Zhu et al., 2016). Navitoclax has demonstrated robust efficacy in reducing senescent cell burden and improving tissue function in animal models of aging and age-related diseases, including cardiovascular dysfunction, pulmonary fibrosis, and radiation-induced senescence (Pan et al., 2017; Chang et al., 2016; Justice et al., 2019).

Other naturally derived senolytics. Fisetin, Piperlongumine, Curcumin, and Epigallocatechin gallate (EGCG) have shown promising selective activity against senescent cells with generally favourable toxicity profiles (Yousefzadeh et al., 2018; Xu et al., 2018; Lewinska et al., 2020). These compounds primarily act by modulating pathways implicated in senescent cell survival, such as the NF-κB and mTOR signalling pathways, as well as inducing oxidative stress specifically in senescent cells (Yousefzadeh et al., 2018; Lewinska et al., 2020). Preclinical evidence supports their potential utility in various age-related conditions, such as osteoporosis, neurodegenerative diseases, and metabolic disorders (Kirkland & Tchkonia, 2020).

Senolytic approaches extend beyond merely clearing senescent cells. By eliminating sources of SASP-driven chronic inflammation and tissue damage, senolytics potentially restore tissue homeostasis and regenerative capacities impaired during aging (Short et al., 2019; Wissler Gerdes et al., 2021). Importantly, intermittent senolytic administration has been sufficient to achieve sustained health benefits, possibly due to prolonged effects of senescent cell clearance and subsequent reduction in inflammation and tissue damage (Zhu et al., 2015; Baker et al., 2016; Xu et al., 2018). Additionally, senolytic therapies may enhance responsiveness to other pharmacological interventions and rejuvenation strategies, such as regenerative stem cell therapies, immunomodulation, and anti-inflammatory treatments (Childs et al., 2017; Kirkland & Tchkonia, 2020).

Clinical translation of senolytic therapies is actively ongoing. Initial human clinical trials have begun to assess safety, feasibility, and preliminary efficacy of senolytic interventions in various age-related conditions, including idiopathic pulmonary fibrosis, chronic kidney disease, osteoarthritis, and frailty syndromes (Justice et al., 2019; Hickson et al., 2019; Kirkland & Tchkonia, 2020). Early results indicate promising potential benefits with manageable safety profiles, underscoring the therapeutic promise of senolytics (Hickson et al., 2019; Justice et al., 2019). However, critical issues remain to be addressed, including optimization of drug delivery methods, treatment frequency and duration, target cell specificity, off-target effects, and individual patient factors influencing response to such interventions (Kirkland & Tchkonia, 2020; Robbins et al., 2021).

The rationale for senolytic interventions is rooted in the targeted elimination of senescent cells, key drivers of age-associated pathologies. **Emerging evidence from preclinical and early clinical trials supports senolytics' potential utility in attenuating senescence-associated phenotypes, enhancing health span, and alleviating various chronic diseases associated with aging**. Continued rigorous investigation and optimization of senolytic interventions promise to provide powerful new geroscience-based strategies to address the health burden of population aging.

- Dasatinib and Quercetin as Potent Senolytics

Among the growing class of senolytic compounds, the combination of D+Q has emerged as one of the most well-characterized and effective senolytic regimens, with proven efficacy across multiple experimental models of aging and chronic disease (Zhu et al., 2015; Xu et al., 2018; Justice et al., 2019). This synergistic pair was identified through hypothesis-driven screening aimed at targeting senescent cell-specific anti-apoptotic pathways, which confer resistance to cell death and enable their persistence in aged tissues (Zhu et al., 2015; Kirkland & Tchkonia, 2020).

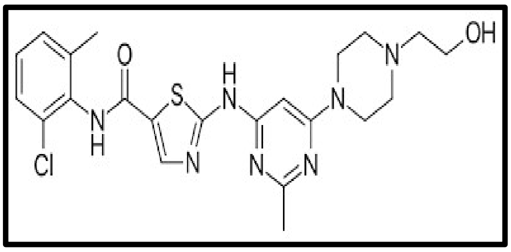

Figure 6. Structure of Dasatinib.
Dasatinib is a targeted therapy medication used to treat certain cases of chronic myelogenous leukaemia (CML) and acute lymphoblastic leukaemia (ALL). Specifically it is used to treat cases that are Philadelphia chromosome-positive (Ph+).
(Source: Wikipedia)

Dasatinib (Figure 6) is a broad-spectrum tyrosine kinase inhibitor originally approved by the FDA for the treatment of chronic myeloid leukaemia (CML) and acute lymphoblastic leukaemia (ALL) (O'Hare et al., 2005). It inhibits multiple kinases, including Src family kinases, BCR-ABL, and ephrin receptors, some of which are overexpressed in senescent cells (Zhu et al., 2015; Yosef et al., 2016). Dasatinib exhibited senolytic activity particularly against senescent human preadipocytes and human umbilical vein endothelial cells (HUVECs), and has been shown to selectively induce apoptosis by downregulating key components of the senescent cell anti-apoptotic pathways (SCAPs) (Zhu et al., 2015; Chang et al., 2016).

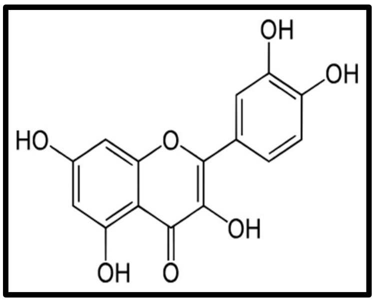

Figure 7. Structure of Quercetin.
Quercetin is a plant flavonol from the flavonoid group of polyphenols. It is found in many fruits, vegetables, leaves, seeds, and grains; capers, red onions, and kale. It has a bitter flavour and is used as an ingredient in dietary supplements, beverages, and foods.
(Source: Wikipedia)

Quercetin (Figure 7) is a naturally occurring plant flavonoid found in various fruits and vegetables, including onions, apples, and capers. It exhibits antioxidant, anti-inflammatory, and anti-carcinogenic properties and has long been used in nutraceutical formulations (Boots et al., 2008; D’Andrea, 2015). In the context of senescence, Quercetin demonstrates senolytic effects in senescent HUVECs and bone marrow-derived mesenchymal stem cells (MSCs), largely through inhibition of PI3K/AKT, BCL-2 family proteins, and other survival pathways involved in SASP regulation and cell viability (Zhu et al., 2015).

The combination of D+Q acts synergistically, broadening the spectrum of senescent cell types targeted by the intervention. While Dasatinib preferentially targets senescent progenitor-like cells, Quercetin is more effective against post-mitotic senescent cells, including endothelial and mesenchymal lineages. Together, D+Q has demonstrated robust in vivo efficacy in selectively clearing senescent cells across multiple tissues without significant toxicity to non-senescent cells (Zhu et al., 2015; Xu et al., 2018; Hickson et al., 2019). This combination has been shown to eliminate senescent cells in adipose tissue, lung, bone, brain, kidney, and liver, resulting in marked improvements in tissue function and organismal health span (Xu et al., 2018; Justice et al., 2019; Roos et al., 2016; Krzystyniak et al., 2022).

Preclinical studies using D+Q have reported numerous benefits:

- In a mouse model of accelerated aging, intermittent **D+Q treatment reduced senescent cell burden, attenuated age-associated physical dysfunction, and extended health span and lifespan** (Baker et al., 2016; Xu et al., 2018).
- In a bleomycin-induced model of pulmonary fibrosis, **D+Q administration decreased lung senescence, fibrosis, and inflammation, demonstrating its therapeutic utility in fibrotic conditions** (Schafer et al., 2017).
- In models of Alzheimer’s disease and neurodegeneration, **D+Q treatment reduced senescent glial cells, lowered neuroinflammatory markers, and improved cognitive function** (Zhang et al., 2019; Bussian et al., 2018).

Importantly, the intermittent administration of D+Q, such as weekly or bi-weekly dosing, has been found sufficient to confer lasting beneficial effects, presumably due to the persistent clearance of senescent cells and the reduced need for continuous exposure, which minimizes potential off-target effects (Zhu et al., 2015; Xu et al., 2018). This intermittent dosing strategy also helps avoid cytotoxicity observed in some continuous chemotherapeutic regimens.

The safety and feasibility of D+Q have also been evaluated in early-phase clinical trials. In a pilot study involving patients with idiopathic pulmonary fibrosis, a short, intermittent course of D+Q significantly improved physical function without severe adverse effects (Justice et al., 2019). Other clinical trials have examined the potential of D+Q in chronic kidney disease, diabetes-related complications, and osteoarthritis, with ongoing studies aiming to confirm efficacy and refine dosage regimens (Hickson et al., 2019; Kirkland & Tchkonia, 2020).

The molecular targets of D+Q remain a topic of active investigation. In addition to BCL-2 family proteins, Quercetin modulates NF-κB, HIF-1α, and MAPK pathways, whereas Dasatinib affects ephrin signalling, c-KIT, and SRC family kinases, many of which contribute to senescent cell survival (Zhu et al., 2015; Yosef et al., 2016). Proteomic and transcriptomic studies have revealed that D+Q treatment alters the SASP profile, reducing expression of key pro-inflammatory cytokines (IL-6, IL-8), growth factors (VEGF, IGF-1), and proteases (MMPs), suggesting not only senescent cell removal but also modulation of the local tissue environment (Kirkland et al., 2017).

Challenges and limitations of D+Q intervention include variability in responsiveness across different cell types and tissues, potential off-target effects, pharmacokinetic constraints, and interactions with the host immune system. Additionally, the long-term consequences of removing senescent cells remain uncertain, as some may play context-dependent beneficial roles in tissue repair, wound healing, and embryonic development (Demaria et al., 2014; Rhinn et al., 2019).

The Dasatinib and Quercetin combination represents a landmark in senolytic research, offering a broadly applicable, low-toxicity intervention to target diverse senescent cell populations. Supported by substantial preclinical evidence and early clinical trials, D+Q continues to serve as a benchmark for developing next generation senotherapeutics, highlighting the clinical potential of targeting cellular senescence to improve health span and combat age-related diseases.

1.4 The Gut-Brain Axis

● Overview of Gut-Brain Communication

The gut-brain axis (GBA) is a complex, bidirectional communication network that integrates neural, hormonal, immunological, and metabolic signalling pathways between the gastrointestinal tract and the central nervous system (CNS) (Figure 8; Mayer et al., 2015; Carabotti et al., 2015). This integrative system plays a central role in maintaining homeostasis and regulating physiological processes including mood, behaviour, cognition, stress response, appetite, and immunity. Disruption of gut-brain axis communication has been increasingly implicated in the pathogenesis of numerous disorders, ranging from gastrointestinal diseases to neuropsychiatric and neurodegenerative conditions (Cryan et al., 2019; Morais et al., 2021).

The primary anatomical and functional components of the GBA include:

- Central nervous system (CNS)
- Enteric nervous system (ENS), often referred to as the "second brain," comprising ~500 million neurons embedded in the gut wall (Furness, 2006)
- Autonomic nervous system (ANS), including the sympathetic and parasympathetic branches (notably the vagus nerve)
- Neuroendocrine signalling, primarily via the hypothalamic-pituitary-adrenal (HPA) axis
- Immune system, including gut-associated lymphoid tissue (GALT)
- Gut microbiota and their metabolites

Together, these components form a distributed sensor–effector network that relays information bidirectionally between the gut and the brain.

Neural signalling via the vagus nerve constitutes a major conduit for real-time communication between the gut and brain. The vagus transmits afferent sensory input from the intestinal lumen to the brainstem and modulates efferent motor responses regulating gastrointestinal motility, secretion, and permeability (Bonaz et al., 2018). Direct signalling via enteric neurons, sympathetic fibres, and sensory ganglia complements this pathway, integrating signals about luminal content, immune status, and visceral pain (Furness, 2012).

The HPA axis represents the neuroendocrine branch of the GBA, acting as the principal stress response system. Activation of the HPA axis during stress induces cortisol release, which affects gut permeability, microbiota composition, mucosal immunity, and intestinal motility (Sudo et al., 2004; O'Mahony et al., 2009). Conversely, alterations in gut microbiota can influence HPA axis activity, as demonstrated in germ-free animals exhibiting exaggerated corticosterone responses to stress that normalize upon colonization (Sudo et al., 2004).

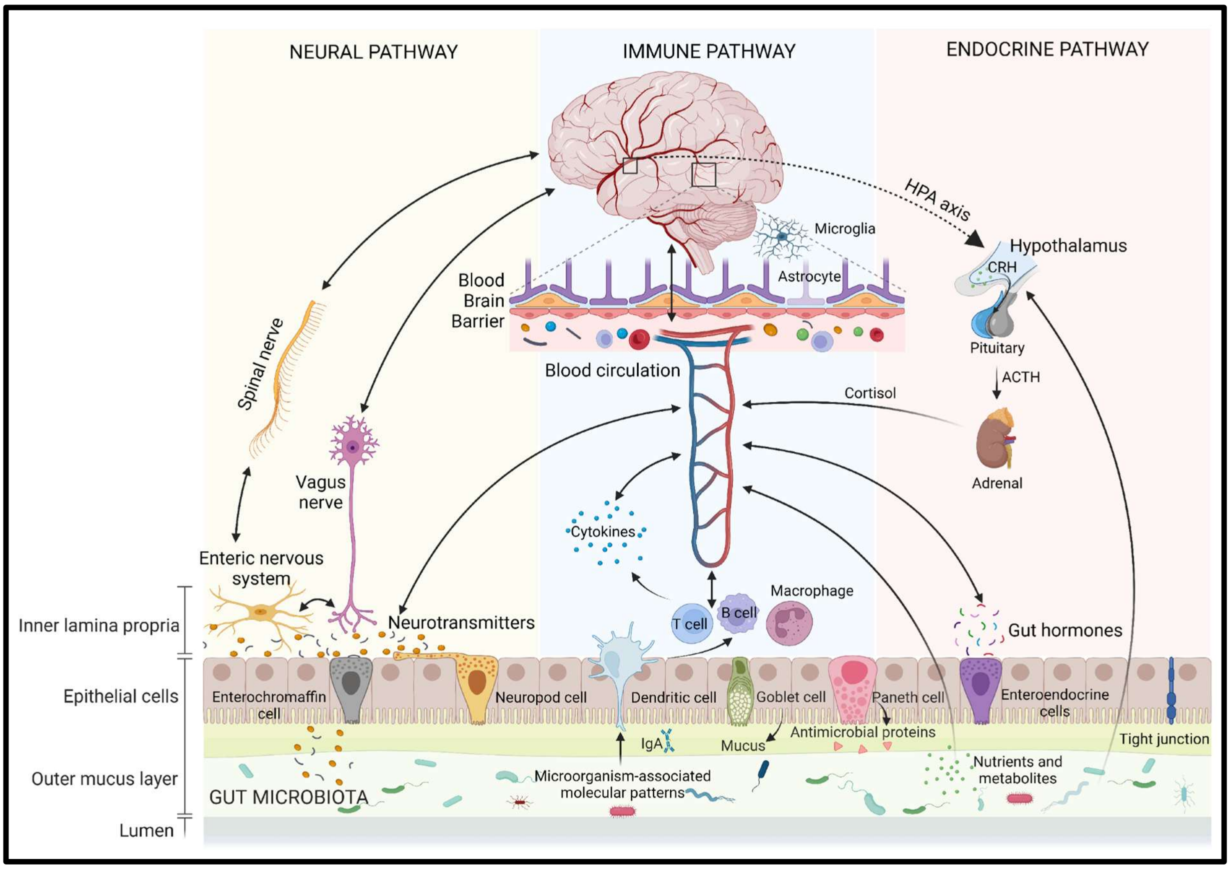

Figure 8. Pathways involved in bidirectional communication within the gut-brain axis. They include neural, immune, and endocrine pathways. Neurotransmitters: dopamine, serotonin, norepinephrine, gamma-aminobutyric acid (GABA), etc. Cytokines: interleukin (IL)-1β, IL-6, IL-10, tumour necrosis factor-α (TNF-α), etc. Nutrients and metabolites: short-chain fatty acids (SCFAs), amine compounds, vitamins, neuroprecursors, etc.

(Source: Zheng Y. et al., 2023)

One of the most significant advances in GBA research has been the recognition of the gut microbiota as a dynamic and essential modulator of gut-brain communication (Cryan & Dinan, 2012; Sharon et al., 2016). The trillions of microorganisms residing in the intestinal tract influence the CNS through multiple mechanisms:

- **Microbial metabolites** such as short-chain fatty acids (e.g., acetate, propionate, butyrate), tryptophan derivatives (e.g., indole, serotonin), and secondary bile acids cross the gut epithelium and modulate immune responses, neurotransmission, and neuroinflammation (Dalile et al., 2019; Agus et al., 2018; Schroeder & Bäckhed, 2016).
- **Production of neurotransmitters**: Gut bacteria can synthesize or modulate bioactive molecules such as gamma-aminobutyric acid (GABA), serotonin (5-HT), dopamine, and acetylcholine, influencing host neurophysiology and behaviour (Strandwitz, 2018).
- **Immune modulation**: The gut microbiota interacts with the immune system, shaping inflammatory tone and impacting neuroimmune homeostasis (Belkaid & Hand, 2014; Erny et al., 2015).
- **Impact on the blood-brain barrier (BBB)**: Microbiota composition influences the integrity of the BBB, as shown in germ-free mice with increased BBB permeability that is reversed upon microbial colonization (Braniste et al., 2014).

Collectively, these mechanisms demonstrate how gut microbes can exert profound effects on brain development, function, and behaviour. Dysbiosis, defined as alterations in microbial composition or function, has been associated with multiple disorders, including irritable bowel syndrome (IBS), major depressive disorder (MDD), anxiety, autism spectrum disorder (ASD), Parkinson’s disease, and Alzheimer’s disease (Morais et al., 2021; Sharon et al., 2016; Vogt et al., 2017).

The GBA is particularly sensitive during critical developmental windows. Early life events such as mode of delivery, breastfeeding, antibiotic exposure, and psychosocial stress can influence microbiota composition and neurodevelopment (Borre et al., 2014; O'Mahony et al., 2009). Perturbations in early-life microbiota are associated with long-term consequences on stress reactivity and cognitive function, suggesting a “microbiota–gut–brain axis programming” effect (Clarke et al., 2013; Luczynski et al., 2016).

In adulthood and aging, the composition and diversity of the gut microbiota undergo significant shifts. Aging is associated with reduced microbial diversity, increased pro-inflammatory taxa, and decreased levels of beneficial SCFA-producing bacteria, changes that have been linked to increased intestinal permeability, systemic inflammation (“inflammaging”), and cognitive decline (O'Toole & Jeffery, 2015; Ghosh et al., 2022; Thevaranjan et al., 2017).

Given its centrality in maintaining neurophysiological homeostasis, the GBA represents a compelling therapeutic target. Strategies under investigation include:

- **Probiotics and prebiotics**: Shown to improve stress resilience, mood, and cognition in animal and human studies (Messaoudi et al., 2011; Allen et al., 2016).
- **Faecal microbiota transplantation (FMT)**: Emerging data suggest FMT can modulate brain function and behaviour, although evidence in humans remains preliminary (Kang et al., 2017; Hazan, 2020).
- **Dietary interventions**: High-fibre, polyphenol-rich, and Mediterranean-style diets support microbiota diversity and cognitive health (Nagpal et al., 2019; Ghosh et al., 2022).
- **Senotherapeutics**: Recent research suggests that interventions targeting senescent cells may also beneficially impact the gut-brain axis, particularly by restoring intestinal barrier integrity and reducing neuroinflammation (Petrazzo et al., 2026).

- ## Role in Cognitive Function and Aging

The gut-brain axis (GBA) plays an increasingly recognized role in regulating higher-order brain functions such as cognition, memory, learning, mood, and emotional processing. Through a complex network of bidirectional signalling between the gastrointestinal tract and the central nervous system (CNS), the GBA influences neurodevelopment, neuroplasticity, and neurodegeneration (Cryan et al., 2019; Morais et al., 2021). Perturbations in this axis have been implicated in cognitive impairments across the lifespan, including those associated with normal aging, mild cognitive impairment (MCI), and neurodegenerative diseases such as Alzheimer's and Parkinson’s diseases (Sharon et al., 2016; Vogt et al., 2017).

Emerging evidence has linked the composition and diversity of the gut microbiota with cognitive outcomes. A healthy and diverse microbiota supports cognitive function through the regulation of metabolic, inflammatory, and neurotrophic pathways (Mohajeri et al., 2018). In contrast, dysbiosis, characterized by a loss of microbial diversity, overgrowth of pathobionts, and depletion of beneficial taxa such as *Faecalibacterium prausnitzii*, *Bifidobacterium*, and *Lactobacillus* spp., is associated with impaired memory, reduced synaptic plasticity, and increased neuroinflammation (Goyal et al., 2021).

Multiple studies in animal models have demonstrated that microbiota manipulations can directly influence learning and memory. Germ-free (GF) mice, which lack commensal microbiota, exhibit abnormal development of the hippocampus, altered expression of synaptic plasticity-related genes, and deficits in recognition and spatial memory (Diaz Heijtz et al., 2011; Luczynski et al., 2016). These deficits can be reversed by colonization with specific bacterial strains or faecal microbiota transplantation (FMT), indicating a causative role for the microbiota in cognitive function (Ogbonnaya et al., 2015).

In humans, several studies have shown associations between gut microbial profiles and cognitive performance, especially in aging populations. For example, reduced microbial richness and lower abundance of SCFA-producing bacteria have been observed in individuals with Alzheimer’s disease and MCI compared to cognitively healthy controls (Vogt et al., 2017; Liu et al., 2019). Moreover, probiotic supplementation with *Lactobacillus* and *Bifidobacterium* strains has been shown to improve cognitive function in patients with MCI and early Alzheimer’s disease (Akbari et al., 2016; Kobayashi et al., 2019).

The microbiota modulates brain function through several interrelated mechanisms:

- **Short-chain fatty acids (SCFAs)**: These microbial metabolites, primarily acetate, propionate, and butyrate, are produced by bacterial fermentation of dietary fibres. SCFAs influence brain function by regulating the blood-brain barrier (BBB), modulating microglial activity, promoting neurogenesis, and enhancing histone acetylation involved in memory formation (Silva et al., 2020; Dalile et al., 2019).
- **Tryptophan metabolism**: Gut bacteria influence the kynurenine pathway of tryptophan metabolism, affecting levels of neuroactive metabolites like kynurenic acid and quinolinic acid, which play roles in excitotoxicity and cognitive function (Agus et al., 2018). They also affect serotonin (5-HT) biosynthesis, with ~90% of peripheral serotonin synthesized in the gut (Yano et al., 2015).
- **Neurotrophic factors**: The microbiota modulates the expression of brain-derived neurotrophic factor (BDNF), a key regulator of synaptic plasticity, memory, and learning. GF animals and those subjected to antibiotic-induced dysbiosis show significantly reduced BDNF levels in the hippocampus and cortex (Bercik et al., 2011; Hoban et al., 2016).
- **Neuroinflammation**: Dysbiosis contributes to systemic inflammation and increased intestinal permeability (leaky gut), leading to elevated levels of circulating pro-inflammatory cytokines (e.g., IL-6, TNF-α, IL-1β) that can cross the BBB and induce neuroinflammation, an established contributor to cognitive decline and neurodegeneration (Franceschi & Campisi, 2014; Colucci-D'Amato et al., 2020).
- **Endotoxemia and BBB integrity**: Microbial lipopolysaccharide (LPS) can enter circulation via a compromised gut barrier and activate toll-like receptors (TLRs) on microglia, triggering neuroinflammation and synaptic loss (Braniste et al., 2014; Cani et al., 2008; Zhao et al., 2019).

Aging is accompanied by profound alterations in both the gut microbiota and the integrity of physiological barriers, contributing to cognitive decline through systemic and neural mechanisms. Age-associated dysbiosis includes a reduction in microbial diversity, SCFA producers, and an increase in pro-inflammatory microbes such as *Enterobacteriaceae* (O'Toole & Jeffery, 2015; Thevaranjan et al., 2017). These changes parallel reductions in tight junction proteins in the gut epithelium and blood-brain barrier, leading to increased permeability and chronic low-grade inflammation, also known as "inflammaging" (Franceschi et al., 2018; Tran & Greenwood-Van Meerveld, 2013).

Recent studies suggest that modulating the aging gut microbiome may delay or reverse cognitive aging. FMT from young to aged mice restores hippocampal function, increases neurogenesis, and improves spatial memory (Colucci-D'Amato et al., 2020; Parker et al., 2022). In humans, dietary interventions such as high-fibre, polyphenol-rich diets and Mediterranean-style patterns have been shown to promote a neuroprotective microbiota profile and are associated with slower cognitive decline (Ghosh et al., 2020; Nagpal et al., 2019).

Given the multifactorial influence of the gut microbiota on brain health, it is not surprising that microbiota-targeted interventions are emerging as promising strategies to counteract cognitive decline. These include:

- **Probiotics and symbiotics**: Several randomized controlled trials (RCTs) have reported cognitive benefits of probiotic supplementation in elderly adults and patients with MCI (Akbari et al., 2016; Kobayashi et al., 2019).
- **Prebiotics and dietary fibres**: Enhancing SCFA production and promoting microbial diversity may counteract neuroinflammation and support cognitive health (Silva et al., 2020).
- **Senescence-targeting interventions**, such as Dasatinib and Quercetin, may act in part through restoring gut barrier integrity and reducing microbiota-driven neuroinflammation, as suggested in recent preclinical studies (Petrazzo et al., 2026).

Microbiome-Derived Metabolites

One of the most powerful ways in which the gut microbiota communicates with the host, including the brain, is through the production of a vast array of bioactive metabolites (Figure 9). These small molecules, primarily short-chain fatty acids (SCFAs), bile acids, tryptophan catabolites, and neurotransmitter-like compounds, act locally in the gut and systemically, modulating metabolism, immunity, epithelial and blood-brain barrier (BBB) integrity, and neuronal signalling (Nicholson et al., 2012; Agus et al., 2018; Dalile et al., 2019). In the context of aging and cognitive decline, altered levels or composition of these microbial metabolites have been implicated as key drivers of neuroinflammation, barrier dysfunction, and behavioural alterations (Thevaranjan et al., 2017; Ghosh et al., 2022).

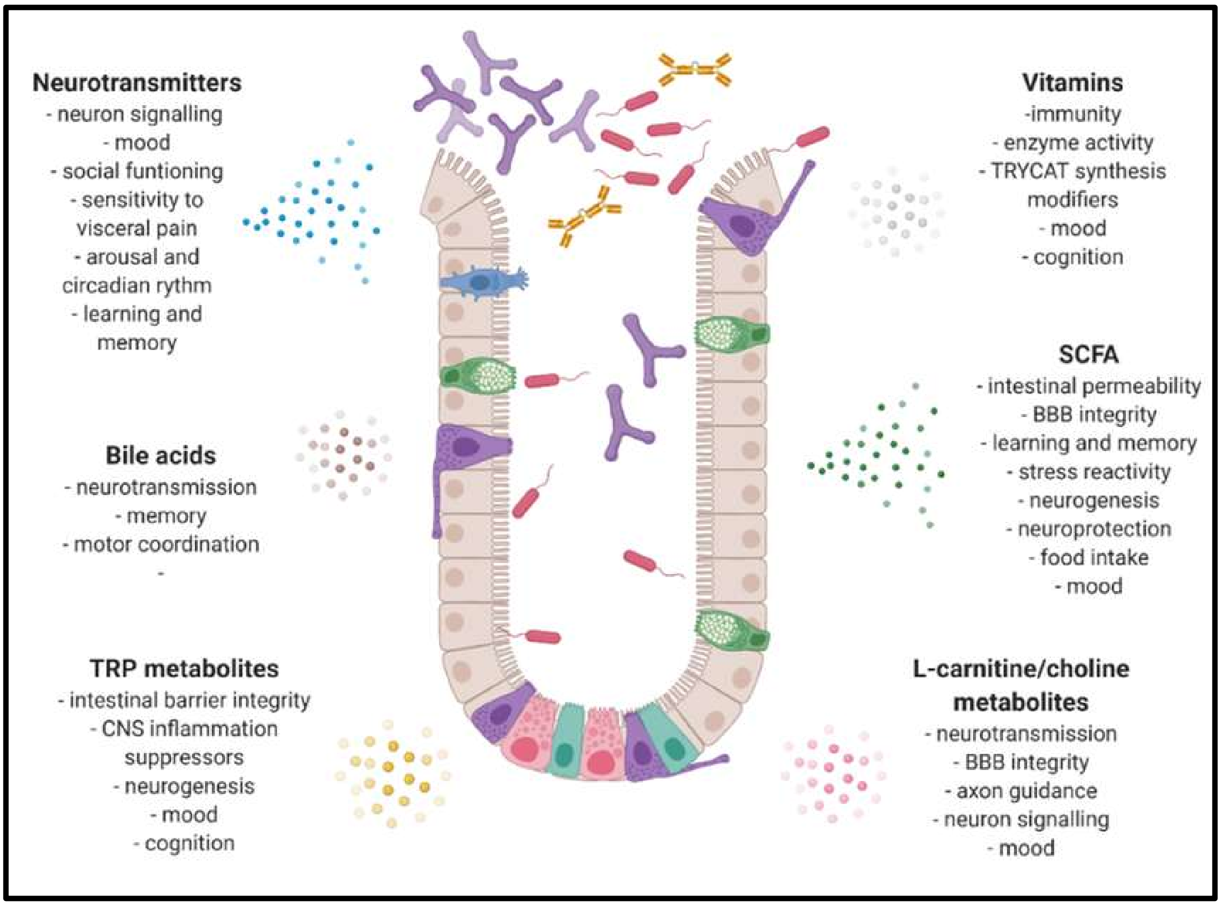

Figure 9. Gut microbiota metabolites and their neuroactive potential
Gut derived metabolites such as neurotransmitters, bile acids, tryptophan (TRP) catabolites, vitamins, short-chain fatty acids (SCFAs), and L-carnitine/choline derivatives are each linked to brain-related functions such as neurotransmission, barrier integrity, mood, memory, and motor coordination.

(Source: Skonieczna-Żydecka K. et al., 2020)

1. Short-Chain Fatty Acids (SCFAs). SCFAs, primarily acetate, propionate, and butyrate, are produced via bacterial fermentation of dietary fibres and resistant starches, primarily by members of the Firmicutes and Bacteroidetes phyla, including *Faecalibacterium prausnitzii*, *Roseburia*, and *Eubacterium* species (Ríos-Covián et al., 2016). These metabolites influence host physiology through multiple mechanisms:

- **Neuroprotection and neuroplasticity**: Butyrate acts as a histone deacetylase (HDAC) inhibitor, promoting the expression of genes involved in neurogenesis, synaptic plasticity, and memory (Stilling et al., 2016; Silva et al., 2020).
- **Modulation of microglial activity**: SCFAs influence microglial maturation and immune surveillance in the CNS. Germ-free mice exhibit defective microglial development that can be restored by SCFA supplementation (Erny et al., 2015).
- **Anti-inflammatory effects**: SCFAs downregulate NF-κB signalling and pro-inflammatory cytokines while promoting regulatory T cell (Treg) differentiation via G-protein-coupled receptors (GPR41/43/109A) (Maslowski et al., 2009; Singh et al., 2014).
- **Gut and BBB barrier integrity**: Butyrate enhances the expression of tight junction proteins (e.g., claudin-1, occludin, zonulin-1) in both intestinal epithelial cells and brain microvascular endothelial cells, contributing to improved barrier function (Braniste et al., 2014; Peng et al., 2009).

In aging, SCFA production is generally reduced due to dietary shifts, decreased microbial diversity, and loss of fiber-degrading bacteria. This reduction is associated with increased gut permeability, systemic inflammation, and neurocognitive decline (O’Toole & Jeffery, 2015; Thevaranjan et al., 2017; Ghosh et al., 2022).

2. Bile Acids. Bile acids (BAs), synthesized from cholesterol in the liver and modified by gut microbes into secondary bile acids (e.g., deoxycholic acid [DCA], lithocholic acid [LCA]), act as metabolic and signalling molecules beyond their traditional role in lipid digestion (Ridlon et al., 2014; Wahlström et al., 2016). Gut bacteria such as *Clostridium*, *Bacteroides*, and *Eubacterium* catalyse deconjugation, dehydroxylation, and epimerization of BAs, thereby shaping the BA pool composition. Bile acids influence cognition and brain health through:

- **Farnesoid X receptor (FXR)** and **Takeda G-protein receptor 5 (TGR5)** signalling, which modulate inflammation, glucose metabolism, and neuronal excitability (Keitel & Häussinger, 2012).
- **Neurotoxicity**: Accumulation of certain hydrophobic bile acids (e.g., LCA) can induce mitochondrial dysfunction, oxidative stress, and neuronal injury (McMillin & DeMorrow, 2016).
- **BBB permeability**: Some bile acids influence the permeability of the BBB, either directly or through inflammatory signalling cascades (McMillin & DeMorrow, 2016).

Recent metabolomic studies in Alzheimer’s disease and mild cognitive impairment show altered serum and faecal bile acid profiles, with elevated secondary-to-primary bile acid ratios and a correlation with cognitive decline (MahmoudianDehkordi et al., 2019; Nho et al., 2019). These findings suggest microbial dysbiosis-driven changes in bile acid metabolism may play a role in neurodegeneration.

3. Tryptophan Metabolites. Tryptophan is an essential amino acid metabolized via three major pathways: serotonin synthesis, the kynurenine pathway, and microbial catabolism to indole derivatives. The latter is heavily influenced by gut microbial composition and activity.

- **Indole derivatives** (e.g., indole-3-acetic acid, indole-3-propionic acid) activate the aryl hydrocarbon receptor (AhR), modulating intestinal immunity, mucosal homeostasis, and even astrocyte function in the brain (Zelante et al., 2013; Rothhammer et al., 2016).
- **Kynurenine pathway metabolites** (e.g., kynurenic acid, quinolinic acid) can be neuroprotective or neurotoxic depending on the balance. Chronic inflammation skews this pathway toward neurotoxic branches, contributing to neurodegeneration and depressive symptoms (Agus et al., 2018; Schwarcz & Stone, 2017).
- **Serotonin**: Though serotonin cannot cross the BBB, ~90% of its production occurs in the gut. Microbial metabolites influence enterochromaffin cell function and circulating 5-HT levels, affecting mood, motility, and platelet function (Yano et al., 2015; Reigstad et al., 2015).

Aging is associated with altered tryptophan metabolism, including elevated kynurenine/tryptophan ratios, decreased indole production, and increased systemic inflammation, all factors linked to cognitive decline.

4. Other Notable Microbial Metabolites.

- **Phenolic compounds**: Microbiota metabolize dietary polyphenols into bioactive molecules like urolithins and phenylpropionic acids that exert neuroprotective and anti-inflammatory effects (Selma et al., 2009; González-Sarrías et al., 2018).
- **Secondary metabolites**: Gut bacteria produce small peptides and secondary metabolites with antimicrobial and immunomodulatory properties, some of which can cross the BBB and influence neuronal signalling (Li et al., 2023).
- **Neurotransmitter analogs**: Certain microbes can synthesize or modulate levels of neurotransmitters such as GABA, dopamine, and acetylcholine, influencing mood and behavior (Strandwitz, 2018; O'Donnell et al., 2020).

These microbiota-derived metabolites form a biochemical bridge between the gut and brain. Their ability to modulate systemic immunity, neural plasticity, barrier integrity, and metabolic regulation positions them as central effectors of gut-brain communication (Sharon et al., 2016; Cryan et al., 2019). Disruption of these metabolic pathways through dysbiosis, poor diet, antibiotic exposure, or age-related changes can lead to a cascade of detrimental effects on cognition, stress reactivity, and overall brain health.

Therefore, intervention strategies aimed at restoring microbial metabolite balance, through diet, pre/probiotics, senolytics, or targeted microbial interventions, hold considerable promise for supporting healthy aging and cognitive function.

1.5 Rationale and Objectives of the Thesis

Age-related cognitive decline arises from interacting processes that extend beyond the brain itself. Among the most relevant are cellular senescence, chronic inflammation, age-associated alterations in the gut microbiota, and dysfunction of the intestinal and blood-brain barriers. Senescent cells accumulate in multiple tissues during aging and promote tissue dysfunction through the senescence-associated secretory phenotype (SASP), while microbiota dysbiosis and altered microbial metabolite production have been linked to impaired barrier integrity, systemic inflammation, and cognitive decline. Although these processes have each been studied extensively, their integration within the aging gut-brain axis remains insufficiently understood.

Senolytic intervention provides a valuable framework to address this question. The combination of dasatinib and quercetin (D+Q) selectively eliminates senescent cells and has shown beneficial effects in models of aging, including improvement of cognitive performance in aged rats. The rationale of this thesis is therefore that senescent cell clearance may influence cognitive aging not only through direct effects on inflammation and tissue homeostasis, but also through modulation of the gut microbiota, microbiota-derived metabolites, and the integrity of the intestinal and blood-brain barriers.

The main objective of this thesis was to determine whether senolytic treatment with D+Q can preserve or restore cognitive function during aging through mechanisms involving the gut-brain axis.

To address this objective, the study aimed to:

1. **Evaluate the effects of D+Q treatment on spatial learning and memory in aged Wistar rats;**
2. **Characterize D+Q-associated changes in gut microbiota composition and predicted functional profile;**
3. **Quantify alterations in microbiota-derived metabolites, including short-chain fatty acids, bile acids, and tryptophan-related metabolites, in faeces and serum;**
4. **Assess the integrity of the intestinal barrier and blood-brain barrier using histological and molecular markers, including mucosal architecture, mucin production, and tight-junction proteins;**
5. **Examine treatment-associated changes in inflammatory status and neurobiological correlates of cognitive function in gut and brain tissues, with particular attention to cytokine expression, hippocampal dendritic spine remodelling, and histone methylation linked to synaptic plasticity.**

Together, these objectives were designed to clarify how cellular senescence, microbiota-dependent signalling, inflammation, and barrier function interact in cognitive aging, and to evaluate the potential utility of senolytic intervention within this framework.

II. Materials and Methods

2.1 Animal Models and Ethical Approval

- ### Animal model

In the present study, adult male Wistar rats were selected as the experimental model for investigating the effects of senolytic intervention on cognition, gut microbiota, and barrier integrity in the context of aging. Wistar rats are a well-established and widely used outbred strain in biomedical research due to their genetic heterogeneity, docile behaviour, and physiological similarities to humans in key systems relevant to neurobiology, metabolism, and immunology.

The rationale for choosing this model lies in several critical characteristics. First, Wistar rats exhibit a clear and measurable age-dependent decline in cognitive performance, including deficits in spatial learning, memory retention, and executive functions. This decline is accompanied by systemic low-grade inflammation and increased levels of circulating pro-inflammatory cytokines, rendering aged Wistar rats a representative model for studying age-related pathologies, including cognitive impairment and gut-brain axis dysregulation.

Second, compared to genetically homogeneous strains such as Fischer 344 or Sprague-Dawley, Wistar rats better capture the heterogeneity of cognitive aging observed in human populations. This heterogeneity is crucial for translational research, particularly in geroscience, where inter-individual variability plays a significant role in the onset and progression of aging-related phenotypes. Notably, aged Wistar rats display variability in the rates of perceptual reasoning decline, synaptic plasticity alterations, and metabolic changes, offering a robust platform for studying differential responses to interventions such as senolytics.

- ### Ethics Statement

Ethical approval for all experimental procedures was obtained prior to the initiation of the study, and animal handling strictly followed the institutional and national guidelines for the care and use of laboratory animals. All efforts were made to minimize animal suffering and the number of animals used, in accordance with the 3Rs principle, Replacement, Reduction, and Refinement. Anaesthesia was performed using isoflurane and ketamine prior to euthanasia, and all procedures involving tissue collection were carried out rapidly and in accordance with protocols aimed at preserving sample integrity while ensuring humane endpoints.

- Experimental Groups and Age Selection

In the Krzystyniak et al. study, both 3-month-old (young) and 18- to 22-month-old (aged) Wistar rats were used to assess the cognitive and behavioural effects of D+Q senolytic treatment. The age of 22 months in rats roughly corresponds to an advanced stage of physiological aging, paralleling human geriatric states in terms of lifespan proportion and incidence of spontaneous pathologies. All animals were housed under controlled laboratory conditions (12-hour light/dark cycle, standard chow, and water ad libitum) and were grouped by age and treatment for the experimental procedures.

Animals were randomly assigned to treatment using a computer-generated block design (block size = 4). Investigators responsible for behavioural scoring, histology and molecular analyses were blinded to group allocation. An a-priori power calculation (G*Power 3.1, $\alpha = 0.05$, power = 0.80) indicated that $n = 8$ animals per group would detect a 30 % reduction in AAPAT shocks (Cohen's $d = 1.2$); we therefore enrolled 8–10 rats per arm to accommodate potential attrition.

Importantly, only male animals were used in both studies due to the availability of aged male Wistar rats and the known hormonal variability in females, which may introduce confounding effects in behavioural and microbiota analyses. While this choice restricts generalizability across sexes, it ensures homogeneity in endocrine influences, particularly in aging-related neuroinflammatory and microbiome outcomes.

Collectively, these attributes make male Wistar rats an appropriate and translationally relevant model for testing senolytic effects on the gut-brain axis.

2.2 Senolytic Treatment Protocol

- Administration of Dasatinib and Quercetin

The senolytic intervention employed in this study consisted of a combination of Dasatinib (D; 5 mg/kg/day) and Quercetin (Q; 50 mg/kg/day), collectively referred to as D+Q. In the context of aging-associated cognitive and physiological deterioration, D+Q has previously shown promising results in alleviating inflammation, enhancing tissue regeneration, and improving spatial memory in aged rodents. Dasatinib (5 mg kg^{-1}) and Quercetin (50 mg kg^{-1}) were freshly dissolved in 60 % Phosal 50 PG, 10 % ethanol and 30 % PEG-400 and administered by oral gavage according to the schedule below.

In our study, Dasatinib (LC Laboratories) and Quercetin (Sigma-Aldrich) were freshly prepared and dissolved together in a specific solvent mixture comprising 60% Phosal 50 PG, 10% ethanol, and 30% polyethylene glycol 400 (PEG-400). The mixture was vortexed until a homogenous solution was obtained, which was then administered via oral gavage to ensure precise and consistent delivery. This route was selected to mimic potential clinical application and maximize gastrointestinal exposure, consistent with previous reports indicating that oral administration is both effective and well-tolerated in aged rats.

- Dosage, Route, and Treatment Regimen

The treatment regimen involved administering D+Q once daily for five consecutive days, repeated every two weeks for a total duration of eight weeks. This cyclic protocol was informed by earlier work demonstrating that intermittent dosing maximizes senolytic efficacy while minimizing potential toxicity or off-target effects associated with continuous administration. The dosing schedule was designed to allow sufficient time for senescent cell clearance, followed by physiological remodelling and restoration of tissue function.

Animals in the control groups received the same volume of vehicle solvent alone, following an identical schedule and handling procedure. All animals were monitored for signs of distress or weight loss throughout the study. Body mass was measured regularly, and no significant differences were observed between the D+Q and vehicle-treated groups, indicating the tolerability of the treatment protocol.

This dosing paradigm was applied consistently across both experimental cohorts. In the first group, D+Q or vehicle treatment occurred between two spatial learning sessions (pre- and post-treatment), allowing for intra-animal comparisons of cognitive performance. In the second cohort, the same protocol was implemented, and animals were left undisturbed for an additional five weeks following treatment cessation, enabling evaluation of the durability of the treatment's cognitive and physiological effects.

This approach not only enabled the assessment of direct effects on senescent cell clearance but also allowed for the exploration of downstream consequences on gut microbiota, microbial metabolite production, intestinal and brain barrier integrity, and behaviour. The study thereby integrates pharmacological intervention with multi-tissue and multi-omic profiling to provide a holistic understanding of D+Q's mechanism of action in the aging gut-brain axis.

2.3 Cognitive Assessment

● Active Allothetic Place Avoidance Task Protocol

The Active Allothetic Place Avoidance Task (AAPAT) is a sensitive hippocampus-dependent cognitive test that demands cognitive coordination, spatial orientation, and executive control. The AAPAT was used as the principal test to measure hippocampus-dependent spatial learning, memory acquisition, and cognitive flexibility. This paradigm is particularly suited for aging studies as it requires coordination between Allothetic cues (external, room-fixed landmarks) and the suppression of misleading idiothetic cues (self-motion and proximal arena cues), thereby increasing the task's sensitivity to hippocampal dysfunction.

The apparatus consisted of an 80 cm diameter, elevated circular arena rotating at 1 rpm, positioned in the centre of a dimly lit (24 lx) 3×4 m room with distal visual cues. A latex harness was mounted on each rat's back and equipped with infrared LEDs to allow for precise tracking of the animal's position using a room-frame infrared camera. A virtual 60° shock zone was fixed in space relative to room cues but rotated with the arena. When the rat entered this sector, it received a mild foot shock (0.2–0.3 mA, 50 Hz, 0.5 s). If the rat failed to exit, the shock repeated every 1.5 s. Current intensity was individually calibrated to elicit an escape response without undue distress.

Each experimental group underwent a 5-day habituation phase during which animals were placed on the arena without shock for 10 minutes per day. This was followed by five days of spatial memory acquisition training, where shocks were delivered in the virtual zone. After D+Q or vehicle administration over eight weeks, the training resumed with a new shock zone location (e.g., from northwest to southwest), thus requiring formation of a novel spatial representation. In the second experimental cohort, a third training session was conducted five weeks after treatment cessation with another new shock zone position (northeast) to test the durability of memory consolidation.

Performance in AAPAT was evaluated using several metrics:

- Number of entries into the shock zone, reflecting spatial learning and task engagement.
- Number of shocks received, indicating the ability to avoid the punished area.
- Shocks per entry (SHs/ENTRs), quantifying learning efficiency.
- Maximum time avoided, interpreted as short-term memory retention.
- Time to first entry, used as a proxy for long-term memory acquisition over successive days.

This approach has been shown to offer superior sensitivity to age- and treatment-related changes in hippocampal-dependent cognition compared to traditional tests like the Morris water maze, which can be confounded by age-related physical impairment or swimming ability.

- Open-Field Test for Locomotor Assessment

To control for possible confounding effects of age or treatment on general locomotor activity and anxiety-like behaviour, an open-field test was performed on all animals following the final cognitive testing session. The test was conducted in a square, opaque-walled arena (100 × 100 × 40 cm) with a matte black floor, placed in a quiet, well-lit room. Prior to testing, the arena was disinfected with 70% ethanol and dried to eliminate olfactory cues.

Each animal was placed in a designated corner of the arena, facing the wall, and allowed to explore freely for a standard observation period. Behaviour was recorded using an overhead video camera, with analysis commencing 15 seconds after placement to allow for acclimatization. Video data were analysed using ToxTrac software (https://sourceforge.net/projects/toxtrac), with detection parameters set to minimize artifacts and ensure accurate tracking (e.g., det.mins = 3000, det.maxs = 50000, det.thr1 = 150, det.thr2 = 240).

Exploration was quantified by dividing the arena into 10 cm^2 virtual squares. Each entry into a square was logged as one unit of exploration, and the exploration rate was computed as the ratio of squares visited over the total possible area. This metric allowed us to determine whether differences in AAPAT performance could be attributed to changes in locomotor ability rather than cognitive impairment.

2.4 Biological Sample Collection

Euthanasia and Initial Handling. Following the completion of behavioural testing and the final treatment cycle, animals were anesthetized using isoflurane inhalation, followed by intraperitoneal injection of ketamine (100 mg/kg). Deep anaesthesia was confirmed by the absence of pedal reflexes. Euthanasia was then performed via intraperitoneal administration of sodium pentobarbital (100 mg/kg). Immediately postmortem, the animals were placed in a supine position and the abdominal cavity was opened along the midline using sterile instruments to expose internal organs.

Intestinal Tissue Collection. The entire gastrointestinal tract was excised with care to avoid rupture of the intestinal wall or contamination of the lumen. The small intestine and colon were separated and further subdivided into anatomically distinct sections: duodenum, jejunum, ileum, caecum, proximal colon, and distal colon. These segments were cut into ~2 cm pieces, gently rinsed in sterile phosphate-buffered saline (PBS) to remove residual contents, and snap-frozen in liquid nitrogen within 15 minutes of removal. Frozen tissues were stored at −80 °C until histological, molecular, and biochemical analyses.

Brain Tissue Collection. Immediately following intestinal tissue dissection, the skull was opened using rongeurs, and the whole brain was carefully extracted and rinsed in ice-cold PBS. The brain was bisected along the mid-sagittal plane. One hemisphere was used for protein and RNA extraction (frozen in liquid nitrogen and stored at −80 °C), while the other was reserved for histological analysis, cryopreserved in OCT medium, and stored at −80 °C. Dissections of hippocampal and cortical regions were performed where necessary using anatomical landmarks on ice-cold dissection plates.

Faecal Sample Collection. Faecal pellets were collected immediately post-mortem from the most distal part of the colon to ensure sample freshness and relevance to microbiota composition. Each pellet was transferred using sterile forceps into pre-weighed, sterile microcentrifuge tubes. The samples were snap-frozen in liquid nitrogen and subsequently stored at −80 °C until DNA extraction and metabolite profiling. Approximately 50–100 mg of faeces were collected per animal, ensuring sufficient material for multi-omic processing including 16S rRNA sequencing, bile acid profiling, and quantification of short-chain fatty acids (SCFAs).

Blood and Serum Collection. Prior to tissue harvesting, whole blood was drawn via cardiac puncture using a heparinized syringe. The collected blood was immediately placed in pre-chilled tubes and allowed to clot at room temperature for 15–30 minutes. Serum was separated by centrifugation at 1,500 × g for 10 minutes at 4 °C. The supernatant was carefully aliquoted into sterile tubes and stored at −80 °C. These serum samples were later used for metabolite quantification (SCFAs, bile acids, tryptophan derivatives) and cytokine profiling.

Sample Integrity and Quality Control. To ensure biological reproducibility and analytical consistency, all collected samples were coded, time-stamped, and handled according to standardized protocols. Snap-freezing in liquid nitrogen was prioritized for preserving metabolic activity and nucleic acid stability. For each tissue type, a master list of sample IDs and corresponding experimental groups was maintained to enable blinded downstream analysis. No significant degradation or quality loss was observed in the collected samples, as assessed by RNA integrity numbers (RIN) and protein concentration assays during preliminary extraction steps.

2.5 Microbiome Analysis

To determine the composition and structure of the intestinal microbiota and explore its potential modulation by senolytic treatment, 16S ribosomal RNA (rRNA) gene sequencing was performed on faecal samples collected from aged Wistar rats post-intervention. The methodology comprised meticulous DNA extraction, library preparation, sequencing, and a robust bioinformatics pipeline optimized for high-resolution microbial taxonomic classification.

DNA Extraction from Faecal Samples. Faecal pellets were collected directly from the descending colon at the time of euthanasia and snap-frozen in liquid nitrogen before storage at −80 °C. Approximately 50–100 mg of faecal material per animal was used for DNA extraction, which was carried out following the IHMS_SOP_07_V2 protocol developed by the International Human Microbiome Standards (IHMS) Consortium and adapted by INRA (France). Samples were homogenized in a lysis buffer containing 5% guanidine thiocyanate and 10% N-lauryl sarcosine, followed by incubation at 70 °C for 1 hour to lyse microbial cells. Homogenization was enhanced using glass beads on an orbital vortex for 10 minutes. Polyvinylpolypyrrolidone (PVPP) was added to adsorb polyphenolic compounds, and the homogenate was subjected to a series of centrifugation and washing steps using Tris-EDTA-NaCl-PVPP buffer to purify the DNA. Following precipitation with isopropanol and ethanol washing, the DNA pellet was dried, resuspended in a phosphate-acetate buffer, and incubated on ice for 90 minutes. After an additional RNAse digestion step and ethanol precipitation, the final DNA was dissolved in Tris-EDTA buffer and stored at −20 °C. DNA quantity and purity were verified using a Nanodrop spectrophotometer.

16S rRNA Gene Amplification and Sequencing. The variable region V4 of the bacterial 16S rRNA gene was amplified for all samples. PCR amplification products were prepared for sequencing using Illumina-compatible library protocols. Sequencing was conducted at the Max Rubner-Institut using the Illumina MiSeq platform, applying v3 chemistry with 2 × 300 bp paired-end reads, which provides sufficient resolution for community-level taxonomic profiling down to the genus and, in some cases, species level.

Bioinformatic Pipeline. Bioinformatic processing was carried out using R version 4.3.2. Raw FASTQ files were processed through the DADA2 pipeline, which enables denoising of reads and generation of amplicon sequence variants (ASVs), a higher-resolution alternative to traditional Operational Taxonomic Units. Chimera removal, quality filtering, and trimming of low-quality reads were performed according to best practices for MiSeq-generated reads. Taxonomic assignment of ASVs was achieved by alignment to the SILVA database version 138.1. The classification algorithm included bootstrap confidence scoring and employed the naive Bayesian classifier method for accurate assignment. Spearman rank correlations were used to assess associations between microbial taxon relative abundances and measured SCFA and bile acid concentrations. To ensure robust estimates, analyses were restricted to taxa detected in ≥80% of samples and reaching >1% relative abundance in at least one sample. Taxa were then grouped according to similarity in their correlation profiles with SCFAs and bile acids by computing Euclidean distances between each taxon’s vector of correlation coefficients, followed by hierarchical clustering with the UPGMA approach (the “average” linkage option in `hclust`). Correlation heatmaps were generated with `heatmap.2` from the **gplots** package (v3.2).

Community Structure and Diversity Analysis. Post-classification, the microbial community structure was analysed across taxonomic levels (phylum, class, genus) using abundance metrics and diversity indices. Alpha-diversity was assessed using Shannon and Simpson indices to evaluate within-sample richness and evenness. Beta-diversity was analysed using Bray-Curtis dissimilarity followed by Principal Coordinates Analysis (PCoA) and hierarchical clustering to assess between-group differences in community composition. The effects of senolytic treatment were visualized by comparing microbial profiles between D+Q-treated and vehicle-treated groups. Raw sequencing data have been deposited in the European Nucleotide Archive (ENA) to ensure transparency, reproducibility, and accessibility for future meta-analyses and comparative studies.

2.6 Metabolomics Profiling

- GC-MS Analysis of Short-Chain Fatty Acids

Faecal and serum SCFAs were analysed using gas chromatography–mass spectrometry (GC-MS), allowing high sensitivity quantification of volatile fatty acids with carbon chains typically ranging from two to six atoms.

Faecal SCFAs: Approximately 25 mg of faeces were weighed into 2 mL reaction tubes. Extraction was initiated by adding 50 µL of 25% hydrochloric acid, 370 µL of deionized water, and 80 µL of an internal standard mixture comprising deuterated analogs of common SCFAs (acetic acid d4, butyric acid d7, propionic acid d6, etc.). After 30 minutes of orbital shaking and 10 minutes of vortexing, the samples were centrifuged at 23,100 × g for 5 minutes. A 300 µL aliquot of the supernatant was transferred into 2 mL amber glass vials with crimp caps and stored at −20 °C until analysis.

Serum SCFAs: Serum samples were prepared according to the protocol by Yao and colleagues (Yao et al., 2022). Shortly, serum samples (200 µL) were thawed and treated with 24 µL of cold 2 N HCl. Following a brief vortex, 360 µL of cold methyl tert-butyl ether (MTBE) was added, and the mixture was vortexed again and centrifuged at 3,000 × g for 10 minutes at 4 °C. Sixty microliters of the upper organic phase were transferred into vials for GC-MS analysis. SCFAs were quantified using a Shimadzu GC-2010 instrument with a QP-2020 quadrupole MS system and ZB Wax Plus column (60 m × 0.25 mm × 0.25 µm). Electron impact ionization spectra were recorded in scan mode (m/z 29–350), and compound identification was based on retention times and fragmentation patterns against reference standards. Temperature and split-ratio programs were tuned to minimize carryover. Technical replicates were analysed when possible, and values were averaged. Compounds with poor reproducibility (e.g., formic acid) or values below the limit of detection (e.g., 3-methylbutyric acid) were excluded from downstream statistical analyses.

Data Processing analysis and Statistical Analysis. Data processing was conducted using MS-DIAL software. Raw spectral data were converted to ABF format, background noise was filtered based on intensity ratios to blank samples, and metabolite peaks were annotated using internal databases and verified manually. Principal component analysis (PCA), Wilcoxon rank-sum tests, and one-way ANOVA were employed in JMP 17.1.0 to assess treatment effects, with α set at 0.05. Features not passing quality filters were excluded from downstream multivariate analyses. Where applicable, metabolite concentrations were correlated with bacterial ASV abundance to identify potential microbial contributors to altered metabolic output. While no correlation passed correction for multiple testing due to sample size constraints, several trends were observed between taxa and SCFA or bile acid levels that suggest structured microbiota-metabolite interactions.

- UHPLC-HRMS Analysis of Bile Acids and Tryptophan Metabolites

Faecal and serum bile acids and **tryptophan catabolites** were analysed using ultra-high-performance liquid chromatography coupled with high-resolution mass spectrometry (UHPLC-HRMS), offering a comprehensive profile of targeted microbial and host-derived metabolites with high precision and specificity.

Sample preparation for faecal bile acids: Faecal samples (89–355 mg) were homogenized in cold methanol containing 18 internal standards using a FastPrep-24 system. After centrifugation, the supernatant was either directly injected or concentrated. A pooled quality control (QC) sample was created by mixing non-concentrated extracts to assess instrument stability throughout the run.

UHPLC-HRMS instrumentation: An Agilent 1290 Infinity UHPLC system coupled with a TripleTOF 5600 mass spectrometer was used. Separation was achieved with a 1.8 µm Acquity UPLC HSS T3 Premier column using a gradient elution of 2 mM ammonium formate (aqueous, phase A) and acetonitrile (phase B). A 2 µL injection volume was applied for each run, and the gradient was optimized to separate structurally similar bile acids. The system operated in both positive and negative electrospray ionization modes to ensure detection of a broad chemical range. Information-dependent acquisition (IDA) mode enabled parallel MS and MS/MS scanning, enhancing confidence in metabolite annotation based on fragmentation patterns and retention times against authenticated standards.

Tryptophan metabolite profiling followed a similar protocol and targeted a panel of 23 compounds, including indoxyl sulfate, trimethylamine (TMA), trimethylamine N-oxide (TMAO), glycerophosphocholine (GPC), carnitine, betaine, and others relevant to gut-brain axis communication. These metabolites were extracted from faeces and quantified under the same UHPLC-HRMS conditions used for bile acids.

2.7 Histological and Morphological Analysis

- Histology of Gut and Brain Tissues

Tissue specimens from the duodenum, jejunum, ileum, caecum, and proximal and distal colon were cryosectioned for histopathological examination. Samples were initially stored at −80 °C and brought to −20 °C shortly before processing. Cryosections of 14 μm thickness were generated at −15 °C using a cryostat and mounted on Superfrost® Plus microscope slides. Sections were dried overnight and stored at −20 °C until staining procedures.

Hematoxylin and Eosin (H&E) Staining was used to evaluate overall tissue architecture, cellular organization, and infiltration. Sections were fixed in precooled methanol for 10 minutes at −20 °C, rehydrated in distilled water for 3 minutes, and stained in hematoxylin solution for 1 minute. Following rinsing under running tap water (3 minutes), sections were dehydrated in 30% and 50% ethanol for 2 minutes each, cleared with two changes of Roti-Histol (5 minutes each), and coverslipped using Entellan. Slides were dried overnight before imaging.

Alcian Blue Staining was employed to visualize acidic mucopolysaccharides and mucins, indicative of barrier function. Cryosections were fixed in 4% paraformaldehyde for 10 minutes at room temperature, rinsed with PBS, and immersed in 3% acetic acid for pH adjustment. Slides were then incubated in 1% Alcian blue solution (pH 2.5) for 30 minutes. Following this, sections were rinsed thoroughly, counterstained with nuclear fast red for 5 minutes, and sequentially dehydrated, cleared in xylene, and coverslipped. Alcian blue-positive mucin regions appeared blue under light microscopy and were digitally imaged for quantification.

Senescence-associated beta-galactosidase (SA-β-gal) was used to quantify the senescence cell burden in the brain. PFA fixed brain of vehicle and D+Q treated rats were cut in 30 μm slices. Hippocampi were isolated and SA-β-galactosidase staining was performed. Astrocytes and neurons were targeted by immunofluorescence prior to SA-β-galactosidase staining with either anti-GFAP or anti-NeuN antibodies to define regions of interest by thresholding the signal intensity using Fiji (ImageJ) software. Within each region of interest, mean grey value was measured using the same software. Representative images for each rat from both treatment groups showing sum projection of NeuN, GFAP and REST staining, thresholding of NeuN and GFAP along with the overlay of their individual and merged regions of interest

- Morphometric Analysis of Mucosal Thickness and Mucin Production

To assess intestinal morphology quantitatively, mucosal thickness was measured using digital histological images from H&E-stained sections. ImageJ software (NIH) was employed for image analysis. For each intestinal section, eight radial lines were drawn through the centre at 45° intervals (0°, 45°, 90°, etc.), yielding up to 16 measurements per sample. These values were averaged and compared across regions to detect local or systemic remodelling effects from D+Q intervention.

Alcian blue-stained sections were analysed to quantify mucin production. Digital images were processed using ImageJ (v1.54f), and the staining intensity was assessed using the Hue-Saturation-Brightness (HSB) threshold method. Distinct threshold settings were applied based on tissue region: for intestinal and caecal tissue, H = 120–215, S = 10–255, B = 0–255; for colonic tissue, H = 120–200, S = 10–255, B = 0–255. The parameters measured included the area of blue pixels, mean blue intensity, and integrated density (area × mean intensity). The final index of mucin production was expressed as the quotient of integrated density to total tissue area.

2.8 Molecular and Biochemical Analyses

- ### Gene expression of Tight-Junctions, Inflammation, and Mucins

RNA Isolation and cDNA Synthesis. Total RNA was extracted using Trizol reagent (Invitrogen, USA) from flash-frozen intestinal and brain tissues. Tissues were homogenized in Trizol (1 mL per 50–100 mg), followed by chloroform extraction and isopropanol precipitation. After ethanol washing and air drying, RNA was resuspended in RNase-free water. RNA concentration and purity were assessed using a NanoDrop spectrophotometer (Thermo Fisher Scientific, USA).

One microgram of purified RNA was treated with DNase I (Thermo Fisher Scientific) and reverse-transcribed to cDNA using the iScript cDNA Synthesis Kit (Bio-Rad, USA), with a mix of random and oligo(dT) primers. Reverse transcription was performed in a thermal cycler with the following settings: 25 °C for 5 min, 42 °C for 30 min, and 85 °C for 5 min.

qPCR Protocol. qPCR was carried out using the SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, USA) on a CFX96 Real-Time PCR Detection System. Each 20 µL reaction included 10 µL of SYBR Green Supermix, 1 µL of cDNA, and 0.5 µM of forward and reverse primers. Cycling conditions were: 95 °C for 3 min, then 40 cycles of 95 °C for 10 s and 60 °C for 30 s. Melt curve analysis was performed to confirm specificity.

Target genes included:

- Tight junction proteins: *tjp1* (Zonulin-1), *ocln* (Occludin), *cldn1* (Claudin-1)
- Inflammatory cytokines: *tnfa* (TNF-α), *ifng* (IFN-γ), *il10* (IL-10)
- Mucin genes: *muc1*, *muc2*
- SCFA receptors: *gpr41*, *gpr43*

Data were normalized to GAPDH and analysed using the $2^{-\Delta\Delta Ct}$ method. All reactions were run in triplicate, and mean Ct values were used for fold-change calculations. Expression changes were compared between D+Q-treated and vehicle-treated groups using CFX Manager Software (Bio-Rad).

- Protein quantification of Tight-Junctions and Inflammation

Protein Extraction and Quantification. After RNA isolation, remaining tissue pellets were washed in 0.3 M guanidine hydrochloride in 95% ethanol, centrifuged, and resuspended in a lysis buffer containing 2% SDS and 50 mM Tris-HCl (pH 7.4). Samples were incubated at 55 °C for 1 hour and vortexed intermittently. Protein concentration was measured using the bicinchoninic acid (BCA) assay (Thermo Fisher Scientific, USA).

Electrophoresis and Transfer. Equal amounts of protein (20–30 μg) were denatured in Laemmli buffer, boiled for 5 min, and loaded on 10% SDS-PAGE gels. Proteins were transferred to PVDF membranes (Bio-Rad) using standard wet transfer protocols.

Immunodetection. Membranes were blocked with 5% non-fat dry milk in TBST for 1 hour and incubated overnight at 4 °C with primary antibodies against:

- Zonulin-1 (ZO-1), Occludin, Claudin-1 (tight junctions)
- TNF-α, IFN-γ, IL-10 (inflammation)
- GAPDH (loading control)

Primary antibodies were diluted 1:1000 unless otherwise stated: ZO-1 (Invitrogen 40-2200), Occludin (Invitrogen 33-1500), Claudin-1 (Invitrogen 51-9000), TNF-α (Invitrogen ARC3012), IFN-γ (ABclonal A12450), IL-10 (Invitrogen 14-7101-85, 1:500) and GAPDH (Santa Cruz sc-166545, 1:5000). Following TBST washes, membranes were incubated with HRP-conjugated secondary antibodies for 1 hour at room temperature and visualized using enhanced chemiluminescence (ECL) on a ChemiDoc XRS+ system (Bio-Rad).

Epigenetic marks. Briefly, tissue was resuspended in 0.4 M H_2SO_4, rotated 1 h at 4 °C, and cleared by centrifugation (16 000 g, 10 min). Proteins were precipitated overnight with 20 % trichloroacetic acid, washed twice with ice-cold acetone, air-dried and redissolved in ddH_2O. Histone aliquots (5 μg) were separated on 15 % SDS-PAGE gels and transferred to PVDF. Blots were probed with antibodies against H3K9me3 (Cell Signaling #13969) and H3K27me3 (Cell Signaling #9733); total H3 (Abcam ab1791, 1:10 000) served as a normaliser. Band intensities were expressed as the ratio H3K9me3/H3 and H3K27me3/H3

Quantification. Densitometry was performed using Image Lab software (Bio-Rad). Band intensities were normalized to GAPDH. Relative expression was calculated against the vehicle group, which was set to 1 for comparison. All protein quantification analyses were conducted in the brain (frontal cortex and cerebellum) and gut tissues (duodenum to distal colon).

- ## Synaptic Plasticity Assessment by DiI-Labelled Spine Imaging

Tissue preparation. Adult male Wistar rats were perfused with 4 % paraformaldehyde, and coronal hippocampal sections (140 µm) were cut on a vibratome. Slices rested for ≥ 1.5 h in phosphate-buffered saline at room temperature to recover membrane integrity before labelling.

Random DiI impregnation. Sparse, unbiased labelling of dendritic arbours was achieved via gene-gun delivery. Tungsten micro-particles (1.6 µm; Bio-Rad) coated with the lipophilic dye DiI (Invitrogen) were fired onto the slice surface, allowing dye to diffuse along the plasma membrane and fill complete neurons.

Confocal imaging parameters. Dendrites located in the CA1 region were imaged on a laser-scanning confocal microscope equipped with a 63 × / 1.4 NA objective. DiI was excited at 561 nm; emission was collected onto GaAsP detectors. Z-stacks were acquired at 1024 × 1024 pixels with a zoom of 3.43, yielding an in-plane resolution of 0.07 µm per pixel.

Spine selection and quantitative analysis. Only secondary and tertiary dendritic segments were analysed to minimise distance-related variability in spine shape. Raw stacks were processed in ImageJ, and spine morphometry was extracted semi-automatically with the custom SpineMagick software. The algorithm fits a fluorescence-weighted skeleton to each protrusion to obtain:

- Spine length – curvilinear distance from the shaft to tip.
- Head width – maximum diameter measured on the proximal two-thirds of the spine, excluding the basal one-third.

A scale-free descriptor, the length : head-width ratio, was used as a robust index of shape variability.

Sampling depth and statistical design. Morphological datasets comprised ~4 400 spines from vehicle-treated animals and ~6 200 spines from D + Q-treated animals, originating from at least three rats per condition. For density estimations, ~900 µm (vehicle) and ~1 500 µm (D + Q) of dendritic length were evaluated. Nested statistics treated the animal as the ultimate unit of replication.

2.9 Statistical Analysis

Description of Statistical Approaches and Software. All statistical analyses were designed to rigorously evaluate the effects of senolytic treatment (Dasatinib and Quercetin) on behavioural, molecular, metabolic, microbiome, and histological endpoints. The choice of statistical tests was determined by the structure of the data, distribution characteristics, and the number of groups being compared. All analyses were conducted under standard significance thresholds ($\alpha = 0.05$) unless otherwise stated and performed using specialized software including GraphPad Prism (v8), JMP (v17.1.0), and R (v4.3.2).

Assessment of Distribution and Data Transformation. Before applying inferential tests, normality and homoscedasticity of data distributions were assessed using:

- Shapiro–Wilk test for normality
- Levene's test for homogeneity of variances. Residual plots and Q-Q plots were visually inspected when appropriate. Data not conforming to parametric assumptions were either log-transformed or analysed using non-parametric methods.

Univariate Statistical Tests. To compare two independent groups (senolytic vs. vehicle), unpaired Student's t-tests were applied when normality assumptions were satisfied. When distributions deviated from normality, Mann–Whitney U tests were used instead. One-tailed tests were used when a directional hypothesis was formulated based on prior evidence (e.g., D+Q is hypothesized to reduce inflammatory markers). Two-tailed tests were used otherwise. For multi-group comparisons (e.g., multiple gut segments, time points, or brain regions), one-way ANOVA was employed when comparing a single factor with three or more levels. If the ANOVA yielded significant results, Tukey's HSD post hoc test was conducted for pairwise comparisons. In experiments where two independent factors were studied simultaneously (e.g., treatment × time), two-way ANOVA followed by Tukey's post hoc test was performed. Data were reported as mean ± standard error of the mean (SEM) for parametric analyses, or median with interquartile range (IQR) for non-parametric data.

Multivariate and Exploratory Analyses. For high-dimensional data such as metabolomic and microbiome profiles, Principal Component Analysis (PCA) was applied to reduce dimensionality and visualize clustering patterns across treatment groups. Prior to PCA, features were scaled and cantered, and variables with missing data or high noise (low signal-to-blank ratios) were excluded from the dataset.

For feature comparisons within metabolomic or microbiota datasets, Wilcoxon rank-sum tests or one-way ANOVA were applied depending on the data type and distribution. Data imputation for missing metabolite values was performed using conservative estimates (e.g., replacing with half of the minimum detectable value) when applicable.

Correlation and Association Analysis. Spearman's rank correlation coefficient was used to assess associations between relative microbial abundances (ASVs) and metabolite concentrations (SCFAs, bile acids), as this method is robust to non-linear and non-normally distributed data. Only ASVs present in at least 80% of samples with ≥1% relative abundance in at least one sample were retained to reduce multiple testing burden. Due to the limited sample size and high dimensionality of the data, corrections for multiple testing (e.g., Benjamini–Hochberg FDR) were explored but not always applied when the primary goal was hypothesis generation rather than confirmatory inference.

Spine imaging analysis. Statistical comparisons among treatment groups used linear mixed-effects models with animal identity as a random factor and Holm–Šidák correction for multiple testing. Normality of residuals was confirmed by Shapiro–Wilk tests; non-parametric alternatives were applied where appropriate. Data are reported as mean ± SEM unless stated otherwise.

Software Tools and Validation used:

- **GraphPad Prism v8** (GraphPad Software, USA): used for behavioural and cytokine data analysis, bar plots, and group comparisons in low-dimensional datasets
- **JMP v17.1.0** (SAS Institute): used for analysis of GC-MS and UHPLC-HRMS metabolite features and PCA
  R v4.3.2: used for microbiome bioinformatics, statistical modelling of ASVs, and ordination plots
- **MS-DIAL**: used for mass spectrometry data processing and feature annotation in metabolomics workflows.

III. Results

3.1 Behavioural and Cognitive Improvement after D+Q Treatment

- Study Design and Testing Timeline

The effect of Dasatinib and Quercetin (D+Q) treatment on spatial learning and memory was assessed in aged and young Wistar rats using the Active Allothetic Place Avoidance Task (AAPAT). Spatial learning and memory were assessed using the AAPAT across three sessions: pre-treatment (1st TRAINING), post-treatment (2nd TRAINING), and follow-up (3rd TRAINING) (Fig. 10). Each training requires animals to form a new spatial representation due to the relocation of the shock zone.

Figure 10. Schematic diagram of experimental design.
Wistar rats are subjected to the training 3 times, each time with a new position of the to-be-avoided place.

- Baseline Cognitive Performance

To establish the baseline of cognitive performance and age-related impairment prior to senolytic intervention, aged and young male Wistar rats were subjected to the first session of the AAPAT, referred to as the 1st TRAINING. This pre-treatment phase enabled a rigorous comparison of cognitive capabilities between age groups and established a reference point for evaluating the beneficial effects of D+Q treatment in subsequent stages.

During this baseline phase, all rats were trained for five consecutive days to avoid a rotating shock sector in a room-frame-fixed position (northwest quadrant), relying exclusively on allocentric spatial cues. Performance metrics, number of entries into the shock zone, number of shocks received, shocks per entry (SHs/ENTRs ratio), maximum time avoided (Tmax), and time to first entry (T1), were recorded and analysed using repeated measures two-way ANOVA.

The results demonstrated that aged rats exhibited significantly impaired spatial learning and memory compared to their younger counterparts across all key parameters (Figure 11):

- Aged rats showed higher entries into the shock zone than young rats (F(1,28) = 33.62; $p < 0.001$).
- Aged rats received more shocks than young rats (F(1,28) = 53; $p < 0.001$).
- SHs/ENTRs ratio was higher in aged rats (F(1,28) = 86; $p < 0.001$).
- The maximum time avoided shocks (Tmax) was lower in aged rats ((F1,28) = 16.83; $p = 0.0003$).
- The time to first entry into the shock sector (T1) was lower in aged rats (F(1,28) = 16.82; $p = 0.0003$).

Despite these impairments, **aged rats showed day-to-day improvement across the five days of training.** A significant effect of training day was detected for all performance metrics (e.g., for shocks: F(1,28) = 53; $p < 0.001$).

Importantly, **no significant differences were observed between aged rats pre-assigned to the D+Q or vehicle group, confirming homogeneity at baseline** and validating the experimental design. This allowed meaningful within-subject comparisons in post-treatment sessions without confounding pre-existing variability.

Overall, these pre-treatment findings confirm that aged rats exhibit hallmark deficits in spatial learning and cognitive flexibility, consistent with age-related decline.

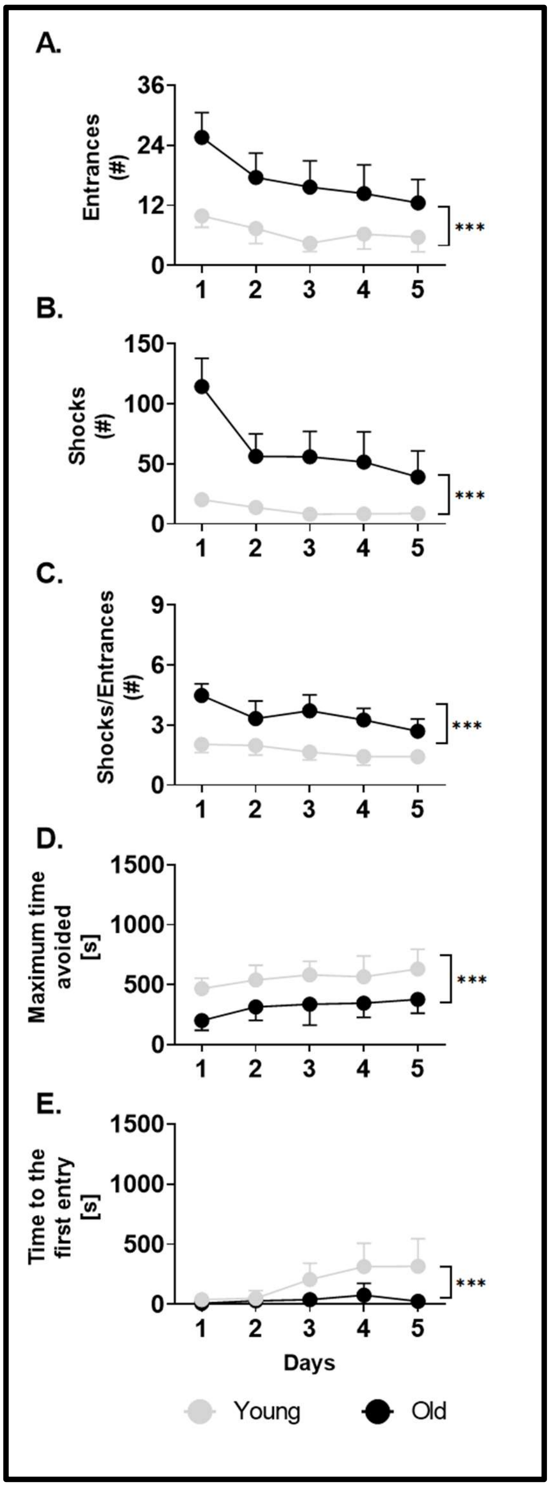

Figure 11. Spatial memory performance of young and aged rats in the AAPAT.
The number of entries into the shock place (# of entries, A), the number of shocks (# shocks, B) and the number of shocks per one entry ratio (Shocks/entrance, C), together with maximum time avoided (D) and time to the first entry (E) during the place avoidance training reflect changes in the learning abilities and memory of young vs aged rats.
Error bar showing 95% CI.
n Young = 16, n Old = 14 animals;
*** = $p < 0.001$.

- Post-Treatment Cognitive Performance

The administration of the senolytic cocktail D+Q led to a significant reversal of cognitive deficits in aged Wistar rats, as demonstrated by their performance in the second session of the AAPAT. This second training, performed after 8 weeks of D+Q administration, involved a new spatial location for the shock zone, thereby requiring the formation of a new spatial memory trace and offering a stringent test for both short- and long-term memory capacities.

In contrast to the vehicle-treated aged rats, those that received D+Q displayed marked improvements across multiple behavioural parameters (Figure 12):

- Number of shocks: Aged D+Q-treated rats received significantly fewer shocks than their vehicle-treated counterparts (F(1,11) = 7.02, *p* = 0.022).
- Skill learning (SHs/ENTRs ratio): Aged animals treated with D+Q also exhibited a significantly lower shocks-per-entry ratio (F(1,11) = 9.1, *p* = 0.012).
- Entrances and avoidance metrics: Although not all parameters reached statistical significance on an individual training day basis, two-way ANOVA comparing 1st (pre-treatment) and 2nd (post-treatment) training sessions across five days showed robust group effects:
    - Entrances (F(1,11) = 9.87, *p* = 0.009)
    - Shocks (F(1,11) = 12.66, *p* = 0.005)
    - SHs/ENTRs (F(1,11) = 8.87, *p* = 0.013)
    - Maximum time avoided (F(1,11) = 6.15, *p* = 0.03)

Overall, **D+Q treated animals outperformed the control group**. Performance gains were especially evident by days 4 and 5 of training, when D+Q-treated animals exhibited a sustained reduction in shock exposure and a higher maximum time of avoidance.

The collected data also revealed that the **cognitive benefits of D+Q were specific to aged animals.** Young rats did not show significant differences between pre- and post-treatment AAPAT performance, consistent with their intact baseline cognitive function and the senescence-targeted mechanism of the intervention.

Interestingly, **no significant changes were observed in the locomotor behaviour between D+Q-treated and control rats**, as confirmed by the open field test (Figure 13). This supports that performance differences in AAPAT stemmed from cognitive rather than motor function alterations.

Taken together, these results provide strong evidence that the **senolytic treatment is sufficient to restore spatial memory and learning abilities in aged rats.** The improvement was measurable in both absolute metrics (shock avoidance, avo5idance duration) and normalized indices of learning efficiency.

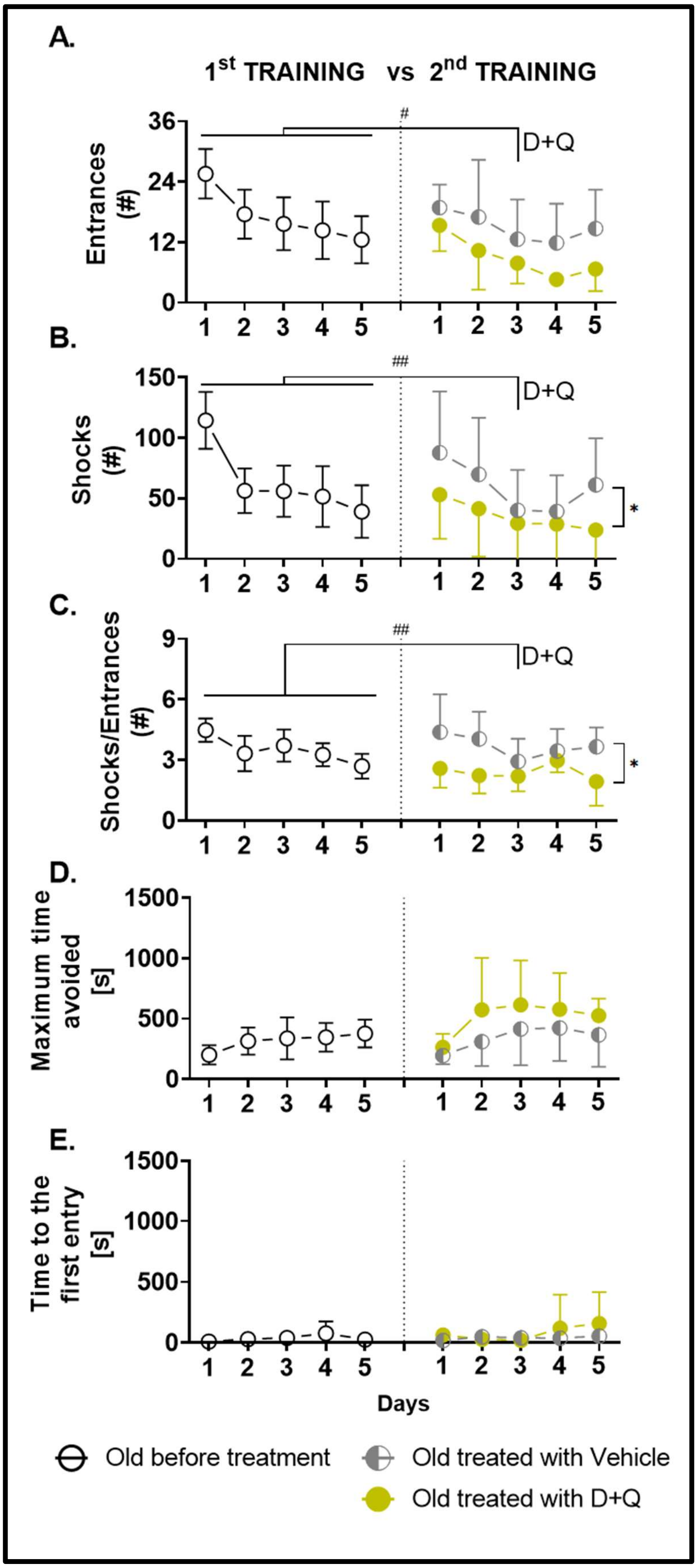

Figure 12. Treatment with D+Q improved cognitive ability in aged rats tested in the AAPAT on spatial memory.
The number of entries into the shock place (# of entries, A), the number of shocks (# shocks, B) and the number of shocks per one entry ratio (Shocks/entrance, C), together with maximum time avoided (D) and time to the first entry (E) during place avoidance training reflect changes in the learning abilities and memory in aged rats after treatment. Error bar showing 95% CI.
n Old before treatment = 14,
n Old treated with Vehicle = 7
n Old treated with D+Q = 6 animals;
# and * = $p < 0.05$;
= $p < 0.01$.

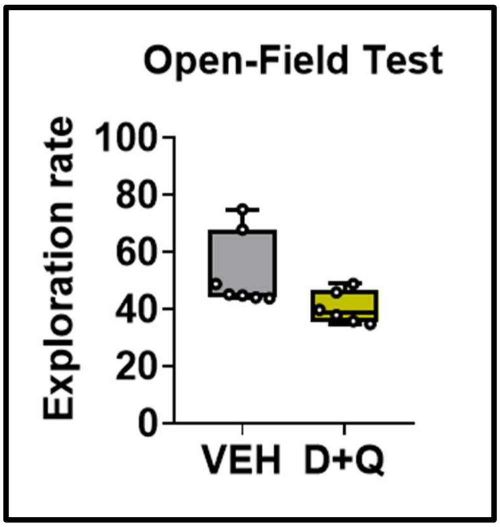

Figure 13. D+Q treatment does not change locomotor activity in male Wistar rats. Exploration rate in open field test was performed after the D+Q or vehicle treatment in aged animals. Exploration ratio was calculated by dividing the open field arena into 10 cm2 squares and calculating the ratio of explored squares recorded with the Toxtrac software over the total number of squares for every individual of both mischiefs during the open field test. Data expressed as box and whiskers showing min. and max. with all points.

- Persistence of Cognitive Effects after D+Q Treatment

To determine whether the cognitive improvements induced by D+Q treatment were sustained beyond the immediate post-treatment period, a third AAPAT session (3rd TRAINING) was conducted five weeks after cessation of treatment. During this period, rats were kept undisturbed in their home cages, with no additional behavioural or pharmacological interventions. This design allowed assessment of long-term memory retention and cognitive stability in the absence of ongoing treatment.

Repeated-measures two-way ANOVA revealed a main effect of days across the five-day training period for all assessed parameters (Figure 14):

- Entrances into the shock zone (F(4, 48) = 27.68; $p < 0.001$)
- Number of shocks received (F(4, 48) = 42.50; $p < 0.001$)
- Shocks-per-entry ratio (SHs/ENTRs) (F(4, 48) = 12.47; $p < 0.001$)
- Maximum time avoided (Tmax) (F(4, 48) = 8.14; $p < 0.001$)

Post hoc comparisons using Tukey's test confirmed that rats exhibited fewer entries and shocks, improved skill learning, and longer avoidance durations on days 3 to 5 compared to days 1 and 2 ($p < 0.01$ for all comparisons).

Critically, no regression to baseline levels was observed, despite the five-week gap between treatment cessation and the onset of the 3rd TRAINING. **Aged rats treated with D+Q demonstrated robust retention of the cognitive improvements** observed in the 2nd TRAINING session. Performance in the 3rd TRAINING closely matched the post-treatment gains and remained significantly superior to baseline (1st TRAINING), confirming durable benefits of senolytic intervention on spatial learning and cognitive coordination.

When comparing the 1st and 3rd TRAINING sessions directly, aged rats displayed a significant reduction in shock zone entries and shocks received, a lower SHs/ENTRs ratio, and a longer maximum time avoided, confirming lasting cognitive enhancement.

- Entrances: F(1,12) = 9.10; $p = 0.01$
- Shocks: F(1,12) = 23.21; $p < 0.001$
- SHs/ENTRs ratio: F(1,12) = 15.13; $p = 0.002$
- Tmax: F(1,12) = 9.96; $p = 0.008$

In contrast, young rats treated with D+Q did not show significant differences between 2nd and 3rd TRAINING sessions, reinforcing that the cognitive benefits of D+Q were specific to age-associated deficits and not due to general enhancement of memory function in healthy animals.

These results support the conclusion that **D+Q treatment has long-lasting effects on cognitive function**, particularly in restoring the capacity for spatial memory formation and retention in aged individuals. The enduring nature of the observed benefits suggests underlying structural and molecular changes, such as those seen in histone methylation and dendritic spine remodelling, that persist well beyond drug administration.

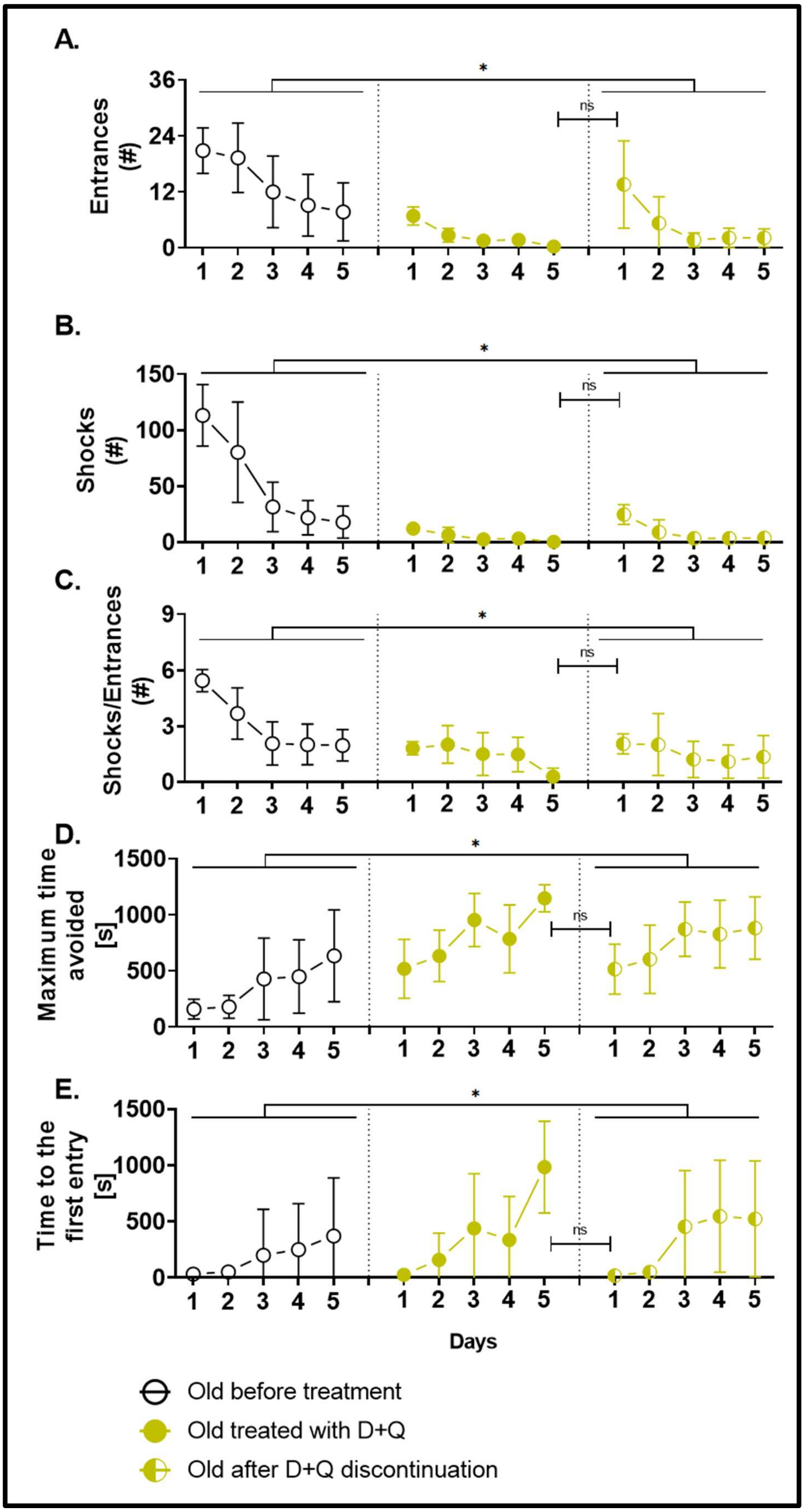

Figure 14. Treatment with D+Q improved cognitive ability in aged rats tested in the AAPAT on spatial memory for at least 5 weeks after treatment termination. The number of entries into the shock place (# of entries, A), the number of shocks (# shocks, B) and the number of shocks per one entry ratio (Shocks/entrance, C), together with maximum time avoided (D) and time to the first entry (E) during place avoidance training reflect changes in the learning abilities and memory in aged rats. Error bar showing 95% CI. n Old before treatment = 7, n Old treated with Vehicle = 7 n Old treated with D+Q = 7 animals; * = $p < 0.05$.

3.2 Hippocampal Chromatin Remodelling

Because the cohort treated with Dasatinib and Quercetin (D+Q) had already shown restored performance in the hippocampus-dependent active place-avoidance task, it was essential to determine whether a corresponding anatomical substrate, namely a change in dendritic-spine number, accompanied this behavioural recovery, as spines represent the principal structural correlate of experience-driven synaptic plasticity.

- Dendritic Spine Density

Synaptic density was measured on 140-μm hippocampal slices stained with the lipophilic carbocyanine dye DiI, which sparsely fills entire dendritic arbours and renders individual spines resolvable under confocal optics. Spine counts were therefore extracted from high-resolution z-stacks, restricted to secondary/tertiary segments situated 70–120 μm from the soma to minimise positional bias. Imaging files were coded so that scoring was blind to treatment, and nested statistics treated the animal, not the individual spine, as the unit of replication.

Quantitative analysis of DiI-filled pyramidal neurons in the hippocampal CA1 region showed that the senolytic regimen did not alter the absolute number of dendritic spines per unit dendritic length in aged animals (Figure 15).

In 24-month-old Wistar males that received only the vehicle, spine packing density remained within the range reported previously for very old rodents, i.e. ~0.60 spines μm^{-1} on proximal basal branches located in the stratum oriens and ~0.75 spines μm^{-1} on proximal apical branches in the stratum radiatum.

Eight weeks of intermittent D+Q administration left total spine counts statistically unchanged in both dendritic compartments (stratum oriens: $p \approx 0.69$; stratum radiatum: $p \approx 0.44$). Thus, the treatment neither rescued an occult age-related synapse deficit nor provoked pruning of existing contacts.

Total spine density in CA1 pyramidal neurons was quantified on DiI-labeled dendritic segments in aged vehicle (N=3) and aged D+Q (N=5) rats. Spine density did not differ between groups in either compartment: stratum oriens (p=0.692) and stratum radiatum (p=0.443). Approximately 900 μm (VEH) and 1500 μm (D+Q) of dendritic length were analysed per group for density estimation.

Despite the behavioural recovery, D+Q did not change total spine density in CA1 basal or apical dendrites. This suggests that the treatment effect is not expressed as large-scale synaptogenesis or synapse loss, but rather through alterations in synaptic structure at the level of individual spines.

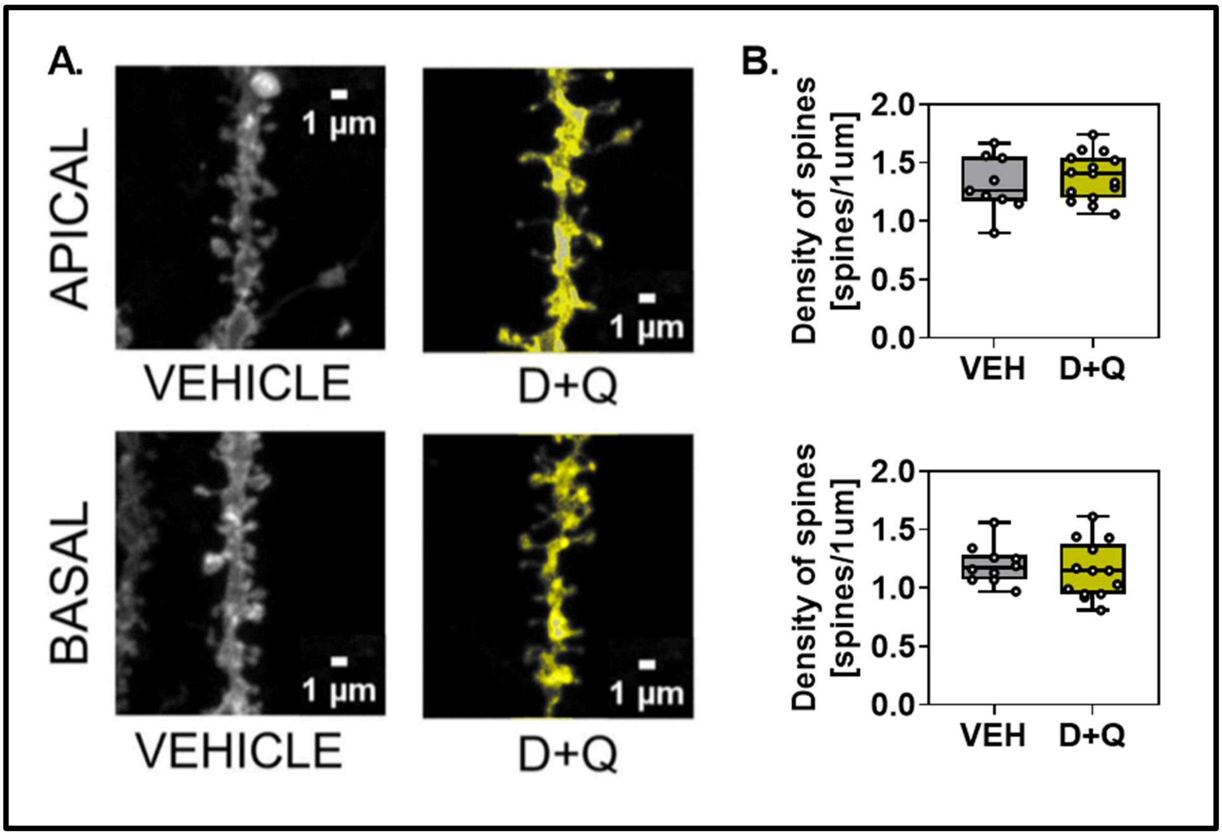

Figure 15. Spine density visualization and quantification following D+Q treatment. Representative images of DiI stained dendrites (A). Spine density were analysed in the CA1 region of the hippocampus (B). DiI stained hippocampal slices from aged vehicle D+Q treated rats were used for the analysis of synaptic plasticity. The analysis was done for two dendritic arbours of stratum pyramidale, namely basal and apical dendrites. Data expressed as box and whiskers showing min. and max. with all points relative to the vehicle group. n VEH = 3, n D+Q = 5 animals.

- Dendritic Spine Morphology

Whereas overall spine numbers remained static, the shape of individual dendritic spines on CA1 pyramidal neurons underwent a pronounced morphological change after senolytic treatment. Detailed semi-automated morphometry of >10 000 DiI-labelled spines using the SpineMagick pipeline revealed that structural plasticity was reinstated on an unchanged numerical scaffold.

Spine morphology was quantified in CA1 basal and apical dendrites using semi-automated analysis of DiI-labeled spines.

In 24-month-old vehicle controls the typical age profile of CA1 apical spines was evident: relatively short protrusions with stocky heads predominated. Eight weeks of intermittent D+Q shifted this profile towards more elongated, filopodium-like forms (Figure 16):

- mean spine length increased by ≈ 12 % ($p = 0.008$)
- the length-to-head-width ratio rose by ≈ 18 % ($p = 0.002$)
- circumference and projected area showed upward trends that did not reach classical significance (both $p < 0.08$)

No significant differences were detected in basal dendrites for length ($p=0.209$), length-to-width ratio ($p=0.453$), circumference ($p=0.364$), or area ($p=0.186$). Across groups, 4400 (VEH) and 6200 (D+Q) spines were analysed (n VEH=3, n D+Q=5 animals).

Because thin and stubby spines are defined by high aspect ratio and small head size, the metric changes above translate into a relative enrichment of thin + stubby spines at the expense of mature mushroom types.

In contrast to density**, D+Q altered apical spine morphology by increasing spine length and the length-to-head width ratio, with trends toward larger spine area/circumference.** These changes are consistent with a shift toward higher-aspect-ratio spine structures, which are often discussed as more dynamic elements of synaptic remodelling, and may therefore be compatible with improved learning performance. Notably, the effect was compartment-specific (apical but not basal dendrites), indicating that distinct dendritic domains may differ in their susceptibility to senolytic-associated remodelling.

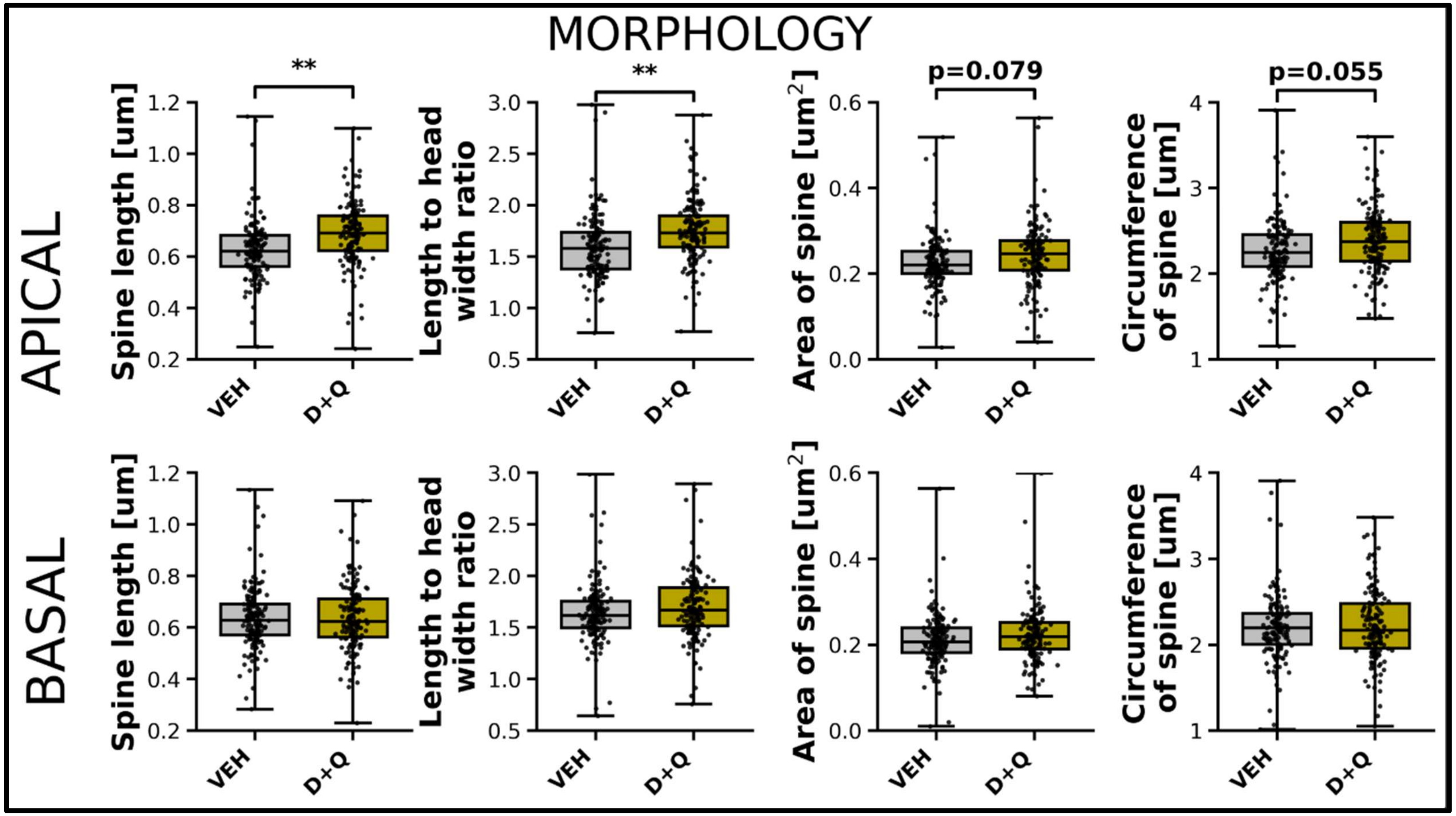

Figure 16. D+Q treatment changes synaptic plasticity in the apical dendrites of neurons of the CA1 region of the hippocampus. Different parameters of the dendritic spine shape (length, length to width ratio, area, circumference) were analysed in the CA1 region of the hippocampus. The analysis was done for two dendritic arbours of *stratum pyramidale*, namely basal and apical dendrites. Data expressed as box and whiskers showing min. and max. with all points.

n VEH = 3, n D+Q = 5 animals; * = $p < 0.05$,** = $p < 0.01$, *** = $p < 0.001$.

3.3 Hippocampal Chromatin Remodelling

● Histone Modifications

To probe whether the structural rescue described in the previous subsections was accompanied by an epigenetic readjustment that favours synaptic plasticity, we quantified two repressive histone marks, trimethyl-lysine 9 (H3K9me3) and trimethyl-lysine 27 (H3K27me3) on histone H3 from dorsal hippocampi. Both modifications are enriched in constitutive or facultative heterochromatin and rise in parallel with organismal and neuronal senescence, where they contribute to transcriptional silencing of plasticity-related loci.

The treatment with the senolytics Dasatinib and Quercetin (D+Q) produced a bidirectional re-equilibration of the two canonical repressive histone marks that drift during physiological ageing. H3K9me3, the hallmark of constitutive heterochromatin, fell markedly in hippocampal extracts from 24-month-old rats after eight weeks of treatment by approximately 50% compared with vehicle ($p < 0.001$, Figure 17.A), whereas the facultative mark H3K27me3 rose towards youthful values by approximately 20% ($p = 0.009$, Figure 17.B).

These reciprocal adjustments restore the epigenetic landscape to a configuration that favours inducible gene expression: a reduction in the densely packed H3K9me3 domains relieves constitutive repression, while replenishment of H3K27me3 sharpens promoter responsiveness by reinstating Polycomb-dependent control. Such a pattern mirrors the chromatin state associated with successful memory encoding and has been repeatedly linked to improved long-term potentiation in independent models of dietary restriction and pharmacological SUV39H1 inhibition. In the present study the same molecular signature co-occurred with the recovery of place-avoidance learning described earlier, suggesting a causal chain that runs from senescent-cell removal through histone remodelling to synaptic renewal.

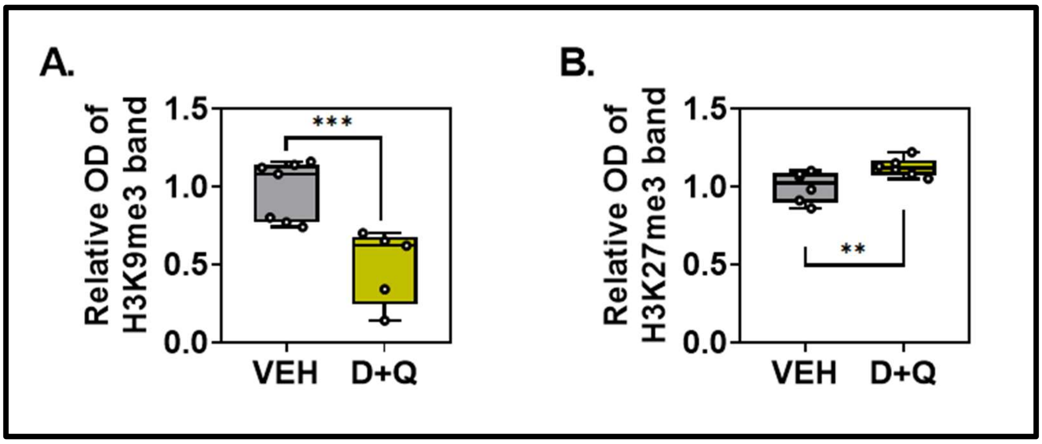

Figure 17. Changes in the levels of H3 histones in the hippocampus of aged rats after D+Q treatment.

Trimethylation of H3K9 (A) and H3K27 (B) relative to the whole H3 in the hippocampus of aged rats after D+Q or VEH treatment measured by Western blot. Data expressed as box and whiskers showing min. and max. with all points.
n VEH/DQ = 7, ** = $p < 0.01$, *** = $p < 0.001$.

- Chromatin Signature

To determine whether broader chromatin-scaffold proteins were influenced, nuclear fractions were probed for Lamin B1 and HMGB1 (Figure 18). **Neither Lamin B1/H3 nor HMGB1/H3 in hippocampal nuclear fractions differed between aged vehicle and aged D+Q groups** (both $p > 0.05$).

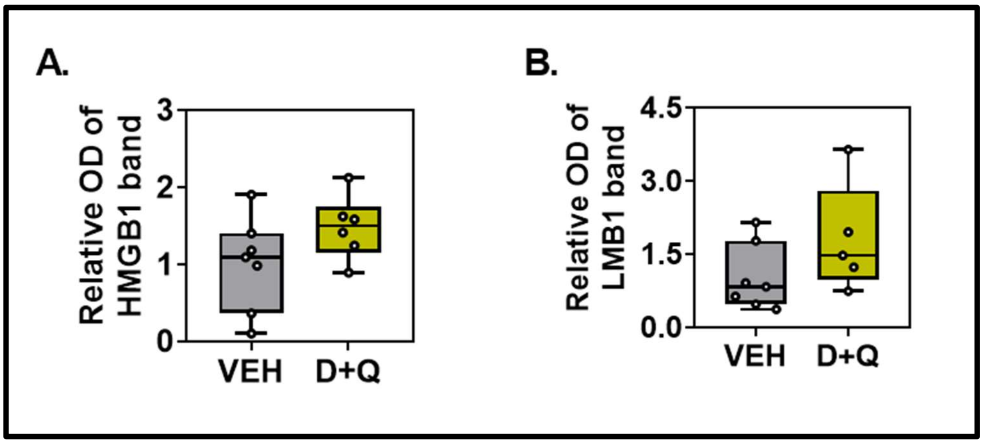

Figure 18. Changes in the levels of LMB1 and HMGB1 in the hippocampus of aged rats after D+Q treatment.

HMGB1 (A) and LMB1 (B) relative to the whole H3 in the hippocampus of aged rats after D+Q or VEH treatment measured by Western blot. Data expressed as box and whiskers showing min. and max. with all points.

3.4 Brain Senescent Cell Burden

Senescence-associated β-galactosidase (SA-β-gal) activity is a widely used histochemical marker of cellular senescence, reflecting increased lysosomal β-galactosidase activity detectable at suboptimal pH (typically pH 6.0). In brain tissue, SA-β-gal staining enables the visualization and quantification of senescent cell accumulation within specific regions, providing an estimate of senescent cell burden. Here, SA-β-gal–positive signal was used as a readout to compare the extent and distribution of senescence in the brain across experimental groups.

SA-β-gal staining and chromatin-related senescence did not differ between groups in CA1–CA3 slices (Figure 19) underscoring that functional benefits arose despite an undetectable shift in overt senescence burden.

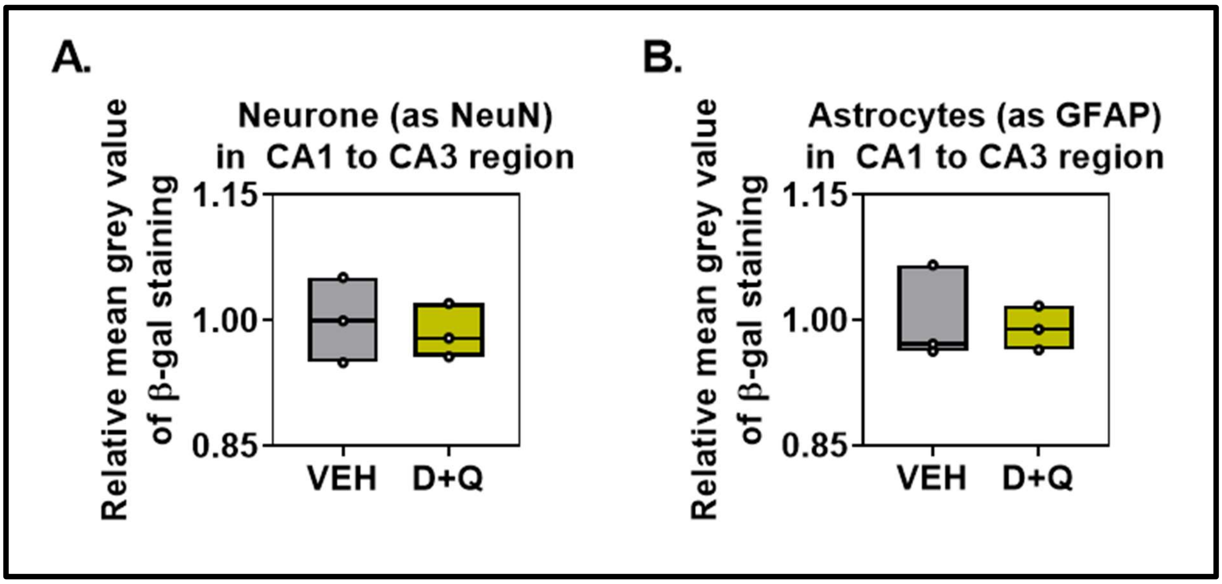

Figure 19. β-galactosidase expression in hippocampal CA1 to CA3 region of vehicle and D+Q treated rats.

A. Mean grey value of SA-β-galactosidase staining in the NeuN (A) and GFAP (B) region of interest from CA1 to CA3 hippocampal region. Data expressed as box and whiskers showing min. and max. with all points.

3.5 Gut Microbiota Composition after D+Q Treatment

Administration of the senolytic cocktail Dasatinib and Quercetin (D+Q) resulted in measurable shifts in the gut microbiota composition of aged Wistar rats, as revealed by 16S rRNA gene sequencing of faecal samples collected post-treatment.

- Taxonomic Composition

The analysis demonstrated both global and taxon-specific effects on the microbial ecology, suggesting that **D+Q exerts microbiota-modulating properties in addition to its known systemic senolytic effects** (Figure 20.A). Multivariate beta-diversity analysis using Bray-Curtis dissimilarity indices revealed a clear separation between the microbiota profiles of D+Q-treated and vehicle-treated aged rats ($p = 0.02$), indicating a treatment-driven restructuring of the microbial community architecture. This separation was visualized via principal coordinates analysis (PCoA) plots and supported by statistically significant PERMANOVA values (Figure 20.B).

While alpha diversity indices did not show significant shifts, **D+Q treatment induced notable changes at the genus and species levels.** Among the altered taxa, the most striking and consistent effect was the **significant increase in the relative abundance of *Lactobacillus acidophilus*** in the D+Q group compared to controls (Figure 20.D). This increase was statistically significant and biologically relevant, considering the well-documented roles of *L. acidophilus* in maintaining gut barrier function, regulating local and systemic immunity, and producing beneficial metabolites such as short-chain fatty acids.

In addition to *L. acidophilus*, D+Q-treated animals also showed increased levels of *Rikenellaceae RC9* and trends toward elevated abundance of *Clostridia vadin B60* and *Lachnospiraceae* members. These changes occurred alongside a reduction in potentially pro-inflammatory or dysbiosis-associated taxa, including certain *Lachnospiraceae* and *Muribaculaceae* species (Figure 20.D).

Interestingly, despite these compositional shifts, **the Firmicutes-to-Bacteroidetes (F/B) ratio, often regarded as a biomarker of gut health, remained stable between groups** (Figure 20.C), suggesting selective rather than broad-spectrum reshaping of the microbiota.

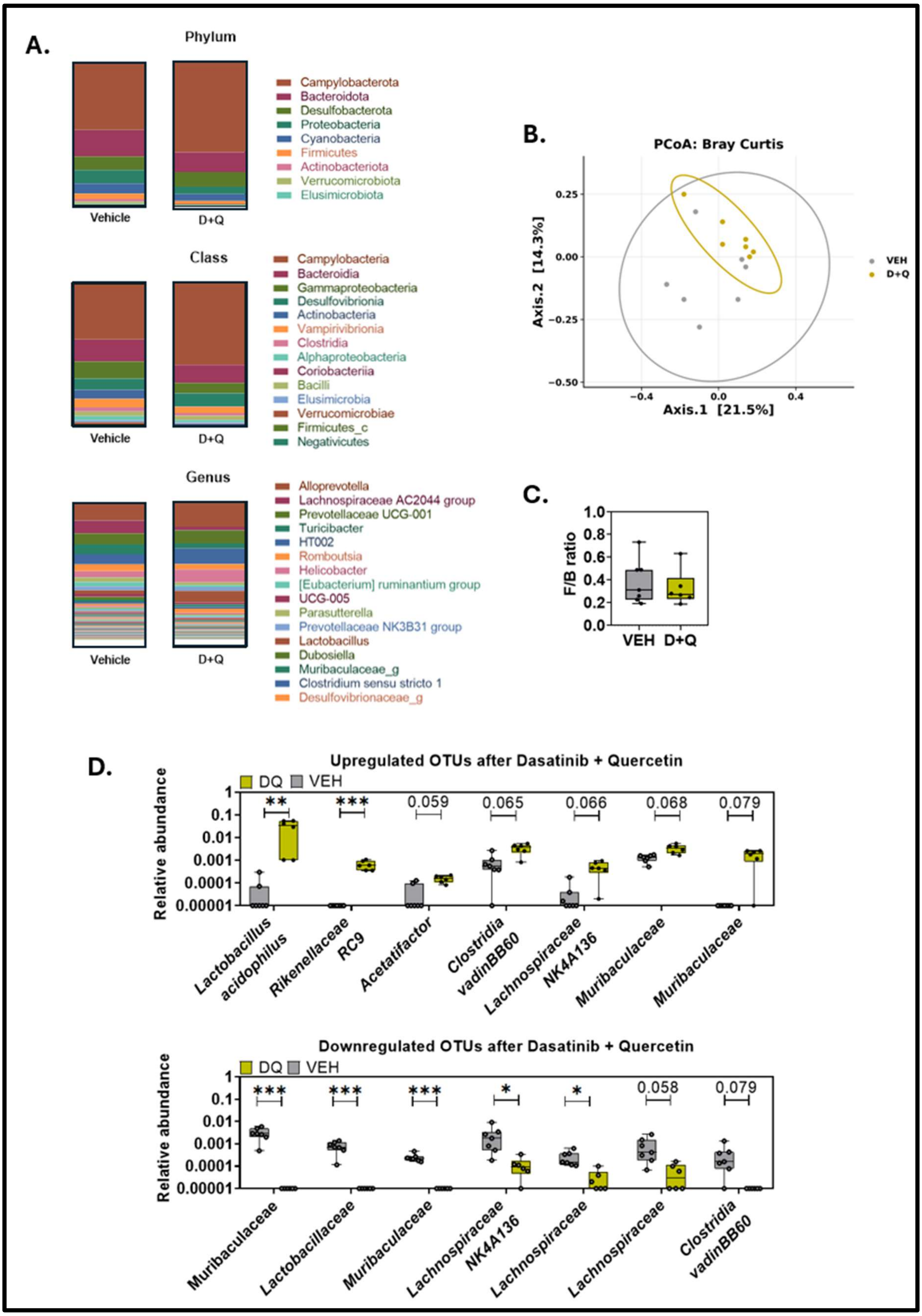
A.
Phylum
Campylobacterota
Bacteroidota
Desulfobacterota
Proteobacteria
Cyanobacteria
Firmicutes
Actinobacteriota
Verrucomicrobiota
Elusimicrobiota
Vehicle
D+Q
Class
Campylobacteria
Bacteroidia
Gammaproteobacteria
Desulfovibrionia
Actinobacteria
Vampirivibrionia
Clostridia
Alphaproteobacteria
Coriobacteriia
Bacilli
Elusimicrobia
Verrucomicrobiae
Firmicutes_c
Negativicutes
Vehicle
D+Q
Genus
Alloprevotella
Lachnospiraceae AC2044 group
Prevotellaceae UCG-001
Turicibacter
HT002
Romboutsia
Helicobacter
[Eubacterium] ruminantium group
UCG-005
Parasutterella
Prevotellaceae NK3B31 group
Lactobacillus
Dubosiella
Muribaculaceae_g
Clostridium sensu stricto 1
Desulfovibrionaceae_g
Vehicle
D+Q
B.
PCoA: Bray Curtis
Axis.2 [14.3%]
Axis.1 [21.5%]
0.25
0.00
−0.25
−0.50
−0.4
0.0
0.4
VEH
D+Q
C.
F/B ratio
1.0
0.8
0.6
0.4
0.2
0.0
VEH
D+Q
D.
Upregulated OTUs after Dasatinib + Quercetin
DQ
VEH
Relative abundance
1
0.1
0.01
0.001
0.0001
0.00001
**
***
0.059
0.065
0.066
0.068
0.079
Lactobacillus acidophilus
Rikenellaceae RC9
Acetatifactor
Clostridia vadinBB60
Lachnospiraceae NK4A136
Muribaculaceae
Muribaculaceae
Downregulated OTUs after Dasatinib + Quercetin
DQ
VEH
Relative abundance
1
0.1
0.01
0.001
0.0001
0.00001
***
***
***
*
*
0.058
0.079
Muribaculaceae
Lactobacillaceae
Muribaculaceae
Lachnospiraceae NK4A136
Lachnospiraceae
Lachnospiraceae
Clostridia vadinBB60

Figure 20. Gut microbiota composition in faeces samples of Wistar rats is altered after administration of D+Q.

A. Stacked bars showing the phylum, class and genus of bacterial communities from D+Q and vehicle-treated animals (A). Beta-diversity with Bray Curtis (B). Firmicutes/Bacteroidetes ratio between D+Q and vehicle treated animals (C). Main upregulated and downregulated operational taxonomic units (D).

Statistical significance represents absence of overlap between D+Q and vehicle bacterial species abundance. Data for F/B ratio and relative abundance of selected species shown as box and whiskers showing min. and max. with all points with p-value for statistical tendency was reported whereas statistical significance was noted as * ≤ 0.05; ** ≤ 0.01; *** ≤ 0.001.

- Predictive Functional Analysis

To assess whether the microbiota compositional changes observed following D+Q treatment translated into functional shifts in microbial metabolic capacity, a predictive metagenomic analysis was conducted. Using 16S rRNA amplicon data from fecal samples, the functional potential of the microbial community was inferred, which estimates Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway abundances based on known gene content of identified bacterial taxa.

The resulting dataset revealed a broad profile of microbial functions across all treatment groups; however, **no statistically significant differences in KEGG pathway abundance were detected between D+Q-treated and vehicle-treated animals** (Figure 21). Importantly, the PCA distribution reveals considerable within-group variance and overlapping confidence ellipses, suggesting high inter-individual variability that may have limited the statistical power to detect discrete functional pathway shifts despite observable compositional trends.

While 16S-based predictive functional analysis did not reveal statistically robust pathway-level changes, the compositional findings suggest that D+Q exerts a selective pressure on microbial communities that may influence host–microbe metabolic interactions.

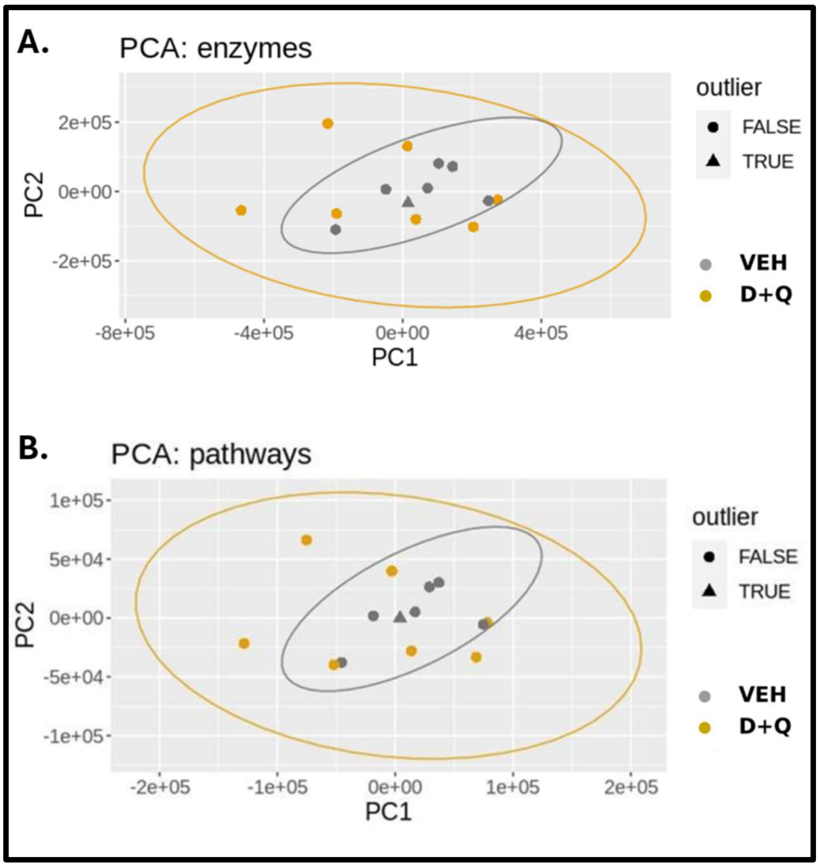

Figure 21. Predictive functional correlative analysis of D+Q treatment on microbial metabolism.

Principal component analysis (PCA) of the predicted abundances at the gene level (A) and at the level of biochemical pathways (B).

3.6 Gut-Derived Metabolite Profiles After D+Q treatment

The metabolomic profile of gut-derived compounds was significantly modulated following Dasatinib and Quercetin (D+Q) treatment in aged Wistar rats. Using GC-MS and UHPLC-HRMS analyses, faecal and serum concentrations of short-chain fatty acids (SCFAs) and bile acids (BAs) were quantified to assess changes in microbial metabolic output and systemic availability. These measurements provided insight into potential functional outcomes of the observed taxonomic shifts in the microbiota.

- Short-Chain Fatty Acid and Bile Acid Profil in Feces and Serum

The administration of D+Q senolytic treatment in aged Wistar rats significantly altered the profile of gut-derived metabolites, particularly SCFAs and BAs, in both faeces and systemic circulation.

GC-MS analysis of faecal samples revealed that **D+Q treatment induced a global reduction in the abundance of several SCFAs.** Specifically, a significant decrease was observed in the concentrations of acetic acid, propanoic acid, 2-methyl propanoic acid, pentanoic acid, 4-methyl pentanoic acid, and 3-methyl butanoic acid when compared to vehicle-treated animals (Figure 22.A). This widespread reduction in SCFAs suggests a decrease in microbial fermentation activity or altered substrate availability following senolytic intervention. Trends toward decreased levels of butanoic acid and 2-methyl butanoic acid were also noted, though they did not reach statistical significance. These findings may indicate a modulation of microbiota metabolic function, which could impact energy harvest and intestinal signalling.

Complementary UHPLC-HRMS profiling demonstrated that D**+Q treatment also significantly impacted faecal bile acid content.** Levels of several bile acid species, including hexanoic acid, taurocholic acid, and tauroursodeoxycholic acid, were significantly reduced in senolytic-treated animals (Figure 25.B). While other bile acids displayed trends, they did not meet significance thresholds. Given that bile acids play key roles in lipid absorption, gut motility, and signalling via FXR and TGR5 receptors, these alterations suggest a substantial shift in host–microbiota co-metabolism and enterohepatic circulation dynamics. The reduced excretion of bile acids could reflect improved reabsorption, diminished microbial transformation, or changes in hepatic synthesis.

To evaluate whether changes in the gut metabolite milieu translated into systemic shifts, serum SCFA concentrations were also measured. Interestingly, **the systemic footprint of faecal SCFA depletion was relatively modest.** Of the four major SCFAs analysed in serum (acetic, formic, butanoic, and propanoic acids), only formic acid exhibited a statistically significant reduction in the D+Q group compared to controls (Figure 22.C). The concentrations of acetic acid, the most dominant SCFA, along with butanoic and propanoic acids, remained unchanged. These results suggest that formic acid may serve as a sensitive systemic biomarker of microbial and metabolic modulation by senolytics, while other SCFAs are either buffered systemically or differentially metabolized and absorbed.

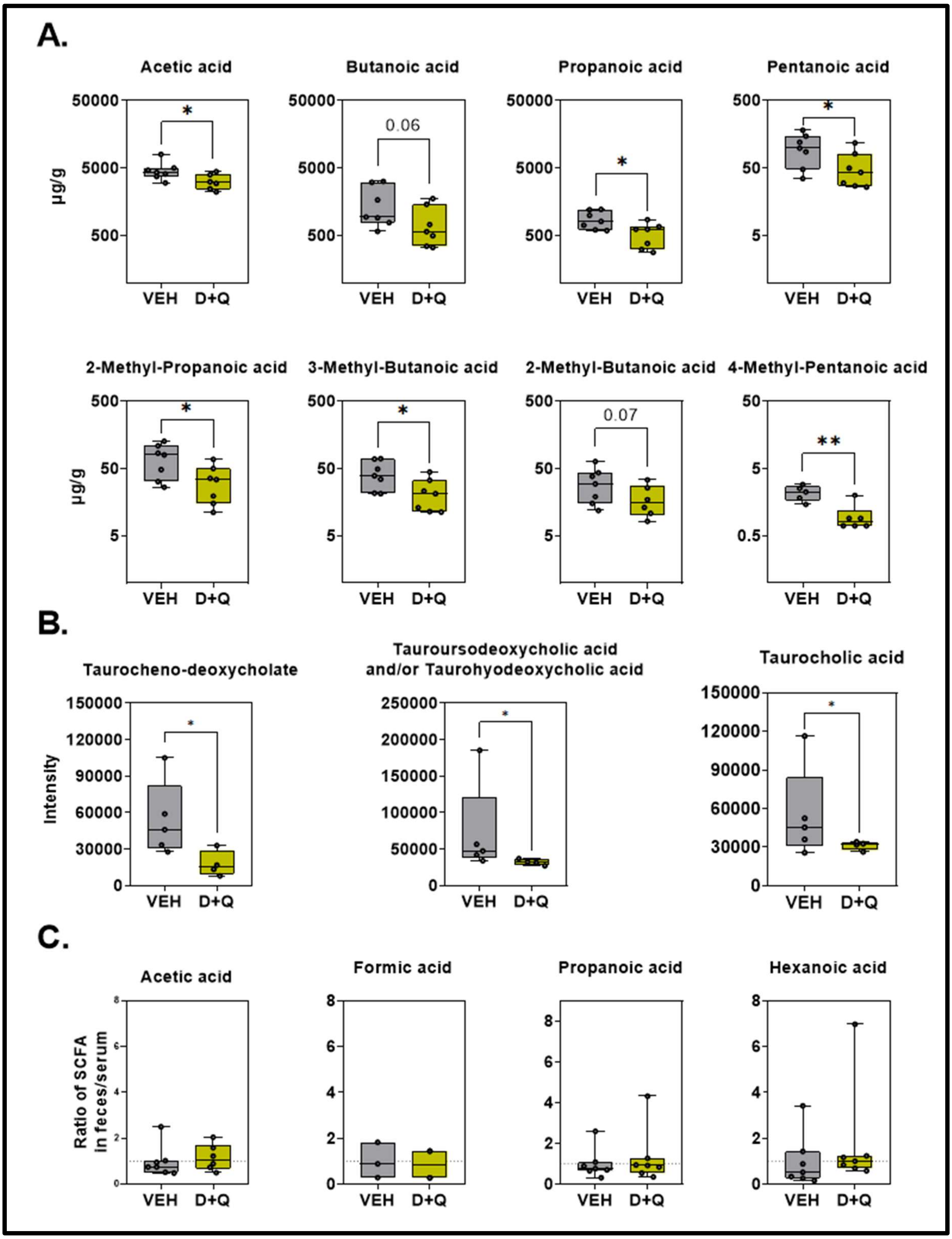

Figure 22. Short-chain fatty acids and bile acids profile in faeces and serum samples of Wistar rats after administration of D+Q.

Box and whiskers showing min. and max. with all points of the significantly different amount of short-chain fatty acids in faeces (A), bile acids in faeces (B) and short-chain fatty acids in serum (C). Statistical significance is reported as p-value, statistical tendency was reported whereas statistical significance was noted as * ≤ 0.05; ** ≤ 0.01.

- Microbiota-Metabolite Correlations

To investigate the functional consequences of D+Q-induced microbial remodelling, we explored the correlation patterns between the relative abundance of prevalent bacterial taxa and key microbial metabolites, SCFAs and BAs, in aged Wistar rats. This analysis aimed to bridge compositional microbial changes with their metabolic outputs, thereby identifying taxa that may act as functional keystone species within the gut ecosystem.

The analysis focused on amplicon sequence variants (ASVs) detected in at least 80% of the animals and reaching a minimum relative abundance of 1% in at least one sample, ensuring robustness and reproducibility of the results. Spearman’s rank correlation coefficients were calculated between these ASVs and the concentrations of individual SCFAs and BAs across all samples. Importantly, this exploratory analysis did not account for treatment group separation and was primarily designed to identify overarching associations, independent of senolytic exposure.

Despite the limited sample size and high dimensionality of the dataset, structured patterns of correlation were evident and visualized through hierarchical clustering heatmaps.

For SCFAs (Figure 23), three dominant bacterial clusters emerged: One group of ASVs demonstrated consistently negative correlations with most SCFAs (depicted in red), suggesting these bacteria may compete with or suppress SCFA-producing pathways. A second group showed minimal or no significant associations (blue), potentially indicating taxa unrelated to fermentative metabolism. The third group revealed positive correlations with the majority of SCFAs (green), implicating them as likely contributors to SCFA production or associated trophic networks.

This clustering was largely consistent across all SCFA species, indicating that **certain taxa might exert general influence on fermentative output rather than specific compound selectivity.** These findings reinforce the ecological view of SCFA production as a networked process involving microbial consortia rather than individual keystone producers.

For BAs (Figure 24), the correlation architecture was notably more complex. Five distinct clusters of bacteria emerged, each exhibiting unique and often metabolite-specific correlation patterns with subsets of bile acids. This modular structure hints at a **higher level of functional specialization, consistent with the known diversity of microbial bile salt hydrolases and BA-transforming enzymes.** Some taxa appeared broadly associated with primary bile acids, while others correlated with specific conjugated or secondary forms, suggesting diverse microbial roles in BA deconjugation, hydroxylation, or epimerization.

While these patterns did not yield statistically validated biomarker associations, they reveal a compelling framework for understanding how senolytic-sensitive microbial shifts may recalibrate metabolic outputs. The findings align with and enrich the compositional observations reported earlier, especially the rise of *Lactobacillus acidophilus*, a known SCFA modulator and BA transformer. Together, these exploratory analyses suggest that D+Q-induced changes in gut microbiota are accompanied by potentially meaningful alterations in metabolic function. Although larger cohorts and integrative multi-omics approaches will be required to validate these associations, the current results highlight the interconnectedness of microbial ecology and metabolite production as key components of the gut–brain axis in aging.

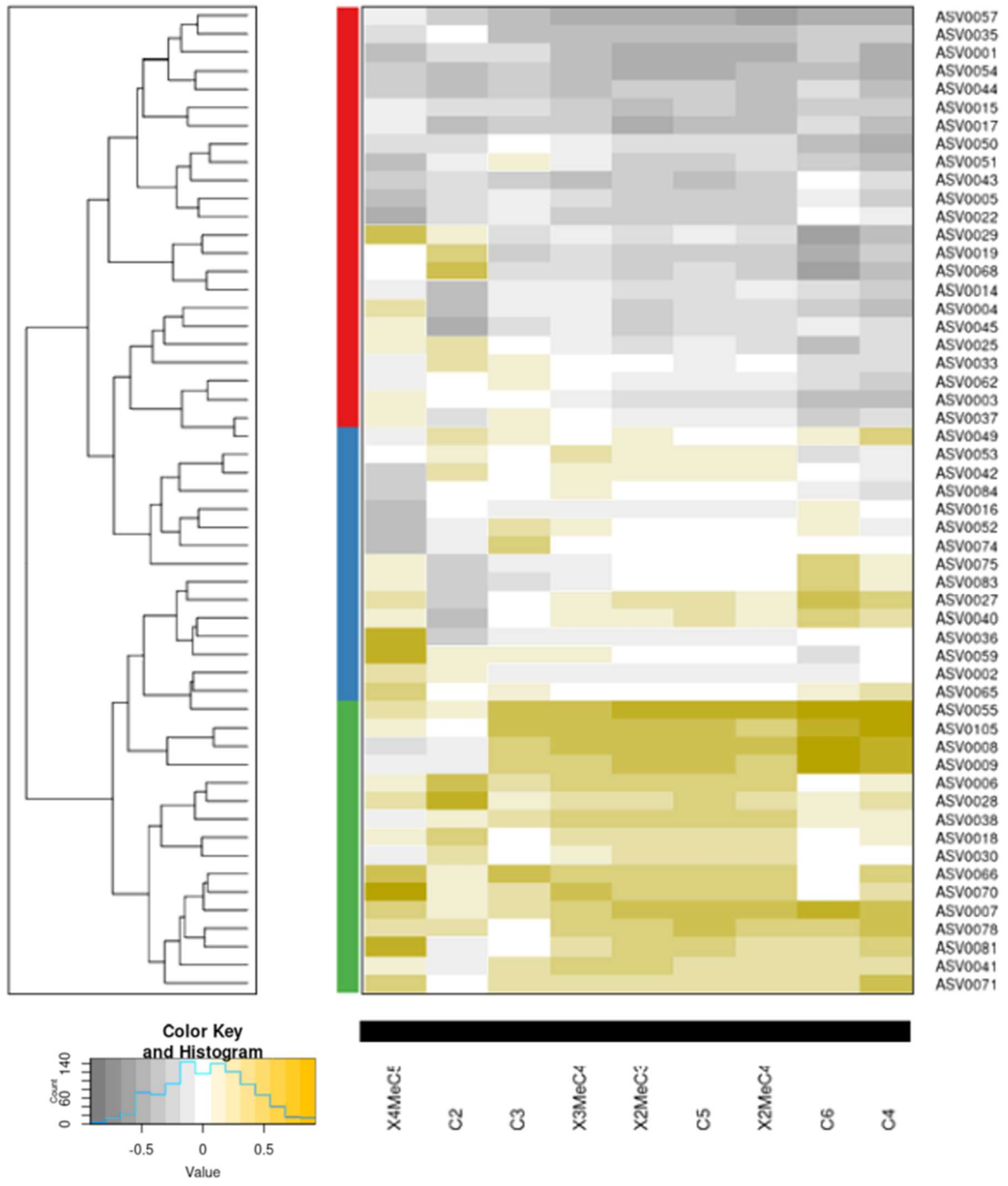

Figure 23. Correlation analysis between microbial communities and short-chain fatty acids.

A. Heatmap of Spearman correlation coefficients between short-chain fatty acids (SCFAs) and relative abundance of bacterial ASVs. Taxa and metabolites were clustered hierarchically, revealing three major bacterial clusters: one predominantly negatively correlated with SCFAs (red), one weakly correlated (blue), and one positively correlated (green). The heatmap illustrates general patterns of association rather than metabolite-specific responses. Heatmap of Spearman correlation coefficients between bile acids (BAs) and relative abundance of bacterial ASVs.

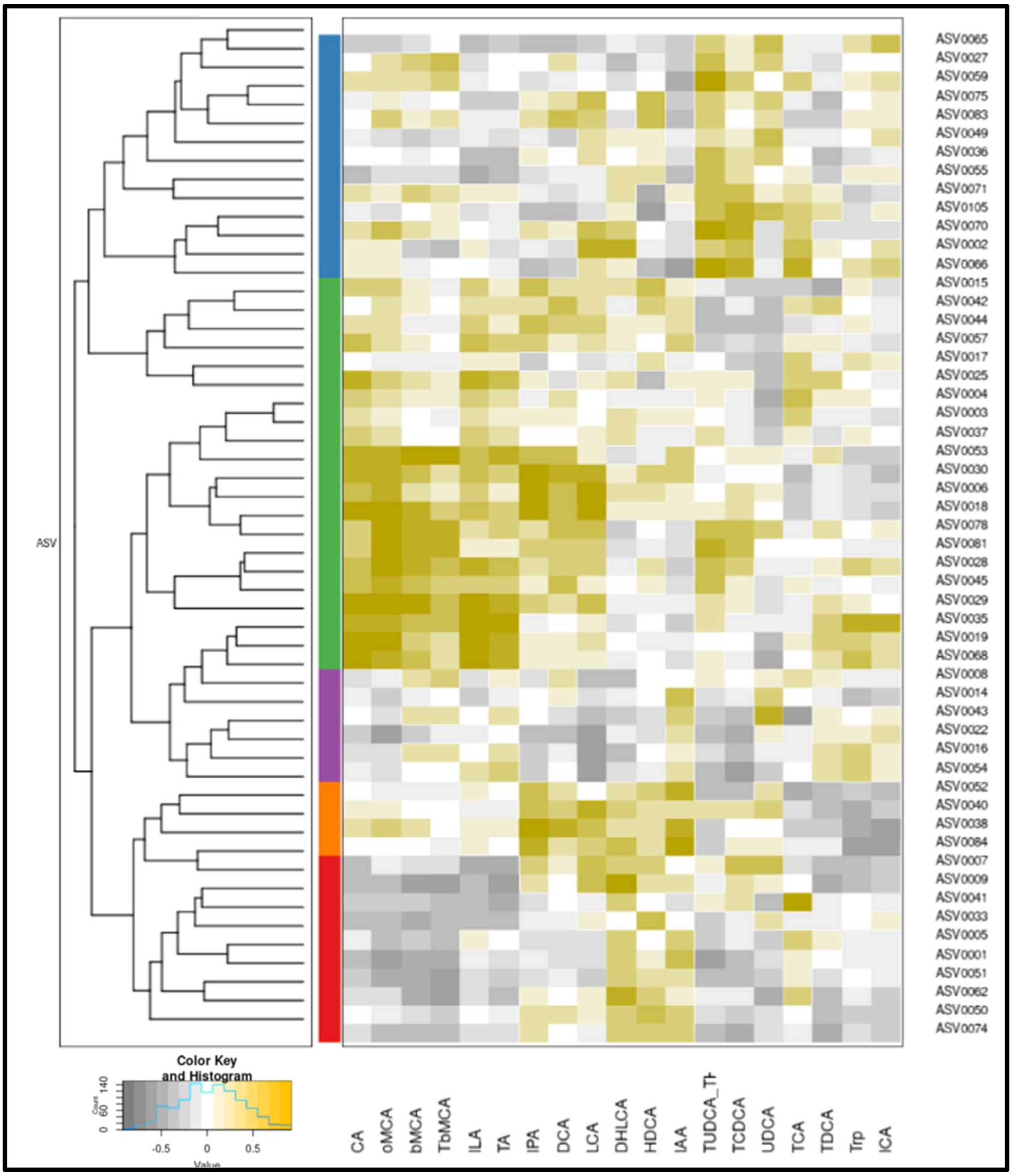

Figure 24. Correlation analysis between microbial communities and bile acids.
A. Heatmap of Spearman correlation coefficients between Bile acids (BAs) and relative abundance of bacterial ASVs. Hierarchical clustering identified five distinct bacterial clusters with differentiated correlation profiles across groups of BAs. This suggests more complex and potentially specialized interactions between gut microbiota and bile acid metabolism.

3.7 Gut Barrier Integrity and Inflammation

The impact of Dasatinib and Quercetin (D+Q) on gut barrier structure and function in aged Wistar rats revealed region-specific modulation of epithelial morphology, mucin production, tight junction expression, and inflammatory markers across six anatomical segments, duodenum, jejunum, ileum, caecum, proximal colon and distal colon, and then pooled into small- versus large-intestine composites for overview statistics,

- Regional Variation in Mucosal Thickness

Histological analysis of intestinal architecture following senolytic treatment with D+Q revealed distinct, region-specific alterations in mucosal thickness across the gastrointestinal tract of aged Wistar rats (Figure 25.C). These findings suggest that the treatment does not exert uniform effects along the gut but rather induces selective remodelling depending on the anatomical location.

Quantitative morphometric analysis of the small intestine (duodenum, jejunum, ileum) showed a statistically significant increase in mucosal thickness following D+Q treatment. In particular, the duodenal mucosa in D+Q-treated rats was markedly thicker than in vehicle controls (Figure 25.B), with mean values increasing from approximately 26.9 µm in controls to 39.8 µm in the treated group ($p = 0.0234$). Together, these findings indicate that D+Q promotes structural improvement in the small-intestinal mucosa. This morphological expansion may reflect enhanced epithelial renewal or barrier fortification, consistent with improved gut homeostasis following senescent cell clearance.

In contrast, the large intestine displayed a different pattern, **the colonic mucosa became significantly thinner compared with vehicle controls** (Figure 25.B). Mostly, the distal colon exhibited a tendency toward reduced mucosal thickness in D+Q-treated animals. Although the observed differences did not always reach statistical significance, the trend was consistent and most pronounced in the distal colon, where the mucosa thinned from an average of 25.8 µm in vehicle-treated animals to 9.5 µm in the treated group ($p = 0.0845$). These findings may reflect a reduced need for compensatory hyperplasia in an environment with lowered inflammatory or microbial burden, or they may indicate a shift in epithelial turnover dynamics specific to the large intestine's distinct microenvironment.

No gross pathological alterations such as ulceration, infiltration, or crypt atrophy were noted in any of the intestinal segments examined (Figure 25.A). These findings suggest that the observed changes in mucosal thickness are not indicative of tissue damage or inflammation but rather represent adaptive structural modifications potentially beneficial to barrier function.

The contrasting responses between the small and large intestines emphasize the compartmentalized effects of senolytic intervention along the gut axis. This regional specificity may be driven by inherent differences in epithelial turnover rates, microbial load, immune surveillance, or metabolic signalling within these segments. Importantly, the thickening observed in the upper small intestine aligns with increased mucin production, suggesting a broader remodelling process that includes both structural and molecular components.

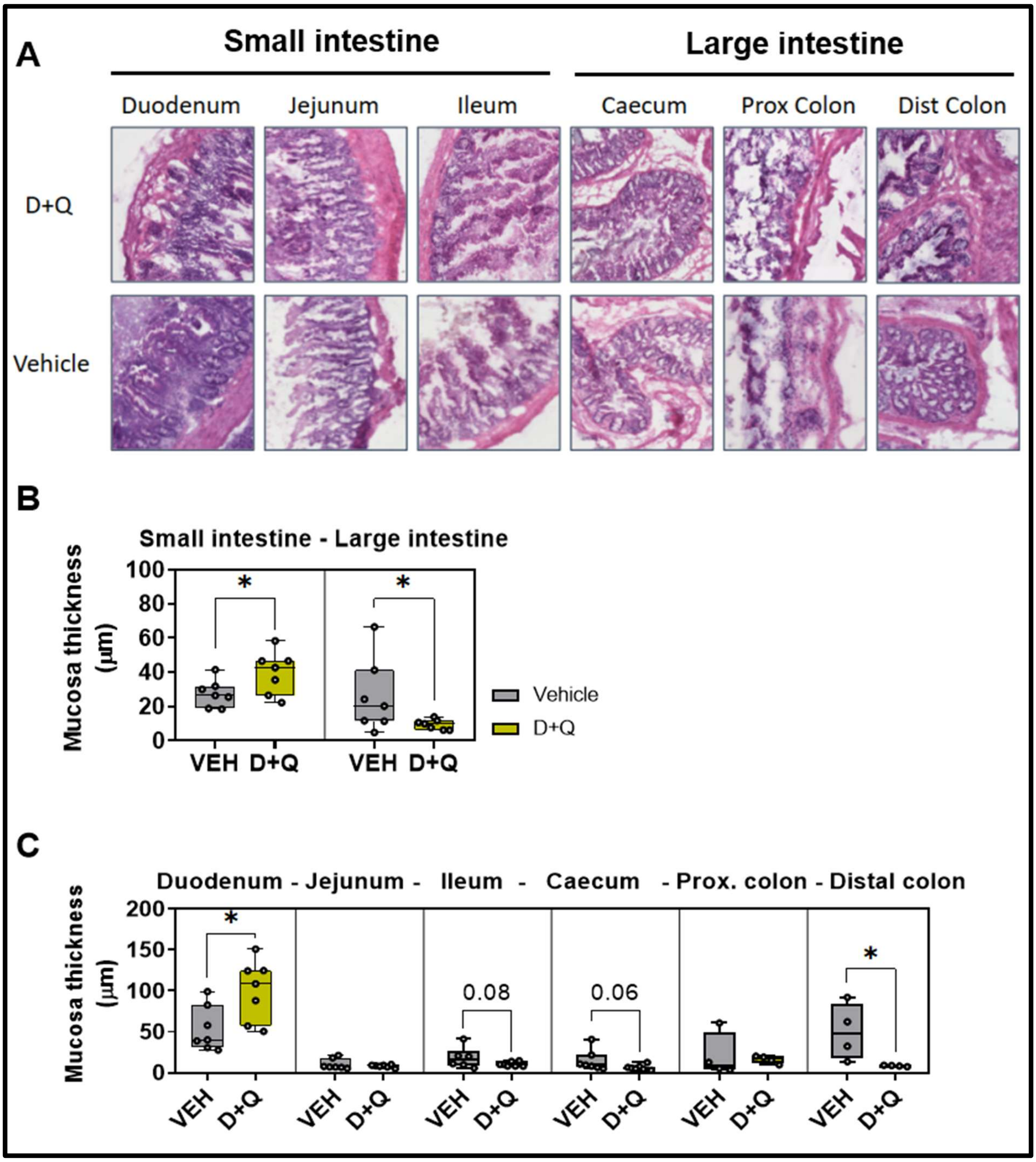

Figure 25. Histological assessment of the gastro-intestinal integrity after D+Q. Representative images of hematoxylin and eosin staining of intestinal, caecal and colonic tissue (A). Quantification of the mucosa thickness in the small and large intestine (B) and in the individual different parts of the gastrointestinal tract (C) in the form of boxes and whiskers showing min. and max. with all points of measured mucosal thickness. Statistical significance is reported as p-value, statistical significance was noted as * ≤ 0.05.

- Mucin-Related Features

A central component of the intestinal barrier is the mucus layer, primarily composed of mucins secreted by goblet cells. These glycoproteins play essential roles in maintaining luminal separation, preventing microbial translocation, and supporting immune homeostasis. In this study, mucin production was assessed through a combination of histological staining with Alcian blue and gene expression profiling of major mucin-encoding genes (*muc1* and *muc2*), offering both morphological and molecular perspectives on how D+Q intervention influences mucosal protection.

Alcian blue staining revealed that D+Q treatment modulates mucin production in a regionally distinct manner along the gastrointestinal tract.

Morphometric quantification using hue-saturation-brightness (HSB) thresholding of digital histology images demonstrated an elevation in acidic mucins along the small-intestinal epithelium after D+Q ($p = 0.06$), whereas the large-intestinal mucin content remained unchanged (Figure 26.B). In the ileum, a significant increase in Alcian blue-positive area was observed in D+Q-treated animals compared to vehicle controls (Figure 26.C), reflecting elevated mucin deposition in this distal part of the small intestine. In contrast, mucin content was significantly reduced in the proximal colon (Figure 26.C), suggesting divergent regulatory mechanisms operating in the upper versus lower gut. These data underscore the compartmentalized nature of the senolytic effect.

At the gene expression level, **the pooled small-intestine *muc1* was significantly upregulated following D+Q treatment** (Figure 27.A). This effect was strongest in the duodenum and ileum, where expression rose more than tenfold compared to vehicle controls ($p = 0.0134$). In contrast, *muc1* expression did not significantly vary in the large intestine. These transcriptional changes aligned closely with histological findings, reinforcing the reliability of the observed regional effects.

Interestingly, ***muc2* expression remained relatively stable across all segments** (Figure 27.A), suggesting that D+Q selectively modulates transmembrane mucins such as *muc1* rather than secretory mucins like *muc2*. Given the distinct functional roles of these mucin types, *muc1* being primarily involved in signalling and epithelial restitution, and *muc2* in forming the gel-like protective layer, these data point to a targeted remodelling of the mucus barrier toward a more regenerative and anti-inflammatory profile in the upper gut.

Taken together, these results indicate that **D+Q treatment enhances mucosal protection where the gut is more permeable and more immunologically active**, such as the small intestine. These region-specific changes likely reflect both local microbial environments and differential epithelial sensitivity to senolytic-induced changes in immune or metabolic tone.

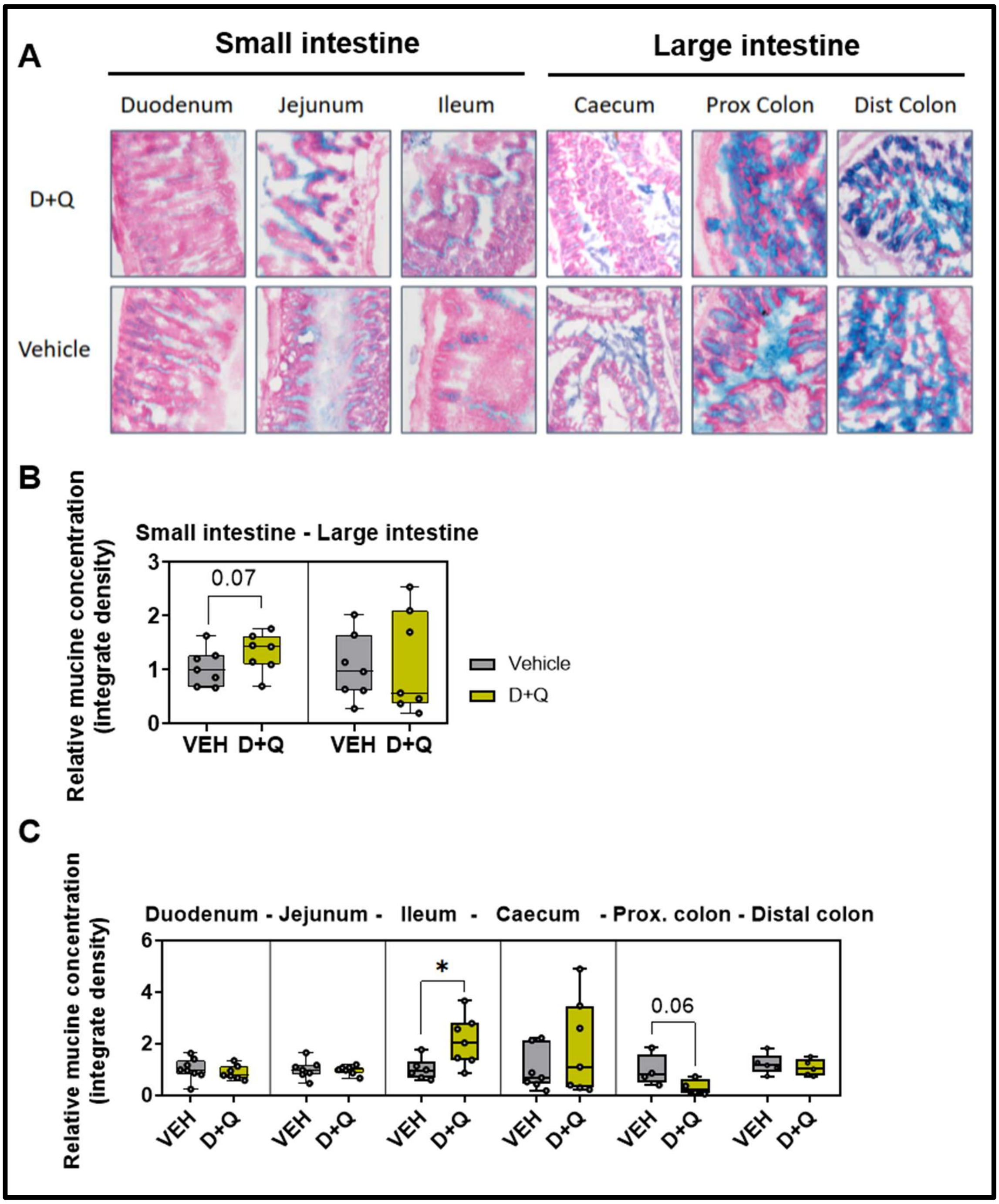

Figure 26. D+Q affects mucin production.
Representative images of Alcian blue staining of intestinal, caecal and colonic tissue for mucin production measurement (A). Quantification of the mucin production in the small and large intestine (B) and in the individual different parts of the gastrointestinal tract (C) in the form of boxes and whiskers showing min. and max. with all points of the integrated density of per tissue surface. Statistical significance is reported as p-value, statistical tendency was reported.

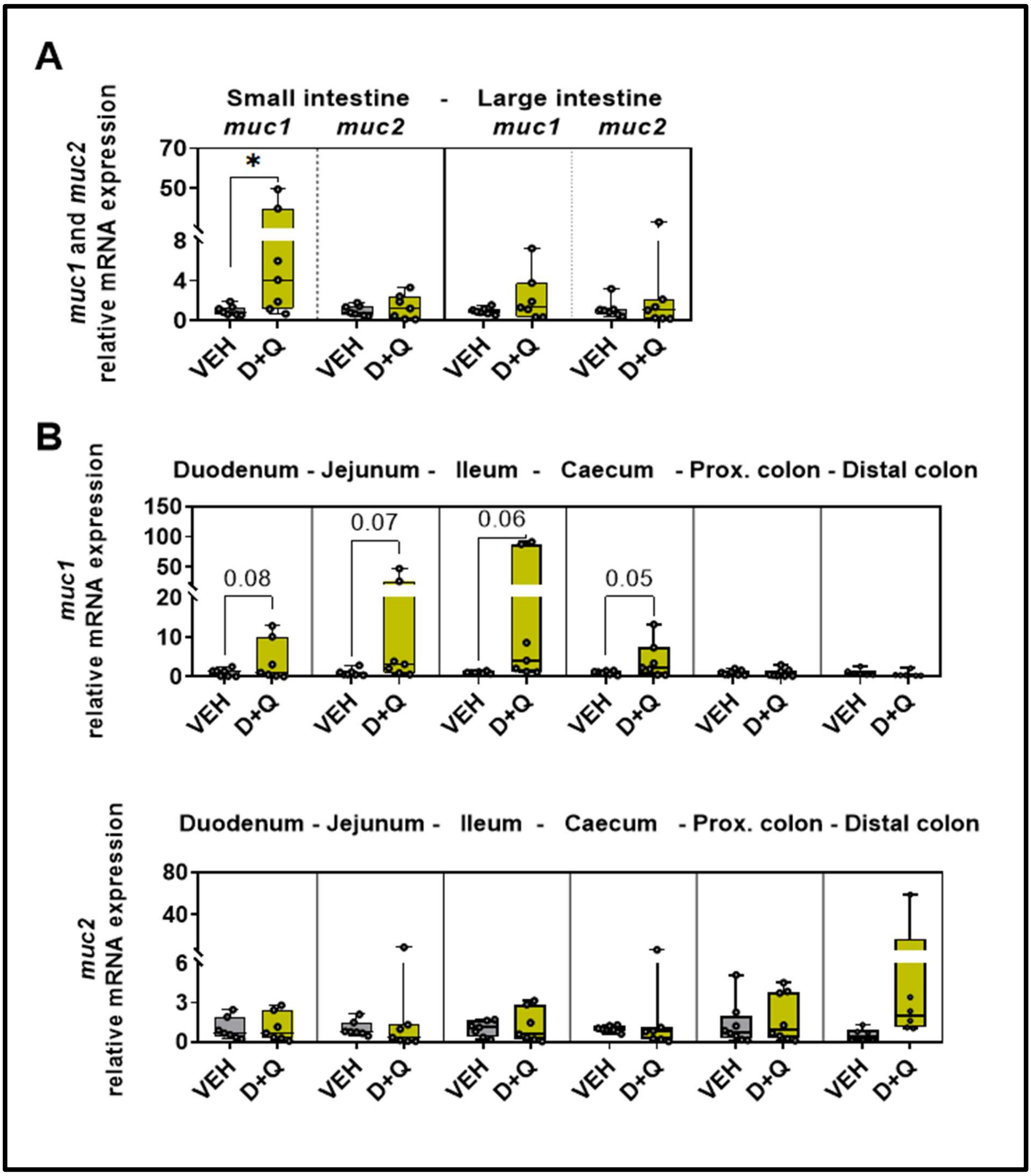

Figure 27. D+Q affects mucin production at transcriptional level.
mRNA expression of mucin markers *muc1* and *muc2* in the small and large intestine (A) and in the individual different parts of the gastrointestinal tract (B) in the form of boxes and whiskers showing min. and max. with all points of ddCt normalized to GAPDH and adjusted for the vehicle group to 1. Statistical significance is reported as p-value, statistical significance was noted as * ≤ 0.05.

- Intestinal Tight-Junction Markers

Senolytic intervention reshaped the molecular sealing apparatus of the aged intestinal epithelium in a region-selective rather than uniform fashion. Transcriptional (RT-qPCR) and protein (Western blot) analyses were all normalised to GAPDH and expressed relative to vehicle-matched aged controls.

All Western-blot bands were quantified from the same membranes, and qPCR melt curves confirmed single amplicons; nevertheless, only three TJ proteins were assayed, and measurements were made in bulk tissue, so cell-type specificity remains undetermined.

Across the three upper-gut segments **D+Q produced concerted upward trends in the scaffold proteins while sharply lowering the sealing claudin** (Figure 28):

- ZO-1 (*tjp1*): mean vehicle = trend towards increase (*p* = 0.06).
  Segment detail: duodenum and jejunum each showed borderline up-regulation (p = 0.07).
- Occludin (*ocln*): vehicle = trend towards increase (*p* = 0.05).
  Expression remained relatively stable at the level of individual segments, indicating a modest pan-intestinal rise rather than a focal spike.
- Claudin-1 (cldn1): robust decrease (p = 0.008).
  The drop was driven chiefly by the jejunum and ileum, where claudin-1 mRNA and protein both fell.

These shifts elevate the ZO-1/Occludin scaffold while loosening the claudin-1 stricture, consistent with a “selectively permeable yet resilient” barrier suited to nutrient absorption.

The colonic pattern was more heterogeneous and less coordinated than that observed in the small intestine:

- ZO-1: yielding no net change when pooled.
  When segregated, the proximal colon showed a mild downward trend (p = 0.07) while distal colon trended upward (p = 0.06).
- Occludin: largely unchanged.
  While the distal colon exhibited a non-significant tendency to fall (p = 0.08).
- Claudin-1: mRNA expression dropped significantly in the large intestine while protein abundance shown a downward trend (p = 0.06).
  The drop was mostly driven by the distal colon.

This pattern tightens the distal barrier against the densest microbial load while avoiding excessive rigidity proximally. Suggesting that, **D+Q re-balances without uniformly reinforcing the intestinal tight junction.**

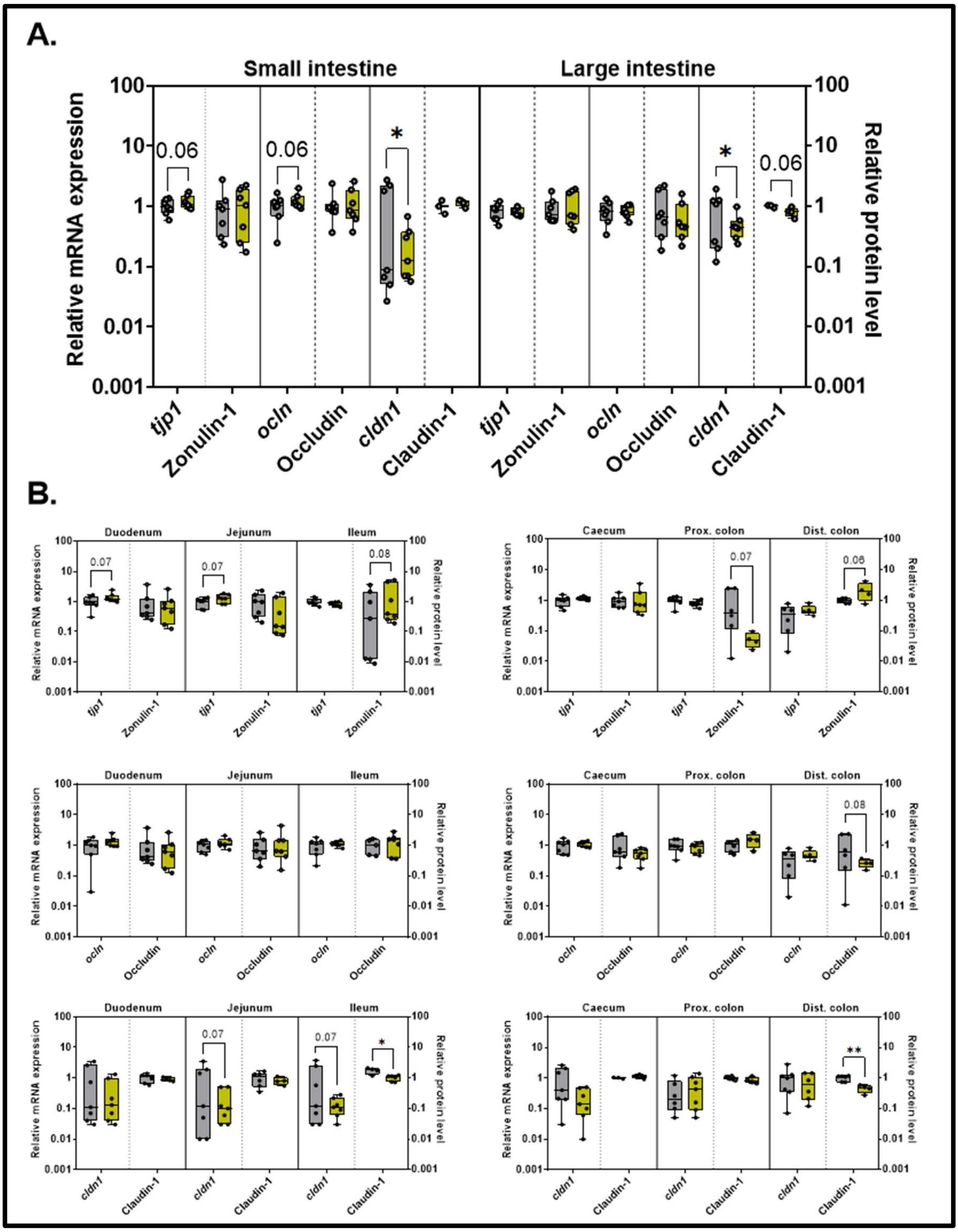

Figure 28. **Gene expression and protein quantification of tight-junction markers in the gastrointestinal tract after treatment with D+Q.**

mRNA expression and protein levels for *tjp1*/ZO-1, *ocln*/Occludin and *cldn*/Claudin-1 small and large intestine (A) or individual segments of the gastrointestinal tract (B) in the form of boxes and whiskers showing min. and max. with all points of ddCt normalized to GAPDH and adjusted for the vehicle group to 1. Statistical significance is reported as p-value, statistical tendency was reported whereas statistical significance was noted as * ≤ 0.05; **≤ 0.01.

- Intestinal Inflammation Markers

Senolytic intervention reshaped the inflammatory tone of the aged intestinal wall in a region-selective rather than uniform manner. Transcriptional and protein analyses of TNF-α, IFN-γ and IL-10 were normalised to GAPDH and expressed relative to vehicle-matched aged controls.

Across the three upper-gut segments, D+Q shifted the inflammatory balance away from a pro-inflammatory profile and toward a more regulatory one (Figures 34–35):

- TNF-α (tnfa): significant decrease in pooled small-intestinal mRNA (p = 0.009) but not at the protein level.
  Transcript levels trended downward in the jejunum and ileum, although the ileum displayed a borderline reduction at protein level (p = 0.06).
- IFN-γ (ifng): no clear change when the small intestine was pooled.
  The duodenum showed significant mRNA downregulation, and ileal protein showed a near-significant decrease (p = 0.06), indicating that the effect was focal rather than pan-intestinal.
- IL-10 (il10): significant increase in pooled small-intestinal mRNA (p = 0.045) but not at the protein level.
  The rise was driven chiefly by the ileum, with an additional upward trend in the duodenum (p = 0.09).

Together, these changes indicate **that D+Q dampens pro-inflammatory signalling in the small intestine primarily at the transcriptional level, while simultaneously enhancing anti-inflammatory tone through IL-10 induction**. The small-bowel response therefore appears coordinated but not uniform, with the ileum emerging as the clearest site of combined TNF-α reduction and IL-10 increase.

The large intestine displayed a more heterogeneous pattern than the small bowel:

- TNF-α: protein abundance was significantly reduce.
  The strongest decreases were seen in the lower-gut compartments, with a clear reduction in the distal colon and an additional downward shift in adjacent colonic/caecal tissue.
- IFN-γ: no robust pooled large-intestinal effect was evident.
  However, the distal colon showed a significant fall in protein abundance, marking it as the principal site of IFN-γ suppression.
- IL-10: no clear net change was observed when the large intestine was pooled. When split by segment, proximal colon il10 mRNA showed an upward trend (p = 0.06), but this was not paralleled by a significant protein-level increase.

This pattern suggests that **D+Q does not uniformly silence intestinal inflammation but rather redistributes inflammatory control along the gut axis**: in the small intestine the dominant effect is transcript-level suppression of TNF-α coupled to increased IL-10, whereas in the large intestine the clearest signal is a reduction of pro-inflammatory proteins, particularly in the distal colon. Such compartment-specific immune recalibration aligns with the broader region-dependent remodelling seen in mucosal thickness, mucin dynamics and tight-junction expression.

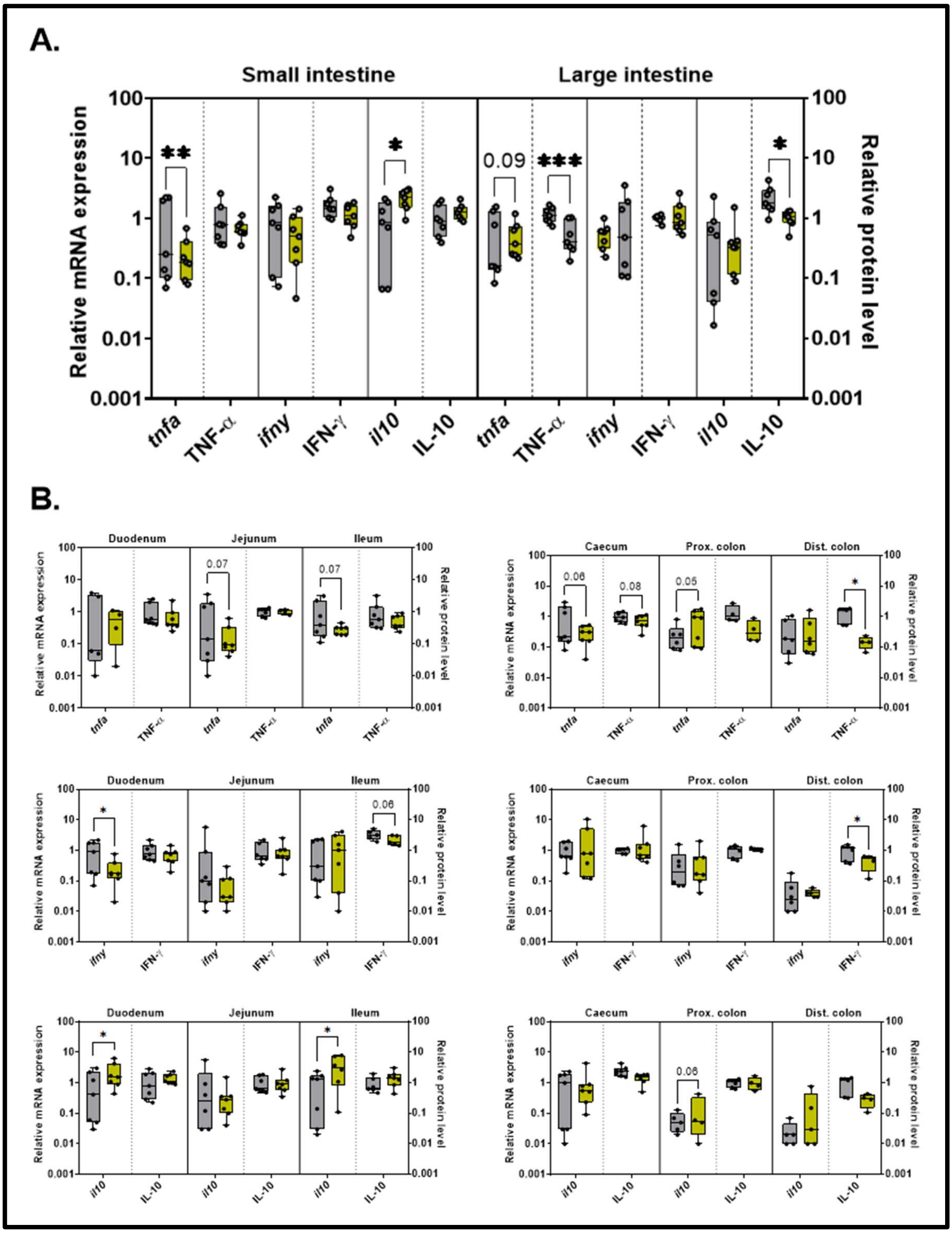

Figure 29. Gene expression and protein quantification of inflammatory markers in the gastro-intestinal tract after treatment with D+Q.

mRNA expression and protein levels for *tnfa*/TNF-α, *ifny*/IFN-γ and *il10*/IL-10 small and large intestine (A) or individual segments of the gastrointestinal tract (B) in the form of boxes and whiskers showing min. and max. with all points of ddCt normalized to GAPDH and adjusted for the vehicle group to 1. Statistical significance is reported as p-value, statistical tendency was reported whereas statistical significance was noted as * ≤ 0.05; **≤ 0.01.

3.8 Peripheral Cytokine and Chemokine Profile

Aging is commonly associated with low-grade chronic inflammation in the gut, often referred to as “inflammaging,” which contributes to epithelial barrier dysfunction, microbial imbalance, and increased susceptibility to disease. Terminal arterial serum was analysed in duplicate using a 32-plex Luminex assay (Millipore) covering the main inflammatory, regulatory and growth-factor mediators, ranging from TNF-α, the IL-1 family and IL-6 to monocyte-recruiting chemokines (MCP-1/CCL2, MIP-1α/CCL3) and the counter-regulatory cytokine IL-10. Raw concentrations were $\log_2$-transformed and normalised to the young-vehicle average to facilitate cross-analyte comparison (Figure 30).

Quantification of these thirty-two soluble mediators in terminal serum samples revealed a bifurcated pattern that neatly segregated pro-inflammatory SASP components from canonical counter-regulators. In agreement with the concept of inflammaging, vehicle-treated old rats exhibited a broad elevation of the former group when compared with young adults: IL-6 registered the largest gain (≈3.4-fold), followed by TNF-α (2.6-fold), IL-1α (2.3-fold) and IL-1β (1.9-fold). Chemokines MCP-1 and MIP-1α rose by ~2-fold, whereas the anti-inflammatory sentinel IL-10 fell to roughly 60 % of the youthful baseline. Analysis segregated aged-vehicle animals into a discrete cluster driven primarily by TNF-α, IL-6 and MCP-1 loadings, underscoring the integrated nature of inflammageing.

Eight weeks of intermittent Dasatinib and Quercetin (D+Q) treatment remodelled the serum secretome. Aged animals display strong upregulation of several pro-inflammatory cytokines (e.g., IL-1α, IL-6, IL-17A, IFNγ, TNFα) compared to young animals. **D+Q treatment attenuates many of these responses, with significant reductions noted for IL-1α, IL-6, IFNγ, and IL-17A, while promoting EGF expression.** These shifts indicate that senolytic treatment blunts the circulating SASP while simultaneously amplifying anti-inflammatory counter-signals. The net contraction of pro-/anti-inflammatory ratios provide a quantitative explanation for the reduced peripheral inflammatory burden reported elsewhere in the study and establishes a plausible molecular link to the cognitive and barrier improvements observed in the same cohort.

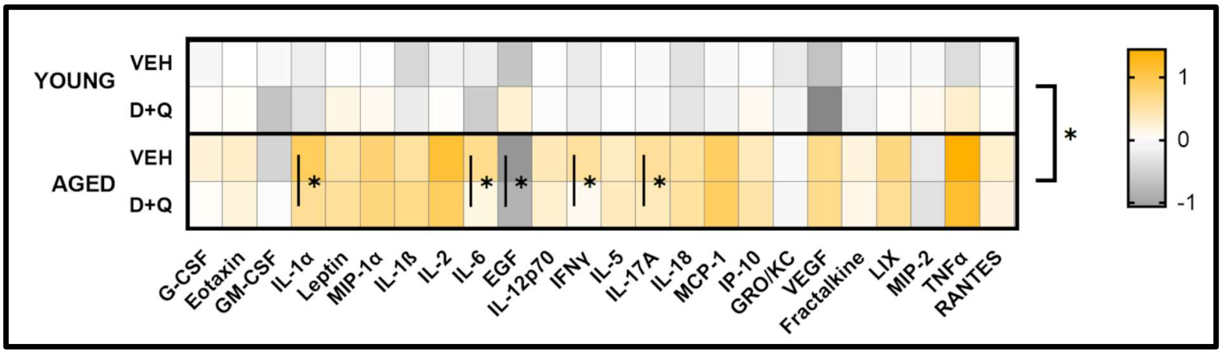

Figure 30. D+Q treatment reduces peripheral inflammation in aged rats.
The heat map depicts cytokines and growth factor levels in blood serum collected after the final behavioural test from young and aged (6-month-old and 25-month-old respectively at the time of collection) rats treated with D+Q or vehicle (VEH). The data were analysed using two-way ANOVA; n = 7–8, * = $p < 0.05$.

3.9 Blood-Brain Barrier Markers and Neuroinflammation

Brain tissue was collected from the frontal cortex and cerebellum for molecular assessment of BBB-associated tight junction proteins and inflammatory markers. These regions were selected based on tissue availability and to sample anatomically and functionally distinct brain areas. This approach enabled evaluation of whether Dasatinib and Quercetin (D+Q) effects on BBB integrity and neuroinflammatory tone were region-dependent or consistent with a broader brain response.

● Brain Tight-Junction Markers

The structural integrity of the blood-brain barrier (BBB) plays a critical role in maintaining central nervous system (CNS) homeostasis by regulating molecular transport, restricting immune cell infiltration, and preventing neurotoxic insult. Age-associated compromise of the BBB has been linked to increased neuroinflammation, impaired neuronal function, and cognitive decline. To assess whether senolytic treatment with Dasatinib and Quercetin (D+Q) could restore BBB function, we evaluated the expression of key tight junction proteins, Zonulin 1 (ZO-1), Occludin, and Claudin-1, in brain tissues (frontal cortex and cerebellum) of aged Wistar rats (Figure 31).

At the transcriptional level, D+Q induced only limited changes across the brain regions examined. mRNA expression of *tjp1*, *ocln* and *cldn1* remained broadly stable when the frontal cortex and cerebellum were considered separately, although pooled brain analysis indicated a significant increase in *cldn1* mRNA. By contrast, the protein-level response was clearer and regionally differentiated.

In the frontal cortex, D+Q shifted the tight-junction profile toward a reinforced barrier-associated signature:

- ZO-1 (tjp1): protein abundance increased significantly following D+Q treatment while level of mRNA remains stable.
- Occludin (ocln): similarly, the protein abundance also increased but not at the transcriptional level.
- Claudin-1 (cldn1): protein expression elevated in the frontal cortex.

Together, these findings indicate that **the frontal cortex displayed the most coordinated tight-junction response to D+Q, with concurrent elevation of the two major scaffold/sealing-associated proteins and partial restoration of Claudin-1.** This profile is consistent with strengthening BBB-associated molecular architecture in a region closely linked to higher-order cognitive processing.

The cerebellum displayed a related but more selective pattern:

- ZO-1: showed a tendency toward increased protein abundance (p = 0.06).
- Occludin: increased significantly and represented the clearest cerebellar response to treatment.
- Claudin-1: remained essentially unchanged, indicating that the cerebellar response did not fully mirror the cortical pattern.

This distribution suggests that **D+Q does not uniformly upregulate all BBB-associated junctional proteins across the brain**. Rather, it preferentially enhances ZO-1/Occludin-enriched tight-junction organization, with additional Claudin-1 reinforcement confined mainly to the frontal cortex. When considered together with the pooled brain analysis showing higher ZO-1 and Occludin protein levels after D+Q, the overall pattern supports a broad improvement in BBB-associated protein architecture with superimposed regional specificity.

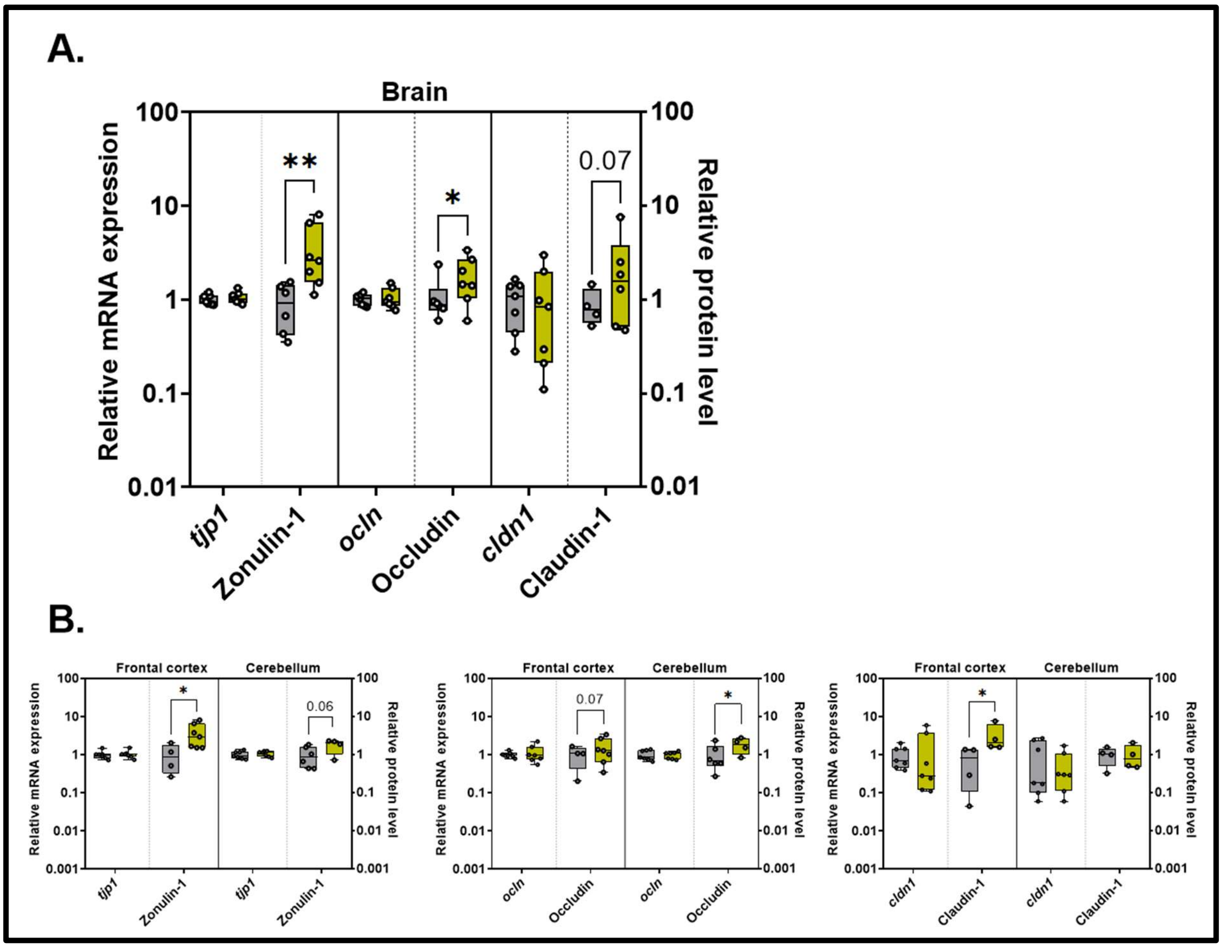

Figure 31. **Gene expression and protein quantification of tight-junction markers in the brain after treatment with D+Q.**

mRNA expression and protein levels for *tjp1*/ZO-1, *ocln*/Occludin and *cldn*/Claudin-1 in the brain (A) or in the frontal cortex and the cerebellum (B) in the form of boxes and whiskers showing min. and max. with all points of ddCt normalized to GAPDH and adjusted for the vehicle group to 1. Statistical significance is reported as p-value, statistical tendency was reported whereas statistical significance was noted as * ≤ 0.05; **≤ 0.01.

- Brain Inflammatory Markers

Neuroinflammation, often exacerbated by aging and barrier dysfunction, is a critical contributor to cognitive decline and neurodegenerative diseases. To evaluate whether D+Q intervention could mitigate neuroinflammation, we measured both mRNA expression and protein quantification of key inflammatory cytokines, tumour necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ), and interleukin-10 (IL-10) in the same tissues as for the tight-junction proteins.

Across the examined brain tissues, D+Q did not produce a broad suppression of classical pro-inflammatory cytokines at the protein level but instead shifted the balance toward a more anti-inflammatory signature dominated by IL-10. At the mRNA level, changes were modest overall: tnfa and ifny showed only limited variation, whereas il10 remained broadly stable. In the pooled brain analysis, *tnfa* mRNA was reduced overall while *ifny* tended to increase, indicating that transcriptional effects were subtle and not uniformly reflected across individual regions.

In the frontal cortex, D+Q produced the clearest anti-inflammatory response (Figures 32):

- TNF-α (tnfa): no robust protein-level change was detected, despite modest variation at the transcript level.
- IFN-γ (ifny): remained largely unchanged at the protein level, indicating no strong local suppression of this pro-inflammatory pathway.
- IL-10 (il10): showed the most prominent response, with a significant increase in protein abundance in D+Q-treated animals, while mRNA remained relatively stable.

Together, these findings indicate that **the frontal cortex did not undergo a generalized cytokine shutdown, but rather a selective reinforcement of anti-inflammatory signalling.** The rise in IL-10, in the absence of major parallel changes in TNF-α or IFN-γ protein, suggests that D+Q promotes immune recalibration more through enhancement of endogenous regulatory tone than through broad repression of pro-inflammatory mediators.

The cerebellum displayed a more muted pattern:

- TNF-α: showed no clear net protein-level change.
- IFN-γ: likewise remained largely stable, with no robust evidence of regional suppression.
- IL-10: did not exhibit the marked increase seen in the frontal cortex, and overall changes in this region remained marginal.

This distribution suggests that **D+Q does not uniformly suppress neuroinflammation across the brain.** Rather, its central effect appears to be regionally biased, with the frontal cortex showing a more evident anti-inflammatory shift and the cerebellum showing comparatively limited cytokine remodeling. When brain tissues were considered together, TNF-α and IFN-γ proteins remained essentially unchanged, whereas IL-10 protein increased, supporting the view that the dominant CNS response to D+Q is anti-inflammatory reinforcement rather than broad inhibition of pro-inflammatory output.

These findings are consistent with parallel observations of improved barrier function and tight junction protein level in the same regions, and they support the hypothesis that **D+Q's neuroprotective effects are mediated not solely by direct senescent cell elimination, but also by downstream modulation of neuroimmune balance.**

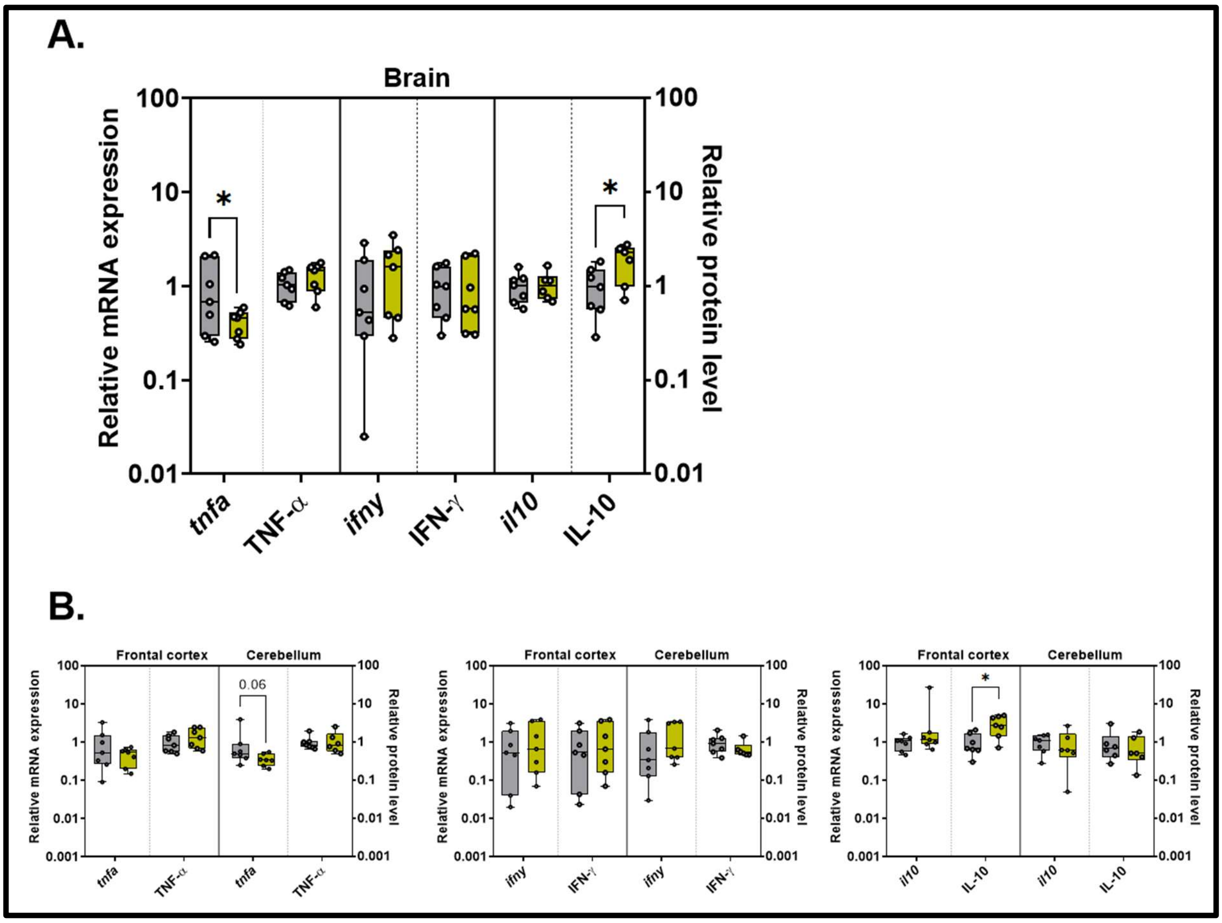

Figure 32. **Gene expression and protein quantification of inflammatory markers in the brain after treatment with D+Q.**

mRNA expression and protein levels for *tnfa*/ TNFα, *ifnγ*/ IFNγ and *il10*/ IL10 in the brain (A) or in the frontal cortex and the cerebellum (B) in the form of boxes and whiskers showing min. and max. with all points of ddCt normalized to GAPDH and adjusted for the vehicle group to 1. Statistical significance is reported as p-value, statistical tendency was reported whereas statistical significance was noted as * ≤ 0.05; **≤ 0.01.

- Short-Chain Fatty Acid Receptor Expression

Short-chain fatty acids (SCFAs), produced through microbial fermentation of dietary fibres in the gut, are key signalling molecules within the gut-brain axis. Their biological activity is mediated in part by specific G-protein coupled receptors, GPR41 (FFAR3) and GPR43 (FFAR2), which are expressed in both peripheral and central tissues. These receptors are implicated in immune modulation, barrier maintenance, and neuronal function. To determine whether senolytic treatment with D+Q alters SCFA receptor-mediated signalling in the brain, the expression of GPR41 and GPR43 was assessed in cerebral tissues using quantitative PCR (Figure 33).

Analysis revealed a **significant downregulation of GPR43 mRNA expression in the brain of D+Q-treated animals, particularly in the cerebellum.** This could be indicating a robust and widespread suppression of this receptor following senolytic intervention.

In contrast, **GPR41 expression showed a modest but consistent trend toward upregulation in the brain of D+Q-treated animals.** Although the increase did not reach significance in individual regions, pooled analysis of all brain samples revealed a near-significant trend ($p = 0.088$). This differential pattern suggests a shift in the balance of SCFA signalling pathways within the central nervous system, possibly reflecting receptor-specific modulation of neuroimmune responses. Similar results were observed in both the small and large intestine.

Taken together, these findings support the hypothesis that senolytic intervention modulates of microbial metabolites through selective regulation of SCFA receptors. **This receptor remodelling may underline, at least in part, the observed improvements in barrier integrity and cognitive performance following D+Q treatment.** Furthermore, the regional specificity of GPR43 suppression in the cerebellum may reflect distinct neuroimmune dynamics in this brain region, possibly related to its role in both motor control and affective regulation in aging.

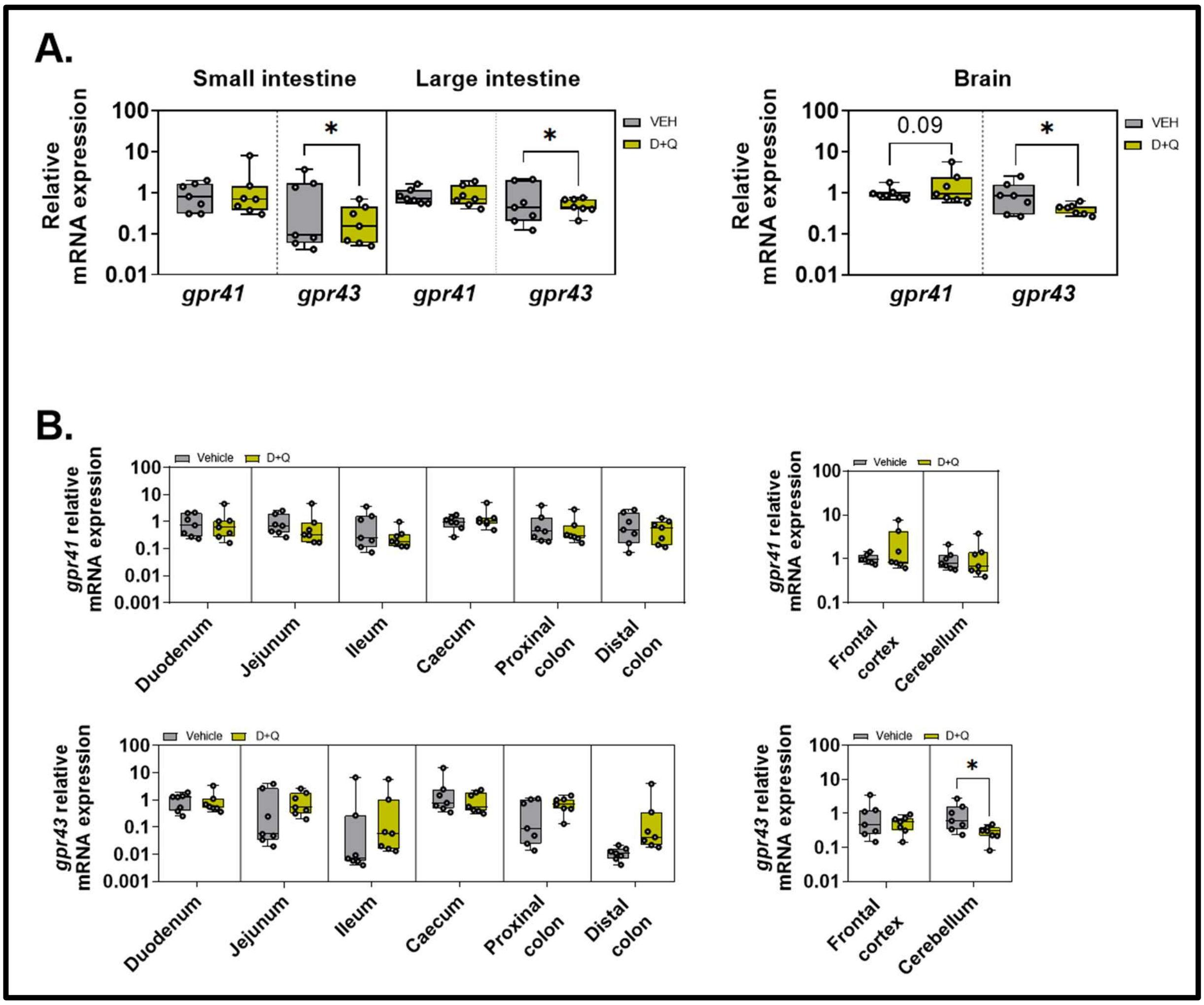

Figure 33. Gene expression of GPR43/FFA2 and GPR41/FFA3 after treatment with D+Q.

mRNA expression levels for *gpr41/ffar3 and gpr43/ffar2* genes in the brain and in the small and large intestine (A) or in the corresponding individual tissues (B) in the form of box and whiskers showing min. and max. with all points of ddCt normalized to GAPDH and adjusted for the vehicle group to 1. Statistical significance is reported as p-value, statistical tendency was reported whereas statistical significance was noted as * ≤ 0.05.

IV. Discussion

4.1 Cognitive Benefits of Senolytic Treatment

Across two independent cohorts of very-old Wistar rats, an eight-week intermittent oral regimen of Dasatinib and Quercetin (D+Q) significantly improved allocentric learning and memory. At baseline, before any intervention, 22–24-month-old rats showed the canonical geriatric pattern in the Active Allothetic Place Avoidance Task (AAPAT): many errors (entry in the shock zone), shortened avoidance intervals and inefficient escape strategies indicating impaired place avoidance, slower adaptation to the aversive stimulus, poor skill acquisition, deficiencies in short-term memory retention, inability to sustain avoidance behaviour and cognitive dysfunction.

Within 48 h of the final D+Q gavage these metrics improved markedly and specifically in aged rats: the maximum avoidance time doubled, the error load halved and the learning curve normalised relative to vehicle controls. This indicated enhanced avoidance and spatial learning, more efficient acquisition of avoidance behaviour, improved cognitive coordination, robust short-term memory formation and learning consolidation.

Five weeks after treatment cessation the gains were still evident, indicating that one eight-week senolytic cycle produces durable functional re-organisation rather than a transient behavioural boost. The effects of D+Q were not merely transient or symptomatic but instead led to meaningful, possibly structural, changes in the cognitive circuitry of aged rats. These results indicated a consolidation of spatial memory over time and an increased capacity for learning across repeated training exposures, consistent with enhanced neuroplasticity which translated to broad and durable functional benefits

No significant differences in total exploration assessed in the Open-Field test between D+Q and vehicle groups were found, supporting the specificity of cognitive outcomes observed in AAPAT. The AAPAT proved to be a sensitive tool to detect these impairments in aged animals without being confounded by decreased locomotor activity. These results set the stage for evaluating the impact of senolytic intervention on cognitive restoration.

These behavioural benefits track with molecular and structural adaptations documented in the same animals: (i) restoration of apical-dendritic spine density and length-to-head ratio in CA1 pyramidal neurons; (ii) reversal of age-linked histone H3K9me3 accumulation and H3K27me3 loss; (iii) reinforcement of blood–brain barrier (BBB) tight-junction architecture (↑ ZO-1, ↑ Occludin, ↑ claudin-1); and (iv) a brain-wide shift from a TNF-α/IFN-γ-dominant milieu toward an IL-10-dominated, anti-inflammatory profile.

The dataset argues that D+Q enhances cognition through a systems-level cascade rather than a single dominant pathway. Our working model integrates four mutually reinforcing processes:

1. **Suppression of systemic inflammaging**. Consistent with the canonical gerotherapeutic mode of action, D+Q markedly reduced circulating SASP mediators. The dampening of peripheral innate signalling could have alleviates chronic microglial priming, lowering neuroinflammatory tone and permitting synaptic remodelling.
2. **Reinstatement of barrier fidelity**. Reinforced intestinal tight junctions and BBB strengthening limits the entry of residual pro-inflammatory moieties. The barrier improvement isolates the CNS from peripheral inflammatory noise and metabolic by-products detrimental to plasticity.
3. **Microbiota-driven metabolite re-programming**. Expansion of *Lactobacillus acidophilus* and concomitant reduction in proteobacterial pathobionts reshape the colonic SCFA and bile-acid pools. Although absolute faecal SCFA output declines (suggesting enhanced absorption), brain GPR41 up-regulation implies more efficient central sensing. SCFAs are recognised epigenetic modulators capable of inhibiting class-I HDACs; their increased utilisation aligns with the literature on histone-mark rejuvenation and BDNF-relevant transcriptional landscape.
4. **Epigenetic facilitation of synaptic plasticity**. The combined fall in H3K9me3 (repressive) and rise in H3K27me3 (developmental pruning) tilt chromatin towards a permissive yet selectively stabilising state, favouring activity-dependent spine turnover and memory consolidation.

Our findings dovetail with, yet extend, prior reports (Table 1) that senolytic interventions can improve cognitive function. In aged mice, Ogrodnik et al. reported that intermittent D+Q treatment reduced errors in the water-escape Stone T-maze, consistent with improved working memory, although BBB integrity and gut microbiota composition were not assessed. In parallel, Dungan et al. showed that D+Q improves age-related physical performance (e.g., rotarod and balance-beam), supporting systemic benefits of senescent-cell targeting but without direct neurobiological or microbiome correlates (Dungan et al., 2022). Importantly, studies that interrogate the gut–brain axis provide complementary support: microbiota-directed interventions have been shown to enhance recognition memory and other cognitive outcomes alongside shifts in microbial composition and inflammatory/metabolic read-outs, strengthening the plausibility of a microbiota component in our mechanism. Moreover, Zhou and colleagues (Zhou et al., 2000) provide supportive context that probiotic administration can be well tolerated in BALB/c mice, reinforcing the safety premise for microbiome-targeted approaches even though cognition was not evaluated in that study.

This thesis aimed to integrate senolytic-induced gut restructuring, barrier fortification, epigenetic re-balancing and behavioural outcomes within the same aged subjects.

Reference	Model	Cognitive test(s)	Outcome	Relevance to the present work
Ogrodnik et al., 2021	Senescence model *INK-ATTAC* $^{+/-}$ transgenic mice	Water-escape Stone T-maze	Clearance of senescent cells alleviates age-associated cognitive impairment, reduce markers of neuronal senescence in the CA3 region of the hippocampus, reduces hippocampal microglial activation, age-related brain inflammation, and infiltration of immune cells	Strong precedent that senolytic interventions can improve cognition in ageing
Bussian et al., 2018	Tauopathy model *PS19;ATTAC* mice	Novel Object recognition	Selective clearance of senescent glia prevents tau aggregation and preserves cognition, mitigates tau pathology and neuronal loss	Establishes that removing senescent cells can be disease-modifying for cognition
Tarantini et al., 2021	Natural aging Aged C57BL/6 mice	Radial-arm water maze	ABT263/Navitoclax treatment improves neurovascular responses and cognitive function in aged mice by restoring endothelial nitric oxide mediation	Supports that senolytics may act via vascular / BBB-adjacent physiology
Fang et al., 2024	Alzheimer's model APP$^{NL-F/NL-F}$ mice	Morris Water Maze	D + Q treatment reduces visceral white adipose tissue, hippocampal senolytic markers, plasma cytokine levels and hippocampal plaque burden, improved spatial learning and memory in female APP$^{NL-F/NL-F}$ mice	Expands senolytic–cognition evidence beyond "normal ageing" into AD-like pathology
Lin et al., 2024	Young and aged C57BL/6J mice	Heat stress model	D+Q reduced heat stress-induced cognitive deficit, stress reaction, endotoxemia, BBB disruption, inflammatory and oxidative injuries to peripheral and central tissues in young adult and aged mice	Ties Senolytic regimen to gut permeability, BBB integrity and cognition in one design

Table 1. Senolytic-targeted interventions influencing cognitive function: evidence supporting a senolytic–brain mechanistic axis.

4.2 The Role of Gut Microbiota in the Gut-Brain Axis

● Significance of Increased *Lactobacillus acidophilus*

A consistent treatment-specific expansion of *Lactobacillus acidophilus* was detected in every 24-month-old rat that received the eight-week senolytic schedule, whereas it was virtually absent or remained at trace levels in vehicle-matched controls. The rise was modest in absolute terms (from ≈1.4 % to ≈6.8 % relative abundance) yet may have significant biological implications.

Lactobacillus acidophilus is a Gram-positive lactic-acid bacterium whose metabolism is typically dominated by lactate production (with additional organic acids depending on substrate and conditions). Its bloom under senolytic treatment may therefore reflect a niche shift toward a less inflammatory, more mucus-associated intestinal environment, consistent with the concomitant reduction in pro-inflammatory cytokines (TNF-α, IFN-γ) and the thickening of the jejuno-ileal mucus barrier observed here.

From a functional perspective, *Lactobacillus* are known to:

- **Enhance epithelial barrier function**, including increases in transepithelial resistance and/or tight-junction gene expression/protein level in intestinal epithelial models (shown for several *Lactobacillus* strains/species).
- **Shape systemic immune tone** via microbially derived metabolites and host-signalling pathways.
- **Modulate tryptophan availability** and downstream kynurenine-pathway metabolites in probiotic contexts; notably, peripheral serotonin itself does not cross the BBB (tryptophan does), so any brain effects are expected to occur via precursor transport and/or immune/vagal signalling rather than serotonin entry.

Thus, the selective bloom of *L. acidophilus* provides a plausible mechanistic link between the gut-centric effects of Dasatinib and Quercetin (D+Q) (reduced permeability, lower luminal irritation) and the distal benefits in the brain (reinforced BBB, restored dendritic architecture, improved memory). It likely acts as both a sensor and amplifier of the newly established anti-senescent milieu.

The observed rise in *L. acidophilus* aligns with its recognized probiotic profile and supports its potential contribution to the cognitive and systemic improvements seen following D+Q treatment. Prior work indicates that *L. acidophilus* supplementation in aged mice can remodel the gut microbiome and improve metabolic/immune-related phenotypes (Jeon et al., 2022). Moreover, independent work in healthy mice reports improved cognitive performance following administration of a defined *L. acidophilus* strain alongside microbiome profiling, supporting the feasibility of targeted microbial interventions in principle. Collectively, published data (Table 2) corroborate the notion that *L. acidophilus* can modulate neuroinflammation, barrier integrity and cognition, yet our study is unique in demonstrating that a host-directed senolytic intervention can secondarily reshape this probiotic niche.

- Interpretation of SCFAs and Bile Acids Modulation

A pathway-level analysis was designed to determine whether the observed enrichment in *Lactobacillus acidophilus* and other taxa following senolytic intervention corresponded to meaningful changes in predicted microbial metabolic functions, such as carbohydrate metabolism, short-chain fatty acid biosynthesis, bile acid biotransformation, or immune-modulatory signalling. The recorded absence of significant pathway-level findings likely reflects a combination of factors:

1. High inter-individual variability, especially common in aged cohorts, may have masked subtle functional differences.
2. Small sample size reduced the statistical power to detect treatment effects with confidence.
3. Limitations of the 16S-based approach, which infers function from taxonomy rather than directly measuring gene expression or metabolite production, inherently restrict the granularity of functional resolution. Many taxa, even closely related ones, may differ in their metabolic repertoires due to horizontal gene transfer or niche-specific adaptations that are not captured in 16S phylogenetic trees.

Importantly, while no consistent functional enrichment was observed at the group level, the compositional shift, most notably the increased abundance of *L. acidophilus*, remains biologically meaningful, as this taxon is well-characterized for its roles in mucin regulation, immunomodulation, and SCFA production (Rastogi et al., 2022). These functional capacities may therefore be better captured through integrative multi-omic approaches, such as shotgun metagenomics, metabolomics, or meta-transcriptomics.

The metabolomic arm of this thesis revealed four convergent phenomena:

- **A broad fall (-25–55 %) in luminal SCFAs**, notably acetate, propionate, isobutyrate and branched-chain pentanoates, after eight weeks of D+Q, whereas butyrate itself showed only a downward trend.
- **A parallel depletion of primary conjugated bile acids** (taurocholic and tauroursodeoxycholic acids) in the caecal and colonic milieu.
- **Minimal spill-over into the systemic compartment**, the sole serum change being a selective drop in formate; other SCFAs and all measured bile acids remained stable in the circulation.
- **Tight correlations with microbial and barrier indices**: diminished faecal SCFA concentrations tracked the bloom of *Lactobacillus acidophilus* ($\rho \approx -0.62$) and the thickening of the jejuno-ileal mucus layer, while lower luminal bile acids associated with up-regulated ileal *tjp1* expression.

One possible interpretation of these findings is that lower faecal SCFA levels reflect impaired microbial fermentation. However, the present dataset does not allow a clear distinction between reduced microbial production and altered host handling of these metabolites. Taken together, the results are more cautiously interpreted as being consistent with altered production, utilization, degradation, and/or absorption of SCFAs, rather than providing direct evidence for microbial failure or enhanced host sequestration alone.

Barrier remodelling may alter luminal metabolite handling. D+Q enlarged villus-crypt units, modulated tight-junction-associated markers, and was associated with reduced indices of paracellular leak, changes that could influence epithelial exposure to and handling of SCFAs. Similar reductions in luminal SCFAs have been described after bariatric surgery or calorie restriction, contexts in which barrier physiology is also altered (Meijer et al., 2022). In the present study, however, faecal measurements alone do not allow us to conclude whether lower SCFA levels primarily reflect increased absorption, altered microbial production, enhanced host utilization, or a combination of these processes.

SCFAs as neuro-metabolites. Acetate, propionate, and related short-chain fatty acids can influence central processes through mechanisms including HDAC inhibition, microglial metabolic re-programming, and vagal afferent signalling (Dalile et al., 2019). These established neuroactive properties make SCFAs plausible mediators within the gut–brain axis. However, because portal or brain concentrations were not measured here, the observed reduction in luminal SCFAs cannot be interpreted as direct evidence of increased cerebral exposure. Rather, the present findings indicate that D+Q alters luminal SCFA availability in parallel with cognitive and hippocampal changes, while the mechanistic relationship between these phenomena remains to be determined.

Lower faecal bile-acid levels are consistent with altered enterohepatic handling. Tauro-conjugated species are actively re-absorbed in the distal ileum via ASBT and recycled to the liver; accordingly, diminished faecal losses may reflect altered reabsorption, intestinal transit, microbial transformation, hepatic synthesis, or combinations of these processes under the senolytic regimen. This remains mechanistically relevant because FXR and TGR5 signalling, both influenced by circulating bile acids, are recognised modulators of neuro-inflammation and energy homeostasis (Chiang et al., 2020). However, the present data do not resolve which component of bile-acid handling is primarily affected.

Receptor crosstalk: the concomitant down-regulation of *gpr43* and the upward drift of *gpr41* mRNA in brain tissue imply a receptor-level remodelling of SCFA sensing, potentially shifting the balance from pro- to anti-inflammatory tone in microglia (Kim et al., 2013).

The observed metabolomic alterations could stem from multiple, possibly converging, mechanisms. One explanation is a reduction in microbial synthesis due to compositional or functional changes in the gut microbiota. Another is altered host handling of SCFAs and bile acids, potentially involving changes in utilization, degradation, reabsorption, and/or absorption across a remodelled intestinal barrier, which could contribute to lower faecal levels despite largely stable serum concentrations. The selective reduction of serum formic acid may reflect increased hepatic clearance or host metabolic utilization. Overall, these data indicate that senolytic treatment modulates the faecal availability and systemic handling of gut-derived metabolites, while the precise balance between production and host processing remains to be established.

Rodent studies using dietary fibre enrichment or direct acetate/propionate supplementation often report behavioural gains alongside SCFA-linked immune and microglial effects, although many such datasets are developmental or disease-context specific (e.g., maternal-obesity offspring paradigms) rather than ageing per se. In parallel, mechanistic BBB work shows that physiologically relevant butyrate and propionate can preserve tight-junction organization and protect barrier integrity in an in vitro BBB model under inflammatory challenge.

By contrast, host-directed interventions cited here as comparators, rapamycin in oral ageing (An et al., 2020), timing of calorie restriction affecting gut microbiota and colonic Zo-1/Occludin transcripts (Zhang et al., 2019), and the *A. muciniphila* protein Amuc_1100 increasing epithelial TEER in vitro (Ottman et al., 2017), do not directly quantify luminal SCFAs and/or bile acids in the same experimental frame, so metabolite directionality should not be inferred from those studies alone (Table 3).

Moreover, centenarians display a distinctive gut microbiome signature enriched for secondary bile-acid biosynthetic pathways and associated bile-acid profiles, supporting bile acids as a meaningful “healthy ageing” axis, though this evidence is primarily microbiome/faecal-metabolite based rather than a direct plasma-vs-faeces redistribution analysis.

Study	Model	Intervention	Outcome	Relevance to the present work
Černá et al., 2024	Old mice receiving FMT from young, exercised donors	Faecal microbiome transplantation	FMT from young-trained donors enhances cognitive function and synaptic plasticity while reducing neuroinflammation, reduces gut permeability and increases ZO-1 levels in the colon, decreases inflammatory markers in the blood, increases SCFA levels in the caecal content and increased representation of bacteria associated to beneficial effects on cognitive function in aged mice	Supports gut–brain axis logic: microbiome and barrier improvements track with cognitive rescue
Lee et al., 2026	C57BL/6 mice	Probiotic administration (including *L. acidophilus*)	Probiotic treatment enhanced memory function, promoted neurotransmitter production, regulated gut microbiota composition of mice	Supports the concept that microbiome-targeted interventions influence cognitive function during aging
Jeon et al., 2022	Young C57BL/6 mice	Oral supplementation of *L. acidophilus* EG004	*Lactobacillus acidophilus EG004* supplementation improved spatial learning and memory in mice. The probiotic altered gut microbiota composition and metabolic pathways related to neurotransmitters and short-chain fatty acids. These microbiome changes suggest cognitive benefits mediated through the gut–brain axis.	Microbial intervention with cognitive outcome example
Erny et al., 2015	Young specific pathogen free and germ-free mice	SCFA reconstitution in microbiota-depleted context	Germ-free animals show widespread microglial dysfunction. Absence or reduction of gut microbiota weakens microglial immune responses. Reduced microbiota complexity impairs microglial maturation and function. Microglial defects can be restored by microbiota recolonization or short-chain fatty acids (SCFAs).	Supports metabolites to brain immune tone bridge

Study	Model	Intervention	Outcome	Relevance to the present work
Liu et al., 2021	Young C57BL/6 mice	High-fibre diet or acetate/propionate supplementation FMT in microbiome depletion model	Maternal high-fibre diet reshapes the gut microbiota of both mothers and offspring. High-fibre intake prevents cognitive and social deficits in offspring caused by maternal obesity. Gut microbiota plays a key role in mediating these behavioural effects. Dietary fibre or SCFA supplementation in offspring restores behavioural deficits induced by maternal obesity.	Acetate/propionate can shape microglia and cognition
Ren et al., 2024	Human sample and longitudinal study Young C57BL/6 mice	Manipulating intestinal BA absorption	Elevated conjugated bile acids and ammonia are associated with age-related cognitive decline. High levels of these metabolites contribute to hippocampal synapse loss. Intestinal bile acid absorption influences brain levels of these metabolites and affects cognitive performance. Reducing bile acid absorption with cholestyramine improves cognition in aged mice.	Supports BA handling ↔ cognition as a plausible pathway
Sato et al., 2021	Human centenarians	Observational: longevity-associated microbiome	Distinct BA/microbiome features associated with healthy longevity and altered colonization resistance	Human-facing context that BA remodelling is a meaningful “aging biology” signature
Huang et al., 2025	Left middle cerebral artery occlusion in mice model	*L. acidophilus* supplementation	*Lactobacillus acidophilus* supplementation improves cognitive performance after cerebral ischemia. Treatment reduces neurological damage, neuroinflammation, and gut microbiota dysbiosis. Improved absorption of linoleic acid enhances microglial peroxisomal function. Enhanced microglial metabolism reduces oxidative stress and shifts microglia toward an anti-inflammatory phenotype. These mechanisms contribute to improved cognitive outcomes in both mice and cerebral ischemia patients.	Feasibility/safety/efficacy of targeted microbial interventions

Table 2. Interplay between microbiota-derived metabolites (SCFAs and Bile Acids) and cognitive outcomes: mechanistic and translational evidence.

4.3 Senolytics and Barrier Integrity

- Impact on Intestinal Mucosa Integrity and Mucin Production

The morphological and molecular analyses performed in the present work converge on the conclusion that Dasatinib and Quercetin (D+Q) remodel the ageing gut epithelium in a region-selective manner. In the proximal small intestine, where luminal exposure to the orally administered drugs is maximal, the mucosal compartment expanded by almost 40 %, crypt depth increased, and villus surfaces became densely carpeted with Alcian-positive goblet cells. Concomitantly, *Muc1* transcripts rose three- to five-fold, whereas *Muc2* levels were preserved, pointing to an enrichment of membrane-bound rather than secreted gel-forming mucins. By contrast, the caecum and distal colon displayed a modest mucosal thinning and a diminution of Alcian-reactive material, accompanied by a downshift in *Muc1*.

These histological data dovetail with the quantitative western-blot evidence of tighter junctional sealing (up-regulated ZO-1 and Occludin) in the ileum but not in the large bowel, suggesting that the overall barrier function is strengthened in the nutrient-absorptive segment even as the distal lumen becomes metabolically leaner.

Notably, D+Q induced region-specific remodelling of intestinal architecture, with an apparent increase in thickness in the upper small intestine contrasted by thinning in the distal small intestine and throughout the large intestine. Given the strong functional compartmentalization along the gastrointestinal tract, these divergent responses are compatible with distinct underlying drivers: the proximal small intestine is predominantly shaped by nutrient and bile exposure, whereas the distal small intestine and colon are more influenced by microbial load and immune activation. In this context, proximal thickening may reflect reinforcement of mucosal structure and/or mucus-associated barrier components, while distal and colonic thinning may represent resolution of age-related inflammatory remodelling (e.g., reduced oedema, immune-cell infiltration, or compensatory hyperplasia). Importantly, this interpretation is supported when architectural changes co-occur with a shift toward a less pro-inflammatory milieu and improved barrier-associated markers.

A plausible mechanistic framework emerges when these findings are integrated with the microbial and metabolic signatures described earlier. Senolytic clearance of p16^Ink4a-positive enterocytes and stromal cells is likely to reduce the SASP burden that drives goblet-cell depletion and hyper-permeability during ageing. The resulting rejuvenated niche supports crypt progenitor activity, accelerates epithelial turnover and favours *Muc1* expression, a mucin that, owing to its transmembrane nature, functions as a first-line sensor and physical shield against bacterial contact. Heightened mucin display would in turn provide additional substrates for the glycan-foraging enzymes of *Lactobacillus*, fuelling the expansion of these taxa observed post-treatment and sustaining a virtuous host–microbiota feedback loop.

These interpretations are consistent with external literature (Table 3). In mice, calorie restriction has been associated with a tighter epithelial barrier tone, including increased colonic Zo-1 and Occludin expression alongside reduced circulating TNF-α, consistent with improved inflammatory–barrier coupling during dietary restriction (Zhang et al., 2019). Probiotic administration of *L. acidophilus* C4 increases PAS-positive mucin staining and up-regulates ZO-1/Occludin while reducing inflammatory mediators in DSS colitis mice (Liu et al., 2023), aligning with the mucus/TJ reinforcement logic discussed here. More broadly, recent work demonstrates that senescent-cell secretomes can impair intestinal epithelial differentiation and crypt formation, and that senescent-cell elimination can improve epithelial integrity, supporting senescence as a plausible upstream driver of age-linked barrier dysfunction (Yun et al., 2023; Eskiocak et al., 2024).

Collectively, the present thesis extends the repertoire of senolytic benefits to include marked reinforcement of the ageing intestinal barrier, driven by coordinated expansion of the mucosal layer and qualitative shifts in the mucin landscape. This barrier fortification likely constitutes a proximal step linking gut microbial remodelling to the systemic, and ultimately neuro-cognitive, remediation associated with D+Q intervention.

- ### Role of Tight-Junction Protein Modulation

In parallel with the mucosal remodelling described above, D+Q treatment exerted a striking, yet region-specific, influence on the molecular architecture of epithelial tight junctions. In the ageing small intestine, the senolytic cocktail augmented both transcript and protein abundance of Zonula-occludens-1 (ZO-1/TJP1) and Occludin, whereas claudin-1 levels fell sharply in the jejuno-ileal axis. The colonic epithelium exhibited the mirror image: ZO-1 and Occludin were unchanged or modestly reduced, but claudin-1 was preferentially preserved. A comparable dichotomy was evident in the central nervous system. Western blots from the frontal cortex and cerebellum showed a robust up-regulation of ZO-1 (≈ +45 %) and Occludin (≈ +35 %), while claudin-1 increased only in the cortex. Concomitantly, serum endotoxin levels dropped by ~25 %, and linear regression revealed a positive correlation between TNF-α suppression and the fall in claudin-1 within gut segments, implying inflammation-tight-junction feedback.

These patterns suggest that senolytic clearance of barrier-resident senescent cells does not rigidly strengthen every component of the junctional complex; instead, it tilts the claudin/Occludin ratio in a compartment-tailored manner. Claudin-1 is well known to confer low paracellular permeability to macromolecules but, when over-expressed, can paradoxically promote epithelial-mesenchymal transition and inflammatory signalling (Bhat et al., 2016). Its selective down-modulation in the absorptive small bowel, coupled with enhanced ZO-1 scaffolding, might therefore ease nutrient flux while preserving the lateral fence, an arrangement reminiscent of the “leaky-but-selective” phenotype reported in calorie-restricted rodents (Zhang et al., 2019). By contrast, colonic maintenance of claudin-1 probably safeguards against microbial translocation in the segment that houses the densest bacterial load. In the brain, the concerted rise of ZO-1 and Occludin, both indispensable for blood-brain-barrier (BBB) tightness, supports the view that the D+Q intervention was associated with improved BBB-associated markers, thereby preventing peripheral cytokines from re-igniting microglial activation and cognitive decline.

Our observations dovetail with evidence from non-senolytic interventions that rejuvenate barrier function (Table 3). Barrier-associated tight-junction programs remain plastic in adulthood and can be shifted by systemic interventions. Four-week calorie restriction increased colonic Zo-1 and Occludin expression and reduced circulating TNF-α in mice, consistent with improved inflammatory–barrier coupling under dietary restriction (Zhang et al., 2019). At the BBB, endothelial senescence has been directly linked to impaired Occludin and BBB dysfunction, and systemic genetic elimination of p16+ cells partially reverses these changes in aged animals (Novo et al., 2024). The present thesis adds nuance by demonstrating a bidirectional, gut-segment-specific modulation under senolytic pressure, suggesting that barrier optimisation rather than uniform reinforcement may be the hallmark of healthy ageing

Our data position tight-junction reprogramming as a central, mechanistically tractable node through which senolytic intervention reconciles gut–brain barrier resilience with metabolic flexibility, opening ground for combined gerotherapeutic-and-microbiome interventions.

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- Relationship Between Gut and Brain Barrier Restoration

Our experimental series indicates that an eight-week oral course of dasatinib + quercetin (D+Q) in aged Wistar rats is associated with region-specific remodeling of the intestinal barrier together with improved blood–brain barrier-associated markers. These changes appear coordinated rather than isolated, but the intestinal response is not uniform across the gastrointestinal tract and is therefore better interpreted as compartment-dependent remodeling than as global epithelial restoration.

- **Gut barrier.** In the proximal small intestine, D+Q increased mucosal thickness, up-regulated ZO-1 and occludin, reduced pro-inflammatory TNF-α and IFN-γ, and boosted IL-10; serum LPS concomitantly fell by ≈25%. However, these effects were not uniform along the gut axis: the large intestine showed mucosal thinning, reduced mucin-associated staining in some segments, and region-specific decreases in claudin-1. Taken together, the intestinal findings support differential, segment-specific remodeling of barrier-associated features rather than a uniform strengthening of the gut barrier.
- **Blood–brain barrier (BBB).** In the frontal cortex and cerebellum the same treatment enhanced ZO-1 and Occludin protein abundance (≈ +40 %), restored claudin-1 in cortex, and shifted the cytokine milieu towards an anti-inflammatory state (IL-10↑, TNF-α↓ trend).

Although the hippocampus would be the most direct region to relate BBB integrity to spatial memory performance, hippocampal tissue was not available for the present analyses. We therefore assessed the frontal cortex and cerebellum to determine whether D+Q exerts broader effects on BBB-associated proteins and inflammatory balance across distinct brain regions. Given the systemic mechanism of senolytic intervention, observing convergent changes in these regions supports the interpretation that D+Q influences brain barrier and inflammatory homeostasis beyond a single memory circuit, while future studies should prioritize hippocampal sampling to more directly connect BBB modulation with the spatial-memory phenotype.

The concurrent improvement in blood–brain barrier-associated markers and region-specific intestinal remodeling after D+Q intervention likely reflects at least two interacting mechanisms.

- **Senescent cell clearance at both sites.** Enterocytes, Peyer's-patch macrophages, brain microvascular endothelial cells and pericytes accumulate p16^Ink4a positivity with age. Their removal would directly permit tight-junction reassembly and mucosal regeneration, as shown in earlier genetic ablation models.
- **Down-stream systemic synchrony.** Gut-leak–driven translocation of LPS, peptidoglycan, and microbially derived metabolites is a recognized trigger of BBB dysfunction and neuroinflammation. In the present study, the reduction in serum LPS together with intestinal and cerebral changes is consistent with the possibility that D+Q modifies peripheral inflammatory signaling in a way that may favor BBB-associated tight-junction expression. Conversely, improved BBB integrity could limit the entry of circulating inflammatory mediators that might otherwise affect enteric and systemic homeostasis. These relationships should be interpreted as a plausible bidirectional model rather than a directly demonstrated feed-forward mechanism.

This bidirectional crosstalk aligns well with the emerging concept of a gut–brain vascular unit in which barrier integrity at one pole is both a driver and a beneficiary of homeostasis at the other (Table 3). The intestine-to-brain barrier linkage has been suggested by antibiotic, probiotic and germ-free studies, yet direct senolytic evidence has been scant. A few key comparisons: Collectively, these studies and our findings converge on the notion that senescence, microbiota and barrier biology are entwined.

Our work adds pharmacological evidence that clearing senescent cells can promote region-specific intestinal barrier remodeling alongside improvement in cerebral barrier-associated markers, supporting a coordinated influence of senolytic intervention on gut–brain barrier homeostasis during aging.

Study	Model	Intervention	Outcome	Relevance to the present work
Budamagunta et al., 2023	Fisher 344 rats	Natural aging	Senolytic clearance of peripheral senescent cells reduces systemic inflammation and preserves blood–brain barrier integrity, thereby limiting neuroinflammation and improving cognitive function during brain aging	Support link between systemic senescence and BBB/cognition
Braniste et al., 2014	Gern- and pathogen-free C57BL/6J and Balb/c mice	Colonization of *C. tyrobutyricum* and *B. thetaiotaomicron*	Gut microbiota regulates blood–brain barrier integrity, influencing tight junction expression and permeability. Microbiota-derived metabolites, particularly SCFAs, contribute to the maturation and maintenance of the BBB	Microbiome/metabolites can causally tune BBB integrity
Sun et al., 2021	Young C57BL/6 mice	Microbiome depletion model by antibiotics	Depletion of gut microbiota disrupts blood–brain barrier structure and function. Restoring the microbiota partially rescues BBB integrity. Microbiota loss promotes infiltration of bone marrow–derived immune cells into the brain. These infiltrating cells differentiate into microglia-like cells, indicating immune alterations in the brain.	Supports microbiome shifts accompanying D+Q could contribute to BBB outcomes
Zhang et al., 2019	Young C57BL/6 mice	30% calorie restriction for 4 weeks, with feeding restricted to light vs dark phase	Feeding time during caloric restriction alters gut microbiota composition. Caloric restriction improves gut barrier integrity, increasing tight junction proteins and reducing inflammation. The timing of feeding influences the persistence of these beneficial effects.	Supports dietary restriction induce tighter barrier and lower systemic inflammation
Ottman et al., 2017	Human HEK cells	Exposure to *A. muciniphila* and recombinant outer-membrane protein	Amuc_1100 activated TLR2 and induced cytokines in PBMCs; *A. muciniphila* and Amuc_1100 increased TEER in Caco-2 monolayers	Mechanistic support for an *Akkermansia*-linked barrier effect

Knox et al., 2022	Brain endothelial cells	Butyrate or propionate supplementation (± LPS challenge)	SCFAs altered actin organization, increased tight-junction “spikes”, protected against LPS-induced TJ mis-localization, improved BBB integrity/TEER, and modulated mitochondrial network dynamics	Support that microbiota-derived metabolites are linked to BBB tightening
Liu et al., 2023	C57BL/6 mice	DSS-induced colitis; intervention with live *Lactobacillus acidophilus*	Lactobacillus acidophilus C4 improves colon integrity and reduces tissue damage. The treatment decreases oxidative stress and inflammatory cytokine levels. It restores intestinal barrier function and increases short-chain fatty acid production.	Expansion of *Lactobacillus* can coincide with stronger junctional sealing and more mucin-associated staining
Suzuki et al., 2009	Intestinal epithelial barrier (Caco-2 cells) model	Quercetin (and myricetin) exposure	Quercetin and myricetin modulate intestinal barrier function and permeability. They alter tight junction protein localization and expression. Quercetin shows a dose-dependent (biphasic) effect on barrier integrity.	Support that the phenotype (↑ TJ organization) is biologically plausible through quercetin-driven TJ assembly, complementing senolysis-based explanations
Novo et al., 2024	Senescent brain endothelial cells (BECs)	Systemic genetic elimination of p16+ cells	Aging and disease are associated with increased senescent cell burden and chronic inflammation. Senolytic treatment reduces senescence markers and inflammatory signalling. Clearing senescent cells improves tissue function and physiological outcomes.	Provides independent evidence that senescent-cell clearance can restore BBB TJ biology (Occludin)

Table 3. Interplay Between Senolytics, Microbiome and Barrier Integrity: Mechanistic and Translational Evidence.

4.4 Inflammation and Immune Modulation

- Impact on Gut Inflammation and Immune Homeostasis

The present work demonstrates that senolytic intervention with Dasatinib and quercetin reshapes the intestinal immune landscape of twenty-four-month-old male Wistar rats in three converging ways.

- **Attenuation of pro-inflammatory tone** – Quantitative PCR and immunoblotting showed a robust down-shift of *Tnf*-α and *Ifng* transcripts and proteins throughout the small intestine, with the largest drop observed in the ileum (≈ 60 % for TNF-α protein).
- **Rise in regulatory signalling** – *Il10* mRNA and IL-10 protein increased two- to four-fold in the same segments, indicating an amplified anti-inflammatory circuit.
- **Systemic reflection** – Multiplex assays revealed an overall contraction of the age-associated cytokine “secretory phenotype” measured in serum.

Taken together these data indicate a net shift from a Th1-skewed, inflammageing intestinal milieu toward an IL-10-dominated homeostatic state.

The dual decrease of TNF-α / IFN-γ and rise of IL-10 after D+Q suggests that clearing senescent cells removes a persistent source of danger-associated molecular patterns (DAMPs) and SASP cytokines that chronically stimulate intestinal macrophages and lamina propria lymphocytes. As senescent enterocytes and mucosal macrophages are eliminated, epithelial damage signals subside, the NF-κB axis is dampened, and regulatory T-cells or IL-10-producing type 2 innate lymphoid cells can expand. Reduced luminal leakage, evidenced by lower serum LPS, further limits toll-like receptor engagement, closing a feed-forward loop. This interpretation is strengthened by the parallel improvement in tight-junction architecture, described in this thesis, better barrier integrity physically curtails the ingress of microbial antigens that would otherwise perpetuate inflammation.

Relative to the literature, the thesis provides the first evidence that a *single* pharmacological senolytic cocktail can synchronously (i) dampen intestinal Th1 cytokines, (ii) raise IL-10, and (iii) reduce systemic endotoxin, therefore bridging cellular senescence biology with classical mucosal immunology.

This regional variability may reflect differences in local immune cell populations, blood-brain barrier permeability, or the degree of senescent cell accumulation.

The overall pattern suggests that D+Q treatment does not uniformly suppress inflammation in the brain but rather enhances endogenous anti-inflammatory signalling, most notably IL-10, while exerting mild or negligible suppression of pro-inflammatory mediators. This may reflect selective clearance of senescent cells that contribute disproportionately to inflammatory signalling in certain brain regions, thereby enabling localized immune recalibration without inducing systemic immunosuppression.

The observed downregulation of GPR43 may indicate a reduction in pro-inflammatory signalling cascades. This is supported by emerging literature linking GPR43 expression to M1-like macrophage (and potentially microglial) activation phenotypes, particularly under inflammatory stress. Given that GPR43 is known to be upregulated during inflammatory polarization and contributes to ROS generation and cytokine production, its suppression by D+Q could represent an adaptive response to curtail neuroinflammation in aging brains. However, it should be noted that GPR43 also participates in barrier-protective and anti-inflammatory responses in epithelial and glial contexts. Thus, the functional implications of its downregulation remain nuanced and context dependent.

On the other hand, the trend toward increased GPR41 expression may reflect a compensatory mechanism aimed at preserving or enhancing SCFA responsiveness through alternative signalling pathways. GPR41 activation has been associated with sympathetic nervous system activity, gut motility, and anti-inflammatory effects in select settings. Its upregulation in brain tissue, although subtle, could suggest a shift toward favouring this receptor in the central processing of SCFA-derived cues.

- Effects of Decreased Inflammation on Cognitive Function

Across both the behavioural and molecular arms of the project a coherent narrative emerged: the senolytic cocktail D+Q not only pruned peripheral and intestinal inflammatory mediators but also dampened brain-intrinsic cytokine output, and this double-edged anti-inflammatory action coincided with striking improvements in hippocampal-dependent spatial learning and memory retention. Specifically:

- **Peripheral compartment** – multiplex cytokine analysis demonstrated a broad collapse of the age-related pro-inflammatory secretome (TNF-α, IL-1β, MCP-1, IP-10) while IL-10 rose; serum endotoxin levels fell in parallel, indicating reduced systemic immune activation.
- **Intestinal wall** – *Tnf* and *Ifng* transcripts dropped by roughly one half, IL-10 mRNA doubled, and mucosal macrophage/microglia markers (Iba-1) were less abundant; barrier restoration (tight-junction up-regulation) suggests a primary locus of inflammatory resolution.
- **Central nervous system** – in the frontal cortex and cerebellum TNF-α and IFN-γ proteins declined, whereas IL-10 increased almost three-fold; enhanced expression of ZO-1 and Occludin pointed to a sturdier blood-brain barrier (BBB).
- **Behaviour** – aged rats showed fewer shock-zone entries, longer avoidance latencies and superior long-term retrieval five weeks after treatment cessation, confirming durable cognitive rescue.

Together these datasets indicate that D+Q curtails “inflammageing” on both sides of the gut-brain axis, and that the blunted inflammatory tone tracks tightly with cognitive benefit.

The intimate relationship between chronic low-grade inflammation and cognitive impairment in ageing is well recognised: circulating TNF-α and IL-6 predict memory decline in elders, and experimental elevation of these cytokines disrupts long-term potentiation within hours. The present thesis supports a causative, rather than merely correlative, link by showing that selective removal of senescent cells, a major nidus of sterile inflammation, rapidly quenches cytokine signalling and restores mnemonic function.

Three complementary mechanisms are likely at play:

1. **Peripheral-to-central cytokine relay.** Reduced intestinal TNF-α and IFN-γ lower vagal afferent and humoral inflammatory inputs to the brain, thereby limiting microglial priming. The observed rise in IL-10 within cortex suggests either enhanced trafficking of anti-inflammatory monocytes or a re-programming of resident microglia toward an M2-like state, both of which promote synaptic plasticity.
2. **Barrier-centric protection.** Strengthening of epithelial and endothelial tight junctions diminishes translocation of lipopolysaccharide and other pathogen-associated molecular patterns. Decreased serum LPS gives tangible evidence for this break in the gut→blood→brain inflammatory cascade, echoing reports that barrier leakiness predicts cognitive frailty in humans.
3. **Senescent cell clearance within the brain.** Although bulk tissue analyses cannot pin-point cellular sources, clearance of p16^Ink4a-positive microglia or astrocytes would directly extinguish local SASP output; the rapid fall in brain TNF-α is compatible with this possibility and accords with mouse studies in which genetic ablation of senescent glia rescues cognition.

Numerous interventional studies provide external scaffolding for the thesis conclusions (table 4). Collectively, these studies corroborate the thesis that attenuating pro-inflammatory cytokines and/or amplifying IL-10 is a viable avenue to rejuvenate cognition; the present work is distinctive in implicating senescent cell removal as the upstream trigger.

Thus, the cognitive gain observed after D+Q can be interpreted as the functional read-out of a multi-tiered anti-inflammatory re-set spanning gut, circulation and brain.

- Possible Implications of GPR43 and GPR41 Modulation

Quantitative PCR performed on gut and brain extracts revealed a bidirectional reshaping of the two prototypical short-chain-fatty-acid (SCFA) receptors after senolytic exposure:

- **Gpr43 (Ffar2)** transcription declined by ≈40 % in the small and large intestine and by ≈30 % in pooled brain samples; the drop reached significance in cerebellum and in the overall gut.
- **Gpr41 (Ffar3)** remained stable in the gut but exhibited a trend toward elevation in the brain (≈25 %), driven mainly by the frontal cortex.

These receptor shifts occurred in parallel with (i) a global reduction of faecal SCFAs, (ii) lower circulating formate, and (iii) a pronounced anti-inflammatory cytokine profile in both periphery and CNS.

SCFA receptors orchestrate a wide spectrum of immune and metabolic pathways. FFAR2/GPR43 is preferentially activated by acetate and propionate and has a context-dependent role: in epithelial cells it promotes barrier fortification and IL-18 production, whereas in macrophages and microglia it can amplify M1 polarisation and reactive oxygen species. FFAR3/GPR41, activated by propionate and butyrate, is more closely linked to sympathetic outflow, intestinal gluconeogenesis and microglial maturation.

The concordant down-regulation of GPR43 in gut and brain therefore suggests that D+Q curtails a receptor system whose over-activity might sustain inflammageing. In the cerebellum, diminished GPR43 expression aligns with our observation of reduced TNF-α and IFN-γ, supporting reports that FFAR2 signalling via the NLRP3 pathway augments neuroinflammation (Tang et al., 2022). Conversely, maintenance, or slight induction, of GPR41 in cortex could preserve SCFA-driven neurotrophic actions, as FFAR3 activation triggers BDNF release and enhances synaptic plasticity (Petersen et al., 2020). Taken together, the receptor pattern points to a functional re-balancing: limiting pro-inflammatory FFAR2 cues while sparing or boosting neuroprotective FFAR3 pathways.

At the gut level, reduced Gpr43 accompanies lower epithelial TNF-α and higher IL-10, echoing studies where FFAR2 knock-down shields against colitis (Maslowski et al., 2009). Because D+Q simultaneously strengthened tight-junction expression, it is plausible that barrier repair lessens luminal SCFA exposure and secondarily down-tunes epithelial FFAR2, closing a positive feedback loop that restrains mucosal inflammation.

Collectively, the external evidence strengthens the idea that differential tuning of SCFA receptors can shift the inflammatory set-point and influence neurodegeneration trajectories (Table 4).

Our data provide the first demonstration that senolytic intervention can enact such tuning *in vivo.*

Study	Model	Cognitive test(s)	Outcome	Relevance to the present work
Saccon et al., 2021	Young and aged mice	Natural aging	Senolytic treatment with D+Q reduce intestinal senescence/inflammatory signalling in ageing and modulate gut microbiome (gut-centric gerotherapeutic immune reset).	Supports mechanistic claim that senolytics can reprogram the gut environment
Madhurakkat et al., 2025	Aged mice	Surgery-induced cognitive impairment	D+Q treatment reduced systemic SASPs and mitigated the orthopaedic surgery-induced neuroinflammation following orthopaedic surgery	Systemic inflammation + senescence drive cognitive vulnerability
Yun et al., 2023	Mouse fibroblast and mouse intestinal organoid	Senescent fibroblast-conditioned media / SASP applied to intestinal organoids	Demonstrates that SASP factors from senescent fibroblasts deregulate stem-cell activity and differentiation and impair crypt formation in organoids.	Senescent-cell burden (SASP) can disrupt crypt architecture and differentiation programs
Thevaranjan et al., 2017	Aged mice	Age-associated dysbiosis in germ-free mice and faecal microbiota transfer	Links dysbiosis to systemic inflammation and macrophage dysfunction; lowering TNF protected old mice from permeability and rescued microbiota changes	Supports framing that ageing-associated microbiota ↔ leakiness ↔ inflammaging can drive systemic and brain-relevant consequences

Baker et al., 2016	Naturally aged ATTAC mice	p16^Ink4a+ senescent cell clearance (INK-ATTAC/AP20187)	Senescent-cell clearance reduces multiple ageing phenotypes consistent with lower sterile inflammation / SASP burden	Removing senescent cells improves tissue homeostasis by reducing inflammageing
Hoyles et al., 2018	Human brain endothelial models	Propionate exposure	Demonstrate BBB-protective effects of propionate by dampening inflammatory signalling in endothelium	Provides mechanistic basis for SCFA receptor signalling at the BBB
Kim et al., 2013	GPR41(-/-) and GPR43(-/-) C57BL/6 mice	SCFA supplementation	Shows SCFA signalling via GPR41/43 can promote inflammatory responses in certain colitis-like challenges (context-dependent).	GPR41/GPR43 can amplify inflammation depending on the model
Maslowski et al., 2009	GPR43(-/-) C57BL/6 mice	Natural aging	Gut microbiota–derived short-chain fatty acids (SCFAs) regulate immune responses. SCFAs, particularly acetate, protect against inflammation through GPR43 signalling. Loss of SCFA signalling leads to increased inflammatory responses.	Shows FFAR2/GPR43 can be anti-inflammatory / pro-resolution depending on context

Table 4. Senescence Clearance, Cytokine Reprogramming, and SCFA Receptor Modulation: Convergent Evidence Linking Immune Reset to Barrier and Cognitive Restoration

4.5 Synaptic Plasticity and Cognitive Recovery

● Structural Synaptic Remodelling

The behavioural data in this thesis demonstrate that intermittent senolytic treatment with Dasatinib and quercetin (D+Q) restores hippocampus-dependent spatial learning and memory in aged rats, with benefits still detectable weeks after treatment discontinuation (Krzystyniak et al., 2022).

The key question, therefore, is whether this functional rescue is accompanied by measurable changes in the micro-architecture that encodes and stores information, namely excitatory synapses on dendritic spines of hippocampal pyramidal neurons (Kasai et al., 2023; Holtmaat et al., 2009). The present results indicate that D+Q does not "rebuild" the aged hippocampus by globally increasing the number of synapses but instead shifts the quality (morphological state) of spines in a compartment-specific manner, consistent with renewed structural plasticity rather than simple synaptogenesis.

Core findings from this thesis (CA1 pyramidal neurons, DiI-based spine analysis):

- **No global increase in spine density** in CA1 apical or basal dendrites after D+Q (i.e., the total number of protrusions per dendritic length is preserved).
- **Apical dendrite–specific spine remodelling**: mean spine length increased by ~12% ($p = 0.008$) and the length-to-head-width ratio increased by ~18% ($p = 0.002$), with non-significant upward trends in area/circumference ($p < 0.08$).
- **Basal dendrites were not detectably affected** (length, ratio, circumference, area all $p > 0.18$).
- The dataset is sizeable at the spine level (thousands of spines analysed) and statistically treated appropriately via nested structure (animal as the unit of replication), strengthening confidence that the observed effect is a consistent morphological shift rather than a sampling artefact.

Aging brains often show relatively modest neuron loss in hippocampus but substantial functional deterioration, particularly in synaptic efficacy, plasticity thresholds, and the ability to form/maintain long-term potentiation (LTP) (Burke et al., 2006). In that context, a null effect on *density* is not a failure of the intervention; it is compatible with a model in which aging primarily disrupts synaptic *state* and *responsiveness* rather than simply eliminating synapses. Indeed, the results presented in this thesis align with a plasticity-cantered interpretation: D+Q appears to increase the prevalence of higher-aspect-ratio spines (operationally consistent with a relative enrichment of thin/stubby profiles at the expense of more stable mushroom-like spines), which are commonly discussed as more dynamic elements of synaptic remodelling (Bourne et al., 2007).

From a learning-theory perspective, thin spines are often framed as “learning spines”, highly motile protrusions that can be recruited, stabilized, and enlarged during encoding, while mushroom spines are often framed as “memory spines”, more stable structures associated with consolidated connections. Aged hippocampus is frequently described as biased toward reduced structural dynamics, with diminished capacity to remodel spines during experience-dependent learning. The present data suggest that D+Q shifts the spine landscape toward a morphology that is *more permissive to remodelling*. This provides a plausible anatomical substrate for the behavioural restoration observed in the active place-avoidance task (AAPAT), particularly because the cognitive improvement persisted beyond the treatment window, implying durable reconfiguration rather than an acute psychostimulant-like effect (Cimadevilla et al., 2001; Wesierska et al., 2005).

The apical/basal dissociation is not merely an anatomical curiosity; it is circuit relevant. CA1 pyramidal neurons integrate distinct information streams across dendritic domains (Spruston et al., 2008). Apical dendrites in stratum radiatum receive major excitatory input from CA3 Schaffer collaterals and are strongly implicated in classic LTP mechanisms supporting spatial memory encoding (Frick et al., 2003). Basal dendrites (*stratum oriens*) receive partially different input ensembles and may exhibit distinct inhibitory gating and plasticity rules. Compartment-specific vulnerability and compartment-specific rescue are therefore biologically plausible, particularly in aging where plasticity “deficits” are not uniform across cell types, layers, or even dendritic branches.

Within the D+Q framework, this compartment specificity can be interpreted in at least three non-mutually exclusive ways:

1. **Selective restoration of plasticity in the dendritic domain most engaged by the behavioural task.** The AAPAT is an explicitly hippocampus-dependent task requiring continuous discrimination of spatial frames (room cues vs rotating arena cues) and ongoing updating of avoidance decisions. This kind of computation is strongly dependent on CA3–CA1 information transfer and plasticity in CA1 apical domains. Thus, apical spine remodelling fits the behavioural phenotype better than a non-specific brain-wide increase in spine density would.
2. **Aging-related “ceiling effects” on density, but not on morphology.** In older animals, spine density may be maintained by homeostatic constraints even while spine shape distributions shift toward less plastic states (Turrigiano et al., 2012). In such a scenario, interventions that lower inflammatory or metabolic constraints may “unlock” remodelling of existing synapses rather than generating new ones. The thesis results are consistent with such a model: density is preserved, but structural parameters shift.
3. **Indirect rescue via systemic changes rather than direct elimination of senescent cells in brain.** Importantly, this thesis (and the linked source study) did not detect a treatment-dependent reduction in SA-β-gal signal in CA1–CA3, nor changes in chromatin/scaffold senescence-associated proteins HMGB1 and Lamin B1 in hippocampal nuclear fractions. This argues against a simplistic “D+Q clears senescent cells locally in hippocampus → spines recover” narrative and instead supports a model in which brain benefits can emerge despite an undetectable shift in overt hippocampal senescence burden, potentially via reduced peripheral inflammaging and improved systemic milieu (vascular function, immune tone, gut-derived signalling).

We establish that D+Q robustly attenuates pro-inflammatory signalling and increases anti-inflammatory tone (notably IL-10) in aged animals. Chronic low-grade inflammation is a known suppressor of synaptic plasticity: inflammatory cytokines can impair LTP induction/maintenance, alter glutamatergic receptor trafficking, and bias microglial function toward excessive synaptic pruning or maladaptive synapse modulation (Curran et al., 2001). Therefore, a systemic shift away from an inflammaging profile provides a mechanistically credible bridge from senolytic intervention to restored learning competence.

In that integrated view, the hippocampus is not treated as an isolated organ but as a node in a network influenced by peripheral immune signals, gut-derived metabolites, and barrier function (Cryan et al., 2019). Under such a model, the apical spine phenotype observed here can be interpreted as the structural "readout" of a more permissive plasticity environment, created by reduced inflammatory constraint and altered metabolic signalling, rather than solely the result of local hippocampal senescent-cell ablation.

A careful interpretation should acknowledge that spine-shape shifts are correlates of plasticity rather than direct measures of synaptic strength. The present study does not include electrophysiological recordings (e.g., LTP magnitude, AMPAR/NMDAR ratios), ultrastructural synapse counts, or cell-type-specific transcriptomics that would definitively map morphology to function. Nonetheless, the convergence of (i) durable behavioural improvement, (ii) apical spine morphological shift toward more dynamic profiles, and (iii) concurrent epigenetic remodelling (next subsection) provides a coherent triad consistent with rejuvenated plasticity.

● Epigenetic Modulation of Plasticity-Related Chromatin States

If synaptic plasticity is the "hardware" of memory, epigenetic regulation is a substantial part of the "firmware": it determines whether neurons can rapidly deploy transcriptional programs required for encoding, consolidation, and adaptive rewiring (Day et al., 2011; Gräff et al., 2013). Aging is associated with epigenetic drift, including altered histone methylation patterns that bias chromatin toward inappropriate repression of activity-dependent genes (Benayoun et al., 2015). The present thesis directly addresses this layer by measuring two canonical repressive histone marks in dorsal hippocampus and assessing broader chromatin/senescence-associated nuclear proteins.

Core epigenetic findings from this thesis (dorsal hippocampus, histone extracts):

- **H3K9me3 decreased by ~50%** after D+Q compared with aged vehicle controls.
- **H3K27me3 increased by ~20%** after D+Q.
- **No detectable change in Lamin B1 or HMGB1** in hippocampal nuclear fractions.
- **No detectable reduction in SA-β-gal signal** in CA1–CA3, reinforcing that benefits can occur without a gross measurable drop in overt hippocampal senescence markers.

H3K9me3 is commonly associated with constitutive heterochromatin and deep transcriptional repression (Nicetto et al., 2019; Becker et al., 2016). High levels can constrain neuronal gene inducibility, particularly problematic in aging, where learning requires rapid, coordinated activation of immediate early genes and synapse-modifying programs (Parkel et al., 2013). In contrast, H3K27me3 is a facultative repression mark often linked to Polycomb-mediated regulation, enabling controlled silencing and poised regulation of developmental and activity-dependent gene sets (Wiles et al., 2017; Margueron et al., 2011). The *bidirectional* change observed here, downward shift in H3K9me3 with an upward shift in H3K27me3, suggests not a uniform "opening" of chromatin, but a rebalancing: reducing excessive constitutive repression while restoring more regulated, promoter-specific control.

This is important because memory-supportive transcription is not simply "more expression"; it is appropriate inducibility with specificity (Day et al., 2011). Excessive H3K9me3 may lock genes into inappropriately silent states, while appropriate H3K27me3 dynamics may help maintain neuronal identity and allow precise switching of gene modules during learning. The pattern described in the thesis explicitly frames this as a configuration that favours inducible gene expression and aligns with signatures reported in models where cognition improves alongside histone-mark normalization.

A notable feature of this dataset is timing and coherence: cognitive improvement in aged animals is sustained well after the end of drug exposure, and the thesis proposes that this endurance implies underlying structural and molecular changes that persist beyond administration.

Histone methylation marks are plausible contributors to such persistence. Unlike acute neurotransmitter changes, histone modifications can stabilize altered transcriptional "set points," thereby supporting prolonged plasticity competence and potentially facilitating the maintenance of newly optimized synaptic configurations.

The synaptic findings in CA1 apical dendrites, morphological shifts consistent with increased remodelling potential, fit naturally into an epigenetic permissiveness model: if chromatin becomes less constrained by constitutive repression (lower H3K9me3), neurons may more effectively engage activity-dependent programs required for spine restructuring, receptor trafficking, and cytoskeletal remodelling during spatial learning.

The absence of detectable changes in Lamin B1, HMGB1, and SA-β-gal activity complicates a strictly local interpretation. Rather than undermining the epigenetic result, it suggests a more nuanced possibility: D+Q may modulate neuronal/glial chromatin state without measurably altering bulk senescence-marker burden in the sampled hippocampal regions. This has two important implications:

1. **Mechanistic route may be indirect (systemic-to-brain signalling).** The linked study and thesis discussion note that senolytic benefits in brain can be mediated by peripheral reductions in SASP/inflammation, even when CNS senescence markers are difficult to detect or not reduced. In this scenario, improved systemic immune tone, vascular function, and gut-derived metabolite signalling could reduce chronic stress on hippocampal cells, enabling chromatin remodelling and improved plasticity.
2. **Cell-type resolution may matter.** Bulk hippocampal lysate measures can mask cell-specific effects. A modest but biologically meaningful change in neuronal chromatin (or in a small but influential glial subpopulation) could shift network function without producing a large change in region-wide SA-β-gal signal. This is consistent with the broader senescence literature in neurodegeneration: in tauopathy and amyloid models, senescence-like phenotypes can be concentrated, in particular, glial subtypes or plaque-associated niches, and senolytic intervention can improve cognition even when effects are spatially restricted.

Multiple independent studies support the conceptual links relevant here (Table 5):

- In a tauopathy model (PS19), clearance of senescent glial cells prevented gliosis, reduced tau pathology and neurodegeneration, and mitigated cognitive decline (Bussian et al., 2018).
- In an Alzheimer's disease model, senolytic therapy removed senescent OPCs in the amyloid plaque environment, reduced neuroinflammation and Aβ burden, and improved cognition (Zhang et al., 2019).
- Whole-body clearance of senescent cells has been shown to alleviate aspects of age-associated brain inflammation and cognitive decline, with specific emphasis on senescent microglia/OPCs, supporting the idea that relatively small senescent populations can exert disproportionate functional influence (Ogrodnik et al., 2021).

On the epigenetic side, prior work (also cited within the thesis source literature) links elevated H3K9me3 with impaired memory and shows that reducing H3K9me3 (e.g., via SUV39H1 inhibition) can improve memory and promote spine formation in aged hippocampus, conceptually resonant with the present observation of lower H3K9me3 alongside improved behaviour and spine remodelling.

That said, it remains important not to over-interpret: the current dataset demonstrates *association* and *co-occurrence* (behaviour + spines + histone marks), not direct causality. The strongest defensible conclusion is that D+Q produces a molecular epigenetic signature in dorsal hippocampus (H3K9me3↓, H3K27me3↑) that is consistent with increased transcriptional permissiveness and coincides with structural remodelling of CA1 apical spines and durable cognitive rescue in aged rats.

Study	Model	Intervention	Outcome	Relevance to the present work
Bloss et al., 2011	Young, middle aged and aged Sprague Dawley rats	Chronic restraint stress	Aging reduces experience-dependent dendritic spine plasticity in the prefrontal cortex. Older animals show a lower capacity to form new dendritic spines after learning. Existing spines in aged brains are more stable but less adaptable to experience. Reduced structural plasticity may contribute to age-related cognitive decline	Establishes that aging can impair spine dynamics even without dramatic neuron loss
Kushwaha & Thakur, 2020	Young and aged swiss albino mice	Natural aging	Aging increases the level of the repressive histone mark H3K9me3 in the hippocampus. Higher hippocampal H3K9me3 levels are associated with poorer memory performance in old mice. These findings suggest that age-related epigenetic repression may contribute to cognitive decline	Supports that age-related epigenetic repression in the hippocampus is associated with cognitive decline
Snigdha et al., 2016	Aged beagles dog	Pharmacological inhibition of H3K9me3 writer (SUV39H1)	Inhibition of the repressive histone mark H3K9me3 improves memory in aged mice. Reducing H3K9me3 increases dendritic spine density in the aged hippocampus. H3K9me3 inhibition elevates BDNF levels associated with synaptic plasticity. These findings suggest that epigenetic modulation can restore hippocampal plasticity and cognitive function during aging	Supports that epigenetic modulation can restore hippocampal synaptic plasticity and improve memory in aging
Gong et al., 2015	Young and aged BALB/C mice	Dietary restriction and rapamycin supplementation	Histone modification patterns in the mouse brain change with aging. Dietary restriction and rapamycin alter age-associated epigenetic marks. These interventions partially reverse aging-related chromatin changes. Epigenetic modulation may contribute to the neuroprotective effects of anti-aging interventions	Supports idea that epigenetic aging signatures can be shifted toward “younger” states

Table 5. Molecular and Structural Correlates of Synaptic Plasticity and Epigenetic Remodelling Following D+Q Treatment

4.6 Critical Assessment of the Study

● Main Limitations

A rigorous evaluation of the present work requires acknowledgment of both its methodological constraints and its conceptual and experimental strengths (Table 6). The study was deliberately designed as a multi-layer investigation of senolytic intervention in naturally aged animals, integrating behavioural, molecular, microbiological and metabolomic readouts. This ambition constitutes both its principal strength and the source of several limitations.

1. Experimental Scope: Sex, Age and Strain

The use of 24-month-old outbred male Wistar rats represents a major strength and a limitation simultaneously.

Strength:

- It increases ecological validity.
- It mirrors the heterogeneity of senescent burden and microbiome composition seen in aging humans.
- It avoids artefacts inherent to transgenic or inbred mouse models.

Limitation:

- Single-sex design obscures known sexual dimorphism in immune aging, gut permeability, and senolytic responsiveness.
- Use of a single extreme age provides a “late-stage snapshot” rather than a developmental trajectory.
- Inter-individual variability in outbred animals reduces statistical power for subtle microbial or pathway-level effects.

Thus, while the model enhances translational realism, it restricts generalisability and temporal interpretation.

2. Temporal Resolution and Causality

The study relied on pre-treatment and terminal endpoints. While this design demonstrates durable effects of D+Q, it limits causal inference. Without intermediate time-points, it is not possible to determine: Whether microbiota shifts precede barrier restoration; Whether barrier repair precedes cognitive rescue; Or whether these processes evolve in parallel.

A rolling time-course with serial faecal, serum and cerebrospinal sampling would allow mechanistic sequencing and reduce ambiguity regarding directionality.

3. Structural and Functional Assessment of Barrier Integrity

A primary limitation concerns the evaluation of intestinal and blood–brain barrier (BBB) functionality. Although tight-junction proteins (ZO-1, Occludin, claudin-1) were quantified at mRNA and protein levels, and mucosal architecture was assessed histologically, direct permeability assays were not performed

Thus, barrier restoration remains inferred rather than functionally demonstrated. Classical approaches such as: FITC-dextran or mannitol transepithelial flux assays; Evans blue or sodium fluorescein leakage; Trans-endothelial electrical resistance, were not feasible in the aged cohort but would provide direct validation of barrier competence.

Similarly, mucin quantification relied on Alcian-blue staining, which does not distinguish between sulphated and sialylated mucins nor between newly secreted versus stored mucin granules. Therefore, while structural reprogramming of the mucosa is strongly supported, the degree of functional sealing requires further confirmation.

4. Microbiome Resolution and Functional Depth

Amplicon-based 16S rRNA sequencing enabled robust taxonomic profiling but limits interpretation to genus level and provides no direct functional annotation.

Consequently: strain-level shifts remain unresolved; SCFA synthesis pathways and xenobiotic metabolism cannot be directly quantified; PICRUSt-based functional inference remains probabilistic.

Shotgun metagenomics or long-read sequencing would refine pathway attribution and metabolic reconstruction. Nevertheless, the coherence between microbial shifts, metabolite changes, barrier markers and cognitive outcomes substantially strengthens biological plausibility.

5. Metabolomic Breadth

The metabolomic approach focused on major SCFAs and taurine-conjugated bile acids. While quantitatively robust, it did not include microbial tryptophan metabolites (indoles); polyamines; endocannabinoid derivatives or secondary bile acid diversity.

Additionally, sampling was restricted to serum and faeces; portal vein or cerebrospinal fluid analysis would refine spatial origin and flux. Thus, metabolite coverage was targeted rather than exhaustive.

6. Behavioural and Neurobiological Read-outs

Cognitive assessment relied primarily on the AAPAT paradigm, a stringent hippocampus-dependent task. This provides strong evidence for spatial learning rescue but does not capture working memory; anxiety-like behaviour; affective state and social cognition.

Furthermore, electrophysiological correlates (e.g., long-term potentiation) were not directly measured in this thesis, leaving a mechanistic gap between barrier repair and network-level plasticity. Importantly, behavioural improvements were replicated across two AAPAT cycles, including delayed follow-up, reinforcing durability and internal consistency.

7. Statistical and Multi-omics Considerations

The modest sample size (n = 7–8 per group) necessitated conservative statistical approaches. False discovery correction reduces Type I error. However, Type II error risk remains. Multi-omic integration analyses (e.g., DIABLO, mixOmics) were not applied.

Despite this, cross-validation between mRNA and protein levels and convergence of independent biological layers enhance confidence in the core findings

Taken together, the study’s strengths lie in its holistic experimental scaffold, confirmation of effect durability, alignment of independent read-outs and coherence with external reports. Limitations are centred on scope (single sex, single age), depth (16S vs. shotgun; limited metabolite panel), resolution (no real-time permeability measures) and causality (absence of intermediate time-points). These caveats do not undermine the principal conclusions but delineate clear avenues for refinement.

A limitation of this work is that hippocampal tissue, arguably the most behaviourally proximate substrate for spatial-memory outcomes, was not available for BBB marker quantification.

- Main Strengths

Despite the above limitations, the study exhibits several major strengths:

1. Holistic Experimental Architecture

Few senolytic studies integrate cognition, microbiota, metabolomics, intestinal barrier, BBB integrity, inflammatory tone and epigenetic remodelling in a single aged cohort.

2. Natural Aging Model

Use of non-transgenic, geriatric rats enhances translational relevance compared to accelerated or engineered models.

3. Cross-layer Convergence

Independent biological layers align coherently:
- Microbial shifts
- Metabolite modulation
- Tight-junction expression
- Cytokine profile
- Epigenetic remodelling
- Synaptic morphology
- Cognitive rescue

Such alignment reduces the likelihood of spurious associations.

4. Durability of Effect

Cognitive improvement persisted after treatment discontinuation, suggesting structural rather than transient pharmacological effects.

5. Protocol Alignment with Established Senolytic Paradigms

Adherence to canonical D+Q dosing enhances cross-species comparability with established murine literature

Domain	Principal caveats	Potential impact
Experimental design	Single sex (male) and single strain; no adult or female arms	Restricts generalisability and masks sex-specific senolytic responses
Temporal resolution	Only pre- and final endpoints; no intermediate time-course	Cannot resolve causality or sequence of barrier vs microbiota vs cognition changes
Functional assays	Tight-junction mRNA/protein quantified, but electrophysiological permeability assays (e.g., FITC-dextran flux) were not performed; SCFA receptor signalling not functionally tested	Leaves degree of barrier sealing and receptor activity inferential
Microbiome depth	16S rRNA sequencing provides limited taxonomic and no direct functional annotation; shotgun metagenomics absent	Underestimates strain-level shifts; hampers pathway attribution
Metabolite coverage	Focused on major SCFAs and bile acids; tryptophan, polyamine and endocannabinoid panels incomplete	May overlook key neuromodulatory molecules modified by D+Q
Behavioural repertoire	AAPAT and open-field only; no anxiety, depressive-like or social recognition tasks	Cognitive rescue could be broader (or narrower) than measured
Statistical and multi-omics integration	Modest sample size limits statistical power	May obscure subtle but biologically relevant associations across datasets

Table 6. Methodological Limitations and Their Potential Impact on Interpretation of the Present Study

- ## Methodological Considerations

The experimental pipeline of this thesis was deliberately ambitious, combining longitudinal senolytic treatment with multi-layer phenotyping in naturally aged Wistar rats. While this integrative design underpins the study's novelty, several methodological issues warrant detailed scrutiny.

Choosing out-bred, 24-month-old male Wistar rats maximised ecological relevance by reproducing the genetic and microbiological heterogeneity of late-life human populations. Nevertheless, three consequences follow. First, heterogeneity inflated inter-individual variance, diminishing statistical power for subtle endpoints such as predicted KEGG pathways or rare microbial taxa. Secondly, single-sex sampling obscures the pronounced sexual dimorphism in gut permeability, immune senescence and response to senolytics (Jašarević et al., 2018; Duong et al., 2022). Thirdly, the use of a single extreme age provides a snapshot rather than a developmental trajectory; earlier "pre-frailty" time-points would clarify when barrier and cognitive benefits first emerge.

We adhered to the canonical gavage schedule of Dasatinib 5 mg kg^{-1} + quercetin 50 mg kg^{-1} for five consecutive days every fortnight, as originally validated by Kirkland's group. Two caveats deserve mention. Oral gavage itself is a stressor that transiently alters corticosterone and gut motility (Balcombe et al., 2004). Although applied to both groups, this intervention could interact with microbiota dynamics. Secondly, we did not include a non-senolytic anti-inflammatory control such as rapamycin or metformin, which would have helped disentangle senescence-specific effects from general antioxidative or metabolic modulation.

The Active-Allothetic Place-Avoidance task (AAPAT) provided a stringent test of hippocampal spatial learning and executive coordination, sensitive to senescence-linked deficits (Stuchlík et al., 2013). Yet reliance on a single paradigm precludes conclusions about non-spatial domains (working memory, affect, social recognition). Moreover, locomotor drive can confound AAPAT performance; although open-field data showed no gross hypoactivity, fine-scale locomotion parameters were not integrated into the learning curves.

Amplicon sequencing (V3-V4) allowed high throughput and cost-efficiency but limited resolution to genus level and excluded functional gene content. Unlike shotgun metagenomics, 16S profiles cannot quantify SCFA synthesis pathways or senescence-related xenobiotic degradation. PICRUSt-based inference, while employed, remains probabilistic and loses precision in aged, highly divergent microbiomes (Douglas, 2020). We also relied on a one-time faecal sampling at sacrifice; continuously collected samples would better capture temporal synchrony between microbial shifts and metabolite excursions.

Targeted GC-MS and UHPLC-HRMS focussed on classical SCFAs and taurine-conjugated bile acids. This strategy delivered quantitative accuracy but overlooked indole, phenylpropionate or endocannabinoid families that participate in gut–brain signalling (Martin-Gallausiaux et al., 2021). In addition, serum sampling alone cannot locate metabolite origin, portal vein or cerebrospinal fluid would refine spatial inference.

Tight-junction expression was assessed by qPCR and western blot. Although changes in ZO-1 and Occludin often parallel functional sealing, they do not equate to permeability reduction. Gold-standard FITC-dextran flux, trans-epithelial electrical resistance or in vivo Evans-blue exclusion were not feasible in our aged cohort but remain desirable for future validation. For the BBB, comparable tracers (e.g., sodium fluorescein) or MRI-contrast agents would extend the molecular evidence.

Because of small sample size (n = 7–8 per group), non-parametric statistics were preferred for microbial and metabolite data, raising the prospect of Type II error. False-discovery correction was applied conservatively; however, the multi-omics nature of the dataset still risks residual multiple-testing inflation. Integrative network analyses (DIABLO, mixOmics) were not attempted but could untangle cross-omic covariation.

Despite these constraints, several methodological decisions enhance confidence in the core findings. Harmonised tissue harvesting safeguarded within-animal correlations; cross-validation between mRNA and protein levels verified barrier markers; and behavioural improvements were reproduced in two independent AAPAT cycles, including a delayed follow-up. Moreover, aligning our protocol with the dosing paradigm widely used in murine studies facilitates cross-species comparison (Xu et al., 2018; Ogrodnik et al., 2021).

Future experiments should introduce (i) female and middle-aged cohorts, (ii) shotgun or long-read metagenomics, (iii) real-time permeability assays, and (iv) causal manipulations such as faecal microbiota transplantation or SCFA receptor antagonists. Such methodological expansions will solidify the mechanistic chain from senolysis to microbial remodelling, barrier repair and cognitive rejuvenation.

4.7 Future Directions

- Future Mechanistic Studies

The next phase of inquiry should move from associative observation to causal dissection. Below is a coherent experimental agenda that extends the present work and targets the most pressing mechanistic unknowns.

1. **Sex- and age-stratified cohorts.**

Rationale. Senescence accumulation, microbiota composition, and BBB permeability are sexually dimorphic and age-stage dependent.
Design. Parallel treatment of middle-aged (12- and 18-month) and old (24-month) male and female rats with identical D+Q schedule. Continuous behavioural monitoring and serial faecal collections every two weeks will chart the temporal hierarchy of microbial, barrier and cognitive changes.
Outcome. Identify critical windows of senolytic responsiveness and sex-specific biomarkers, allowing translational tailoring.

2. **Shotgun metagenomics with metabolite flux tracing.**

Rationale. 16S data give taxonomic snapshots but cannot specify functional gene shifts or microbial origin of circulating metabolites.
Design. Long-read (Oxford Nanopore/HiFi) metagenomes from sequential faecal samples, coupled with stable-isotope-labelled substrates (^13C-inulin, ^2H-choline) and targeted LC-MS/MS of SCFAs, indoles and bile acids in portal vein, liver, serum and CSF.
Outcome. Reconstruct strain-resolved metabolic pathways, quantify actual flux into host circulation and map which taxa contribute to barrier-modifying metabolites.

3. **Causal microbiota manipulations.**

Rationale. Increased Lactobacillus acidophilus richness coincided with improved cognition, but causality is unproven.
Design. a) Broad-spectrum antibiotics followed by D+Q ± recolonisation with an *L. acidophilus* monoculture or a control consortium. *b)* Faecal microbiota transplantation: donors = D+Q-treated or vehicle-treated aged rats; recipients = germ-free middle-aged rats.
Outcome. Determine whether the microbial shift alone can reproduce barrier repair and cognitive rescue, or whether senescent-cell clearance is obligatory.

4. **Barrier functionality assays in vivo.**

Rationale. Transcript/protein changes in ZO-1 and Occludin imply tighter barriers, but permeability was not measured directly.
Design. a) *Intestine:* FITC-dextran gavage with serial plasma fluorescence; high-resolution confocal imaging of epithelial tight-junction continuity in cleared intestinal segments. b) *Brain:* Dynamic contrast-enhanced MRI or intracardiac injection of 70 kDa Texas-Red dextran followed by quantitative fluorescence in brain homogenates.
Outcome. Provide real-time functional validation that D+Q reduces trans-epithelial and trans-endothelial leakage, and correlate level of restoration with metabolite absorption and behavioural metrics.

5. **Cell-specific senescence mapping and depletion.**

Rationale. Which senescent populations drive gut and brain pathology remains unclear.
Design. 1. Single-nucleus RNA-seq and ATAC-seq on ileal epithelium and hippocampus before/after D+Q to identify senescence signatures in Paneth cells, enteric glia, microglia and endothelial cells. 2. Conditional p16-Ink4a-DTR or p21-Casp9 mouse models to ablate senescent epithelial or endothelial cells selectively, without systemic D+Q.
Outcome. Pinpoint the cellular origin of deleterious SASP and reveal whether microbiota and BBB recovery depend on specific senescent niches.

6. **Mechanistic dissections of SCFA-receptor signalling.**

Rationale. D+Q down-regulated GPR43 and modestly up-regulated GPR41 in the brain; the functional meaning is unresolved.
Design. CRISPR-Cas9 knock-out rats for Gpr43 or Gpr41; treat with D+Q and assess permeability, microglial activation and cognition. Complement with CNS-targeted pharmacological agonists/antagonists (e.g., AR420626 for GPR43).
Outcome. Define whether altered SCFA sensing is protective, compensatory or incidental in senolytic-mediated neuroprotection.

7. **Comparative senolytic screens.**

Rationale. Other senotherapeutics (fisetin, navitoclax, FOXO4-DRI) may differ in gut–brain efficacy and toxicity.
Design. Head-to-head trial of three senolytics at equi-effective senescent-cell clearance; endpoints identical to current study.
Outcome. Establish whether gut–brain rejuvenation is a class effect or unique to D+Q, guiding selection for clinical translation.

8. **Translational biomarkers and human pilot studies.**

Rationale. To bridge from rodents to humans, non-invasive markers of barrier integrity and senescence reduction are essential.
Design. Measure circulating lipopolysaccharide-binding protein, zonulin, SASP factors (IL-6, PAI-1) and gut permeability dyes in aged volunteers enrolled in short D+Q pilot trials (Justice et al., 2019). Incorporate neurocognitive batteries and faecal metagenomics.
Outcome. Validate mechanistic read-outs that predict cognitive benefit and are practical for clinical monitoring.

Collectively, these experiments will dissect the causal chain from senescent-cell ablation to microbial re-programming, barrier repair and neural resilience, thereby refining senolytic strategies for geroprotection.

- ## Translational Potential and Clinical Implications

The constellation of findings produced in this thesis, behavioural rejuvenation, restoration of gut and brain barrier integrity, recalibration of the microbiota-metabolite axis, and a systemic shift away from pro-inflammatory states, positions the D+Q cocktail as a realistic candidate for translation into geriatric medicine. Several layers of evidence already favour clinical feasibility. First, both compounds are orally bio-available, already licensed for human use (Dasatinib) or sold as a nutraceutical (quercetin), and their intermittent administration paradigm minimises cumulative toxicity, an important consideration for an ageing population that is typically poly-medicated. Second, phase-I/II senolytic trials in idiopathic pulmonary fibrosis, diabetic kidney disease and Alzheimer's disease have reported target engagement with acceptable short-term safety profiles, even in participants in their late seventies (Justice et al., 2019; Hickson et al., 2019). Our pre-clinical demonstration that a two-month course normalises cognitive flexibility and reduces lipopolysaccharide-binding protein, TNF-α, and IFN-γ provides a mechanistic rationale for extending these trials to neuro-geriatric indications, especially conditions in which chronic low-grade inflammation and barrier leakiness are recognised accelerants of decline.

Translating the gut-centred benefits requires parallel attention to microbiome-directed endpoints. In humans, Lactobacillus acidophilus can be delivered safely in capsule form and has shown modest efficacy in improving insulin sensitivity and depressive scores in middle-aged cohorts; the marked enrichment of this species in D+Q-treated rats suggests that it could serve as both a biomarker of response and a co-therapeutic that might permit dose-sparing of Dasatinib. Serial faecal metagenomics and plasma metabolomics, already validated in large epidemiological studies, can be incorporated into early-phase trials without excessive participant burden, allowing the microbiota signature identified here (higher *L. acidophilus*, lower Proteobacteria, compressed faecal SCFA pool but stable or elevated circulating formate) to be prospectively tested as a correlate of cognitive gain.

Blood–brain-barrier (BBB) repair offers another measurable translational outcome. Dynamic contrast-enhanced MRI, along with cerebrospinal fluid (CSF)/serum albumin quotient, can capture BBB permeability non-invasively and has been accepted by regulatory agencies as an exploratory biomarker in multiple-sclerosis and Alzheimer drug studies. The present data showing up-regulation of ZO-1 and Occludin in the frontal cortex after D+Q provide the molecular underpinning for hypothesising that measurable reductions in contrast leakage will be observed in treated patients. Importantly, a decline in BBB permeability would also reduce central exposure to circulating drugs and metabolites, emphasising the necessity of careful pharmacodynamic monitoring when D+Q is added to complex medication regimens.

From a public-health perspective, the intermittent senolytic schedule lends itself to adherence models similar to osteoporosis treatments such as zoledronate, where once-yearly dosing is sufficient. A "hit-and-run" protocol could therefore be offered during existing geriatric health assessments, enhancing uptake. Nevertheless, the bidirectional relationship between senescent-cell clearance and host–microbe symbiosis demands that dietary habits, antibiotic exposure and probiotic use all be documented rigorously in forthcoming trials, as they may modulate benefit or exacerbate off-target effects.

In regulatory terms, Dasatinib's myelosuppressive capacity, though minimal at the low, pulsed doses proposed for geroprotection, mandates haematological surveillance and exclusion of patients with cytopenias. Quercetin's interaction with warfarin and cyclosporine via OATP inhibition likewise calls for personalised drug-interaction screening. Developing a companion diagnostic panel that includes senescence markers (p16^INK4a T cell assay), gut-derived metabolites (trimethylamine-N-oxide, secondary bile acids) and barrier biomarkers (zonulin, Occludin fragments) would accelerate both regulatory review and real-world implementation.

Finally, the broad clinical implications extend beyond cognitive frailty. The same pathophysiological triad addressed here, immuno-senescence, barrier dysfunction and microbiota dysbiosis, drives sarcopenia, late-life depression, and vaccine hyperresponsiveness. A single geroscience-based intervention that ameliorates all three therefore holds promise not only for extending life span but also for compressing morbidity in the decades preceding death. Large-scale pragmatic trials with multi-domain endpoints, gait speed, executive function, infection frequency, should therefore form the vanguard of translational efforts. Elucidating precise dosing windows, interaction with caloric restriction mimetics, and the additive value of adjunctive probiotic formulations will define the contours of near-future clinical guidelines.

V. Summary and conclusions

5.1 Key Findings and Their Implications

The present thesis demonstrates that intermittent senolytic treatment with Dasatinib and Quercetin (D+Q) improves cognitive performance in aged Wistar rats and is associated with coordinated changes across the gut-brain axis.

- **D+Q improved cognitive performance in aged animals.** Treatment enhanced spatial learning and memory in the Active Allothetic Place Avoidance Task, and these benefits remained detectable after treatment cessation, indicating that the effects were not limited to an acute pharmacological response.
- **Cognitive improvement was accompanied by neurobiological changes consistent with renewed plasticity.** In the hippocampus, D+Q was associated with changes in dendritic spine morphology and with altered histone H3 methylation patterns, supporting a link between senolytic treatment, synaptic remodeling, and epigenetic regulation.
- **D+Q was associated with selective remodeling of the gut microbiota.** The treatment did not induce a uniform restructuring of the microbial community, but rather specific compositional shifts, most notably an increased abundance of *Lactobacillus acidophilus* and changes in several additional taxa, suggesting a targeted microbiota response to senolytic intervention.
- **Microbiota-derived metabolite profiles were altered after treatment.** Several fecal short-chain fatty acids and bile acids were reduced following D+Q administration, whereas serum changes were more limited. These observations indicate that senolytic treatment modifies microbiota-host metabolic signaling, although the precise mechanisms underlying these changes remain to be established.
- **Senolytic treatment induced region-specific intestinal changes.** In the proximal small intestine, D+Q was associated with increased mucosal thickness, enhanced mucin-related features, and increased expression of selected tight-junction markers. In contrast, effects in the large intestine were mixed, indicating that intestinal responses to treatment were not uniform along the gastrointestinal tract.
- **D+Q improved brain barrier-associated markers and shifted inflammatory balance.** In brain tissue, treatment increased the expression of tight-junction proteins associated with blood-brain barrier integrity and promoted a less inflammatory profile, particularly through increased IL-10 expression, supporting a protective effect on the aging brain environment.

Taken together, these findings support the conclusion that intermittent D+Q administration can influence cognitive aging-associated dysfunction through interconnected effects on inflammation, microbial composition, metabolite signaling, and barrier-associated features. This work therefore identifies senescent cell clearance as a promising mechanistic strategy for modulating the aging gut-brain axis.

5.2 Senolytics as an Intervention to Promote Healthy Aging

The results of this thesis support the concept that senolytic intervention may represent a promising approach to promote healthy aging and delay senescence-associated dysfunction. By targeting senescent cells, Dasatinib and Quercetin influenced several hallmarks of aging simultaneously, including chronic inflammation, barrier dysfunction, microbial dysregulation, and cognitive decline. Importantly, these effects were observed after intermittent treatment, which is consistent with the practical appeal of senolytics as a non-continuous intervention paradigm.

In aged Wistar rats, D+Q improved cognitive performance and was associated with beneficial changes in gut, brain, and immune-related parameters. These findings support the idea that senescent cell burden contributes to multisystem dysfunction during aging and that its reduction may improve organismal resilience beyond a single tissue or endpoint.

At the same time, these findings remain preclinical. Further work is required to clarify causal mechanisms, define optimal treatment schedules, assess long-term safety, and determine whether similar effects occur in females and in other aging models. Thus, senolytics should be viewed not as a definitive anti-aging intervention, but as a promising geroscience-based strategy that warrants continued investigation.

5.3 Potential Clinical Applications

The findings of this thesis suggest that senolytic approaches may have future clinical relevance in age-related disorders and syndromes where cognitive decline, chronic inflammation, and barrier dysfunction coexist during aging. In particular, senolytics may prove valuable in disorders characterized by multisystem deterioration, where targeting an upstream aging mechanism could be more effective than treating isolated downstream symptoms.

Potential areas of application include age-related cognitive decline, frailty, and selected chronic disorders, in which senescence-associated inflammation and tissue dysfunction are prominent contributors. The broad, system-level effects observed in this work also support interest in senolytics as adjunctive interventions in older adults with multimorbidity.

However, translation to the clinic will require careful evaluation of dosing schedules, treatment timing, patient selection, biomarkers of response, and safety. Because the ongoing and completed clinical studies are already summarized in Table 7, this section can remain brief and serve mainly to connect the present findings with that broader translational context.

Trial Name	Condition(s) Targeted	Intervention	Phase	Status	Key Findings / Objectives
NCT02874989	Idiopathic pulmonary fibrosis ≥ 50 years old	D+Q	1	Completed	Justice et al., 2019
NCT02848131	40-80 years old Chronic kidney disease Diabetes mellitus	D+Q	2	Enrolling by invitation	Hickson et al., 2019 Bian et al., 2025
NCT04313634	≥ 60 years old Women	D+Q or Fisetin	2	Completed	Farr et al., 2024 Farr et al., 2025
NCT04063124	Mild/early Alzheimer's disease ≥ 65 years old	D+Q	1-2	Completed	Gonzales et al., 2021; 2023; 2025 Sarkar et al., 2023 Garbarino et al., 2024 Andrews et al., 2025
NCT04685590	Mild/early Alzheimer's disease ≥ 60 years old	D+Q	2	Active, not recruiting	Gonzales et al., 2021 Guerrero et al., 2021
NCT04733534	Adult survivors of childhood cancer ≥ 18 years old	D+Q or Fisetin	2	Active, not recruiting	Outcomes pending
NCT02652052	Hematopoietic Stem Cell Transplant survivors ≥ 18 years old	D+Q	1	Completed	Safety, feasibility, and effect on senescent cell burden and physical function in HSCT survivors (first translational senolytic study in cancer-treatment–related premature aging)

Trial Name	Condition(s) Targeted	Intervention	Phase	Status	Key Findings / Objectives
NCT05506488	Non-alcoholic fatty liver disease ≥ 18 years old	D+Q	1-2	Completed	Evaluate safety and efficacy of intermittent D+Q on liver fibrosis progression, inflammatory markers, and senescence burden
NCT07144293	HIV-1 for 10 years Frail or prefrail ≥ 18 years old	D+Q	2	Recruiting	Assess feasibility, safety, and impact on mobility, frailty metrics, and senescence-associated biomarkers
NCT07025226	Previously treated glioma and residual disease ≥ 18 years old	D+Q or Fisetin	1	Recruiting	Evaluate safety, dosing schedule, and biomarker response of sequential senolytic therapy including D+Q
NCT05838560	Schizophrenia or schizoaffective disorder and treatment-resistant depression ≥ 50 years old	D+Q	2	Active, not recruiting	Schweiger et al., 2025
NCT05422885	Adults at risk for Alzheimer's disease ≥ 65 years old	D+Q	1-2	Completed	Millar et al., 2023
NCT04946383	Epigenetic aging in adults ≥ 40 years old	D+Q	2	Unknown status	Hickson et al., 2019 Cavalcante et al., 2020 Ermogenous et al., 2020
NCT04785300	Alzheimer's disease Mild cognitive impairment ≥ 55 years old	D+Q	1-2	Active, not recruiting	Andrews et al., 2025 Guerrero et al., 2021

Trial Name	Condition(s) Targeted	Intervention	Phase	Status	Key Findings / Objectives
NCT05724329	Resectable head and neck squamous cell carcinoma 18-80 years old	D+Q	2	Active, not recruiting	Liu et al., 2025
NCT07270120	Secondary progressive multiple sclerosis 50-85 years old	D+Q	1	Not yet recruiting	Outcomes pending
NCT06018467	Osteopenia / osteoporosis / age-related bone loss 60-90 years old	D+Q	2	Active, not recruiting	Outcomes pending
NCT07000734	Motor, cognitive and immune functions in older adults 50-70 years old	D+Q	2	Recruiting	Outcomes pending
NCT06355037	Chemo-resistant triple-negative breast cancer	D+Q	2	Recruiting	Outcomes pending
NCT06940297	Relapsed or refractory multiple myeloma ≥ 18 years old	D+Q	2	Recruiting	Outcomes pending
NCT05653258	Obesity 18-65 years old	D+Q	2-3	Recruiting	Outcomes pending
NCT07025226	Previously treated glioma with residual disease ≥ 18 years old	D+Q	1	Recruiting	Outcomes pending

Table 7. Ongoing and Completed Clinical Trials Investigating Dasatinib plus Quercetin (D+Q) as Senolytic Therapy

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